Article

Exploring Mediterranean and Arctic Environments as a Novel Source of Bacteria Producing Antibacterial Compounds to be Applied in Aquaculture

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Featured Application: The study provides insights on the potential of bacteria of underexplored marine origin as a source of compounds useful for the treatment of fish diseases caused by bacteria.

Abstract: The need to discover new natural compounds has become urgent as a possible alternative solution to contrast the spread of antibiotic resistance, also in the aquaculture field. Bacterium–bacterium inhibitory activity against bacterial pathogens relevant in aquaculture was evaluated on agar plates for bacteria isolated from cold Arctic (Kongsfjorden, Svalbard Islands; i.e., seawater and sediment samples) and temperate Mediterranean (Lake Faro in Messina and the Straits of Messina, Italy; i.e., brackish water and benthic filter-feeding organisms) environments. Cell-free supernatants (both pure and concentrated 10-fold) were further assayed and, in the case of a positive response, crude extracts were obtained and tested. After the pre-screening procedures, about 30% of the bacterial isolates inhibited the growth of at least one pathogen used as a target. The 10-fold concentrated supernatants of two Arctic *Salinibacterium* spp. strains and the Mediterranean *Bacillus* sp. PS62 (associated with the pennatulacean *Pteroeides spinosum* Ellis, 1764) resulted in being active against P. *damselae* subsp. *piscicida*. The crude extracts obtained from *Bacillus* sp. PS62 also showed inhibitory activity against the same pathogen. Our findings suggest that tested bacteria could represent a novel source of compounds to be applied to overcome pathogenesis in the aquaculture field.

Keywords: antibacterial activity; bioactive molecules; mediterranean; svalbard; associated bacteria; aquaculture

1. Introduction

In the last few decades, as a consequence of the strong over-fishing actions and the subsequent pauperization of ichthyic resources (with serious economic losses for the ichthyic national market and ecological damage), the aquaculture field has gained interest in supporting and providing complementary activity to fisheries, in order to increase profits both qualitatively and quantitatively. However, farming activities of aquatic species are not exempt of problems, such as the exploitation of environmental and biological resources, the spillage of effluents rich in residual nutrients, and the emergence of fish diseases by bacterial etiological agents with huge economic losses [1–4]. In this latter regard, different bacterial strains are known to be responsible for diseases in farmed fish, mollusks and crustaceans. This is the case, for example, of *Photobacterium damselae* subsp. *piscicida* (responsible

of fish photobacteriosis) [5], *P. damselae* subsp. *damselae* (pathogen for both fish and humans) [6], *Pseudomonas fluorescens* and *Aeromonas hydrophila* (both associated with fish septicemia and ulcerative status) [7], *Vibrio harveyi* (pathogenic for paenaid shrimps) [8,9], and *Listonella* (*Vibrio*) *anguillarum* (causing several illness conditions in different species of fish and shellfish) [10]. Antibiotic treatment of bacterial diseases in fish cultures has been applied for many years, causing increasing of antibiotic resistance in bacterial pathogens, with the total absence of protocols for their safe use worsening this issue. According to Bansemir et al. [11], this worrying aspect has projected the attention of researchers to the discovery of new metabolites as a possible alternative to commercial antibiotics. In this regard, the marine environment, and its great variety of living organisms, including microorganisms, seems to be a rich and yet underexplored source of promising bioactive polymers [12], with a multiplicity of biological activities and unique and diversified chemical structures [13], to be tested as pharmaceutical agents [14]. As defined by ZoBell as far back as 1936 [15], and subsequently documented by several authors, the bactericidal power of marine waters derives from bacteria producing bioactive compounds as a result of the interactions with the environment, thus linking their ecological roles and potential benefits for humans. Despite this, the bacterial production of bioactive molecules (including both broad-spectrum and species-specific activities) [16,17] against pathogens with relevance in aquaculture remains scantly investigated.

In this study we proposed a step-by-step screening procedure (by assaying from pure strains to cell-free supernatants and crude extracts) to detect bioactivity in bacterial strains of different (and yet unexplored) origins (Arctic water and sediment, and Mediterranean benthic organisms and brackish waters) against bacterial pathogens with relevance in aquaculture.

2. Materials and Methods

2.1. Bacterial Strain Origins and Phylogenetic Characterization

The 59 bacterial isolates assayed in this study derived from previous projects and activities, and were selected on the basis of their potential in the bioprospecting field. The strains were previously isolated from Arctic and Mediterranean biotic and abiotic matrices and phylogenetically characterized, as follows: 33 strains were from seawater and sediment samples collected from the Arctic Kongsfjorden (Svalbard Islands, 79° N, 12° E; summer 2009), as reported in Papale et al. [18]; 26 strains were isolated from the Mediterranean polychaetes *Megalomma claparedei*, *Sabella spallanzanii* and *Branchiomma luctuosum* and the sponge *Halichondria panicea* from the brackish Lake Faro (38°16′ N, 15°38′ E; Messina, Italy) as described by Rizzo et al. [19–21], and the sea-pen *Pteroeides spinosum* from the Straits of Messina (37°50′41.34″ N, 15°17′31.41″ E; Italy), as detailed by Porporato et al. [22] and Graziano et al. [23] (Table 1).

In this study, we also report on the isolation of an additional 22 strains from water samples of the Lake Faro (coordinates: 38°16′ N, 15°38′ E) at depths ranging from 0.6 to 0.8 m, according to the procedures previously described [19]. Briefly, water samples were serially diluted, and spread-plated onto Marine Agar (MA; Difco, Italy) plates, which were then incubated at 28 °C for 48 h. Bacterial colonies grown on MA were isolated at random and streaked at least three times before being considered pure. Bacterial cultures were then maintained on MA in the dark at 4 °C under aerobic conditions.

The phylogenetic characterization of the sole active strains (see below for details) was performed as described by Rizzo et al. [19]. Briefly, after cell lysis, the amplification of 16S rRNA gene was performed by using the bacteria-specific primer 27F and 1385R [24]. Sequencing was carried out at the GATC Biotech Laboratory (Konstanz, Germany). Next, relatives of isolates were determined by comparison to 16S rRNA gene sequences in the NCBI GenBank and the EMBL databases using BLAST, and the "Seqmatch" and "Classifier" programs of the Ribosomal Database Project II (http://rdp.cme.msu.edu/) [25]. Sequences were further aligned using the program Clustal W [26] to the most similar orthologous sequences retrieved from database. Each alignment was checked manually,

corrected and then analyzed using the neighbour-joining method [27], according to the model of Jukes–Cantor distances.

Table 1. List of Arctic and Mediterranean strains screened for inhibitory activity in this study.

Origin		Strain	AN [a]	Origin [b]	Reference
Arctic Fjord (Svalbard Islands)		*Microbacterium* sp. C3W9B	KX000039	Water	[18]
		Microbacterium sp. C3W23	KX000040	Water	[18]
		Microbacterium sp. C3W24A	KX000038	Water	[18]
		Salinibacterium sp. C2W9	KX000033	Water	[18]
		Salinibacterium sp. C3W3	KX000034	Water	[18]
		Salinibacterium sp. C3W19	KX000035	Water	[18]
		Salinibacterium sp. C3W22	KX000036	Water	[18]
		Pseudomonas sp. C1W5	KX000004	Water	[18]
		Pseudomonas sp. C1W9	KX000003	Water	[18]
		Pseudomonas sp. C1W15	KX000006	Water	[18]
		Pseudomonas sp. C1W16	KX000011	Water	[18]
		Pseudomonas sp. C2W15B	KX000005	Water	[18]
		Aequorivita sp. C1W3A	KX000015	Water	[18]
		Aequorivita sp. C3W18A	KX000016	Water	[18]
		Devosia sp. DS1	KX000002	Sediment	[18]
		Pseudomonas sp. BS19	KX000007	Sediment	[18]
		Pseudomonas sp. BS23	KX000008	Sediment	[18]
		Pseudomonas sp. C1S1	KX000009	Sediment	[18]
		Pseudomonas sp. C1S12B	KX000012	Sediment	[18]
		Pseudomonas sp. C1S20	KX000013	Sediment	[18]
		Pseudomonas sp. C1S21A	KX000010	Sediment	[18]
		Nocardioides sp. AS7	KX000042	Sediment	[18]
		Salinibacterium sp.BS9	KX000027	Sediment	[18]
		Salinibacterium sp.C1S5	KX000031	Sediment	[18]
		Salinibacterium sp.C1S22B	KX000030	Sediment	[18]
		Salinibacterium sp.C1S23B	KX000037	Sediment	[18]
		Bizionia sp. BS8B	KX000021	Sediment	[18]
		Bizionia sp. BS22A	KX000022	Sediment	[18]
		Bizionia sp. BS24	KX000023	Sediment	[18]
		Gelidibacter sp. DS10	KX000024	Sediment	[18]
		Planococcus sp. AS5	KX000045	Sediment	[18]
		Planococcus sp. AS9	KX000046	Sediment	[18]
		Microbacterium sp.C1S13A	KX000041	Sediment	[18]
Mediterranean Sea	Strait of Messina	*Bacillus* sp. PS62	JX966213	*P. spinosum*	[22]
		Enterovibrio sp. PS152	JX966212	*P. spinosum*	[22]
		Brevibacterium sp. PBE178	KR185336	*P. spinosum*	[23]
		Brevibacterium sp. PBE181	KR185337	*P. spinosum*	[23]
		Brevibacterium sp. PBE190	KR185338	*P. spinosum*	[23]
		Brevibacterium sp. PBE209	KR185339	*P. spinosum*	[23]
		Vibrio sp. PBN295	KR185340	*P. spinosum*	[23]
	Faro Lake	*Bacillus* sp. SpN3	LT604457	*H. panicea*	[21]
		Thalassospira sp. A46	JX298566	*S. spallanzanii*	[19]
		Thalassospira sp. A57	JX298539	*B. luctuosum*	[19]
		Idiomarina sp. A19	JX298543	*M. claparedei*	[19]

Table 1. *Cont.*

Origin	Strain	AN [a]	Origin [b]	Reference
	Cellulophaga sp. A49	JX298548	*B. luctuosum*	[19]
	Cellulophaga sp. A50	JX298550	*B. luctuosum*	[19]
	Cellulophaga sp. A51	JX298549	*B. luctuosum*	[19]
	Cellulophaga sp. A55	JX298551	*B. luctuosum*	[19]
	Cellulophaga sp. A60	JX298552	*B. luctuosum*	[19]
	Cellulophaga sp. Mc108	KF032925	*M. claparedei*	[24]
	Cellulophaga sp. Ss85	KF032927	*S. spallanzanii*	[19]
	Cellulophaga sp. Ss88	KF032928	*S. spallanzanii*	[19]
	Cellulophaga sp. Ss91	KF032928	*S. spallanzanii*	[19]
	Joostella sp. A8	JX298555	*M. claparedei*	[19]
	Joostella sp. A9	JX298556	*M. claparedei*	[19]
	Joostella sp. A11	JX298557	*M. claparedei*	[19]
	Joostella sp. A15	JX298558	*M. claparedei*	[19]
	Joostella sp. A17	JX298559	*M. claparedei*	[19]
	Tenacibaculum sp. Mc99	KF032923	*M. claparedei*	[24]

[a] **AN**, Accession Number. [b] Arctic fjord water and sediment samples were from the Kiongsfjorden (Svalbard Islands); *S. spallanzanii*, *M. claparedei*, *B. luctuosum* and *H. panicea* specimens were from the Lake Faro (Messina, Italy); *P. spinosum* specimens were from the Straits of Messina (Messina, Italy).

2.2. Pre-Screening for Bacterium–Bacterium Inhibitory Activity

Both the cross-streaking method (CSM) and overlay assay (OLA) with soft agar were used to preliminary screen all bacterial strains for inhibitory activity [28,29] against fish and shellfish pathogenic bacteria previously isolated from local marine and brackish environments [30]. Lab strains used in this study were: *Aeromonas hydrophila* (AA5), *A. salmonicida* subp. *salmonicida* (13M), *Photobacterium damselae* subp. *piscicida* (Pdp), *Vibrio alginolyticus* (Ag37), *V. harveyi* (G5), *V. anguillarum* (Van), and *V. parahaemolyticus* (L12G). The strains were identified using standard biochemical and physiological characterization and phylogenetic analyses, as previously reported by Gugliandolo et al. [30,31]. All strains are housed in the Culture Collection of the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences at the University of Messina (Italy). They were maintained at −20 °C in Tryptone Soy Broth (TSB; Oxoid, Milan, Italy) supplemented with 1% (*w/v*) NaCl (TSB1) and 50% (*v/v*) glycerol. Uninoculated media were used as control during the screen experiments.

2.2.1. Cross-Streaking Method (CSM)

Each strain was streaked across one-third of a MA plate and incubated at its optimal growth temperature, i.e., 25 °C for mesophiles or 15 °C for cold-adapted bacteria. After good growth was obtained (generally in 3–4 days for mesophiles, and 7–10 days for cold-adapted bacteria), bacterial pathogens were streaked perpendicular to the initial streak and plates were further incubated at 37 °C (with the only exception of *P. damselae* subsp. *piscicida* that was incubated at 25 °C) for 48 h and checked afterwards for inhibition zones. The antagonistic effect was indicated by the failure of the target strain to grow in the confluence area. Inhibition had to be observed at least twice to be considered positive. If the first two assays showed ambiguous results, an additional assay was performed to re-assess inhibitory activity.

2.2.2. Overlay Assay (OLA)

Isolates were pre-cultured in Marine Broth (MB; Difco) with constant shaking at 120 rpm and allowed to reach an optical density OD_{600} = 1.2 prior to be processed (about 48–72 h). Suitable aliquots (100 µL) of each pre-culture were spread on MA plates and incubated at 25 °C for 3–4 days for mesophiles, and at 15 °C for 7–10 days for cold-adapted strains. Then, 8 mL of soft Tryptone Soy Agar (TSA1: 0.7% agar) mixed with 400 µL of an overnight culture in TSB1 (OD_{600} = 1.2) at 37 °C (or 25 °C in the case of *P. damselae* subsp. *piscicida*) of individual bacterial pathogens were poured over MA plates. Inhibitory activity was defined by the absence of the growth of pathogens around the colonies of bacteria isolates after 18–24 h incubation, and was expressed as presence/absence of inhibitory activity [22].

2.3. Inhibitory Activity of Cell-Free Supernatants and Crude Extracts

Based on results from the pre-screening tests described above, active isolates were selected to evaluate inhibitory activity by using the standard disk diffusion method (DDM) (Kirby Bauer test), as accepted by the National Committee for Clinical Laboratory Standards (NCCLS 2000) and commercially available disks (6 mm in diameter, Oxoid), by testing cell-free supernatants (CFS), 10-fold concentrated supernatants (CFSc) and crude extracts (CE), as described below.

2.3.1. Cell-Free Supernatants (CFS and CFSc)

To obtain CFS and CFSc, selected bacterial isolates (resulting active by the CSM or OLA methods described above) were preliminarily inoculated into TSB with and without an additional carbon source (final concentration 2%, *w/v*), as follows: glucose (TSB-Glu), sucrose (TSB-Suc) or glycerol (TSB-Gly) Bacterial cultures were incubated under constant agitation for 48 h at optimal growth temperature of each isolate and then centrifuged at 10,000 rpm at 4 °C for 30 min. Obtained CFS were filter-sterilized (on nitrocellulose membranes; pore diameter 0.22 µm) prior to be tested and ten-fold concentrated (CFSc) by using a concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany), as described by Simonetta et al. [32]. The inhibitory activity of each CFS and CFSc was tested by the DDM. Briefly, bacterial pathogens were suspended in 3 mL of a saline solution (NaCl 0.9%, *w/v*) until a turbidity of McFarland 0.5 standard (containing around 1.5×10^8 cells/mL) was obtained. The suspensions were then spread-plated in triplicates on TSA1 plates, and aliquots of each CFS and CFSc (20 µL and 60 µL, respectively) were used to soak sterile discs (6 mm diameter). The discs were finally laid on the plates previously inoculated with pathogenic strains. Discs with distilled water (20 µL) were used as a negative control, while discs containing tetracycline (30 µg; Oxoid, Italy) were used as a positive control. The plates were incubated for 24 h at the optimal temperature for each pathogen.

2.3.2. Crude Extracts (CE)

To obtain CE from active bacterial cultures, active isolates were grown in 300 mL of TSB at optimal growth temperature (i.e., 25 and 15 °C for mesophilic and cold-adapted strains, respectively) under agitation until the stationary phase of growth was achieved. After 48 h of incubation, 50 mL aliquots of CFS (obtained as described in the section above) were acidified with phosphoric acid (85%, *v/v*), and bioactive molecules extracted twice in ethyl acetate (cell-free supernatant: ethyl acetate ratio 1:1.25). Ethyl acetate was totally evaporated at room temperature and extracts were collected [33]. Prior to be tested by DDM, 100 mg of each extract were dissolved in 1000 µL of ethyl acetate. Briefly, 20 µL of each extract were applied to sterile filter paper disks (6 mm in diameter, Oxoid). After the complete solvent evaporation, the disks (containing each 2 mg of the extract) were placed onto TSA1 plates inoculated with the target pathogens. Disks soaked with ethyl acetate and submitted to evaporation were used as negative controls, and disks containing tetracycline (30 µg; Oxoid, Italy) (were used as positive controls. Plates were incubated overnight at 37 °C (or 25 °C in the case of *P. damselae* subsp. *piscicida*). The diameter of complete inhibition zones was measured and means and standard

deviations (n = 3) were calculated. The results were codified as strong activity (inhibition zone of 15 mm), moderate activity (inhibition zone ranging from 9 to 14 mm) and weak activity (inhibition zone inferior to 8 mm) [34]. Minimum inhibitory concentration (MIC) values were determined for the most active extracts, by serial diluting each extract in order to obtain different extract concentrations (0.25, 0.5, 1, 2, 4, 8, 16 mg/mL).

2.4. Cytotoxicity Assay

As a preliminary cytotoxicity test, blood agar assay was performed on CFS, CFSc and CE obtained by most promising strains. Ten µL of supernatants and extracts were placed on Blood Agar (BA, Difco) plates and dried. After 48 h of incubation at 37 °C the presence of halos was verified, and classified in α, β and γ haemolysis [35]. Sodium dodecyl sulfate (SDS, Sigma-Aldrich, Milan, Italy; 0.1% *w/v*) and sterile phosphate buffer saline (PBS 10X; Sigma-Aldrich, Milan, Italy) were used as a positive and negative control, respectively.

3. Results

3.1. Bacterial Isolation and Phylogenetic Identification

A total of 22 strains were isolated from the water of Lake Faro. Among them, three isolates (namely W14, W19, and W24) showing inhibitory activity after the pre-screening procedure (see the following sections) were identified by the 16S rRNA gene sequencing. They were affiliated to the genera *Tenacibaculum* (i.e., isolates W14 and W19) and *Pseudoalteromonas* (i.e., isolate W24). Nucleotide sequences have been deposited in the GenBank database under the accession nos. MK636534 (strain W19), MK636535 (strain W14) and MK636536 (strain W24).

3.2. Preliminary Screening for Inhibitory Activity by CSM and OLA

Bacterial isolates (25 out of 81) exhibiting antibacterial activity against at least one pathogenic strain used as a target are reported in Table 2. The preliminary screening carried out by the CSM allowed individuating 21 strains, with most of them that were able to inhibit *P. damselae* subsp. *piscicida* (20 isolates). Two isolates (*Pseudomonas* sp. BS23 and *Microbacterium* sp. C1S13A, both from Arctic sediment) were active against *V. anguillarum*, while *A. hydrophila* and *V. parahaemolitycus* were inhibited by a single isolate each (*Salinibacterium* sp. C3W3 and *Microbacterium* sp. C1S13A from Arctic water and sediment, respectively).

Table 2. Pre-screened isolates from Arctic fjord and Mediterranean Sea environments against pathogens relevant in aquaculture. Isolates in grey boxes were selected for further analyses. +, activity; (+) weak activity; - no activity.

Origin		Isolate	*Ah*		*As*		*Pdp*		*Va*		*Vh*		*Vp*	
			CSM	*OLA*	*CSM*	*OLA*	*CSM*	*OLA*	*CSM*	*OLA*	*CSM*	*OLA*	*CSM*	*OLA*
Arctic fjord	*Water*	*Pseudomonas* sp. BS23	-	-	-	-	+	+	+	(+)	-	-	-	-
		Pseudomonas sp. C1S1	-	+	-	+	+	+	-	-	-	-	-	-
		Pseudomonas sp. C2W15B	-	-	-	-	+	-	-	-	-	-	-	-
		Microbacterium sp. C3W24A	-	-	-	-	+	-	-	-	-	-	-	-
		Salinibacterium sp. C2W9	-	-	-	-	+	+	-	-	-	-	-	-
		Salinibacterium sp. C3W3	-	-	-	-	+	-	-	-	-	-	+	-
	Sediment	*Microbacterium* sp. C1S13A	+	+	-	-	-	-	+	(+)	-	-	-	-
		Salinibacterium sp. BS9	-	-	-	-	+	-	-	-	-	-	-	-

Table 2. *Cont.*

	Origin	Isolate	*Ah*		*As*		*Pdp*		*Va*		*Vh*		*Vp*	
Mediterranean Sea	*M. claparedei*	*Idiomarina* sp. A19	-	-	-	-	+	+	-	-	-	-	-	-
		Cellulophaga sp. Mc108	-	-	-	-	+	+	-	-	-	-	-	-
		Joostella sp. A8	-	-	-	-	+	+	-	-	-	-	-	-
		Joostella sp. A11	-	-	-	-	+	-	-	-	-	-	-	-
		Joostella sp. A15	-	-	-	-	+	+	-	-	-	-	-	-
		Joostella sp. A17	-	-	-	-	+	-	-	-	-	-	-	-
		Tenacibaculum sp. Mc99	-	-	-	-	-	+	-	-	-	-	-	-
	Lake Faro water	*Pseudoalteromonas* sp. W24	-	-	-	-	-	+	-	-	-	-	-	-
		Vibrio sp. PBN295	-	-	-	-	+	+	-	-	-	-	-	-
	P. spinosum	*Bacillus* sp. PS62	-	-	-	-	+	+	-	-	-	-	-	-
		Brevibacterium sp. PBE178	-	-	-	-	+	+	-	-	-	-	-	-
		Brevibacterium sp. PBE181	-	-	-	-	+	+	-	-	-	-	-	-
		Brevibacterium sp. PBE190	-	-	-	-	+	+	-	-	-	-	-	-
		Brevibacterium sp. PBE209	-	-	-	-	+	+	-	-	-	-	-	-
	H. panicea	*Bacillus* sp. SpN3	-	-	-	-	-	+	-	-	-	-	-	-
	Lake Faro water	*Tenacibaculum* sp. W14	-	-	-	-	-	+	-	-	-	-	-	-
		Tenacibaculum sp. W19	-	-	-	-	+	+	-	-	-	-	-	-

CSM, cross-streaking method; **OLA**, overlay assay; **Ah**, Aeromonas hydrophila; **As**, A. salmonicida; **Pdp**, Photobacterium damselae subsp. piscicida; **Va**, Vibrio anguillarum; **Vh**, V. harveyi; **Vp**, V. parahaemolyticus.

Overall, the OLA confirmed the results obtained by the CSM, even if it seldom revealed also an inhibitory activity that was not observed by the method above (Table 2). In detail: *Pseudomonas* sp. C1S1 from Arctic sediment inhibited *A. hydrophila* and *A. salmonicida*; *Pseudoalteromonas* sp. W24, *Tenacibaculum* spp. W14 and Mc99, *Bacillus* sp. SP3 (all from Lake Faro) inhibited *P. damselae* subsp. *piscicida*. As shown in Table 2, 10 strains were selected for further analyses due to their capability to produce inhibition halos of diameter ≥ 1 mm in the overlay assay (data not shown).

3.3. Antibacterial Activity Cell-Free Supernatants

A weak response (not measurable halos) was generally obtained from TSB cultures without sugar addition and by using CFS (inhibition halos < 8 mm, data not shown), whereas better results were obtained by testing CFSc obtained after growth in TSB supplemented with sugars. Two isolates (*Tenacibaculum* sp. W14 and *Bacillus* sp. PS62) resulted particularly active against *P. damselae* subsp. *piscicida*, as they produced inhibition halos of 8.3 ± 0.6 and 12.3 ± 0.6 mm in diameter, respectively, when growing in TSB-Suc and/or TSB-Glu. With regard to cold-adapted strains, CFSc obtained from *Salinibacterium* spp. C3W3 and C2W9 cultures resulted in being active against *P. damselae* subsp. *piscicida* after growth in TSB-Suc (10 ± 0.0 mm). CFSc obtained from *Idiomarina* sp. A19 cultures after growth in TSB-Glu and TSB-Suc produced an inhibition halo of 10 ± 0.0 mm against the same pathogen. All results are shown in Table 3.

Table 3. Inhibitory activity exhibited by concentrated supernatants (CFSc) of selected isolates.

	Origin	Isolate	*Photobacterium damselae* subsp. *piscicida*		
			TSB+Glu	**TSB+Suc**	**TSB+Gly**
Arctic Fjord	Water	*Pseudomonas* sp. C1S1	-	-	-
	Water	*Salinibacterium* sp. C2W9	-	10.0 ± 0.0	-
	Water	*Salinibacterium* sp. C3W3	-	10.0 ± 0.0	-
Mediterranean Sea	*M. claparedei*	*Cellulophaga* sp. Mc108	-	-	-
	M. claparedei	*Joostella* sp. A8	-	-	-
	M. claparedei	*Idiomarina* sp. A19	10 ± 0.0	10 ± 0.0	-
	H. panicea	*Bacillus* sp. SpN3	-	-	-
	P. spinosum	*Bacillus* sp. PS62	12.3 ± 0.6	12.3 ± 0.6	-
	P. spinosum	*Brevibacterium* sp. PBE190	-	-	-
	Lake Faro water	*Tenacibaculum* sp. W14	8.3 ± 0.6	8.3 ± 0.6	-
	Control	Tetracycline		37 ± 0.0	

Glu, glucose; **Suc**, sucrose; **Gly**, glycerol.

3.4. Crude Extracts (CE)

CE were obtained from CFS of *Tenacibaculum* sp. W14, *Salinibacterium* sp. C2W9 and *Bacillus* sp. PS62. This latter was the most promising isolate as the CE yield was 20 mg/mL, obtained after one-week incubation in TSB-Glu under its optimal growth conditions.

Inhibition halos were produced against *P. damselae* subsp. *piscicida*, when testing both 2 and 4 mg of the CE from *Bacillus* sp. PS62, which showed weak and moderate inhibitory activity, respectively (7.0 ± 1.4 and 12.0 ± 1.0 mm halos diameter, respectively).

The minimum inhibitory concentration values were obtained for the active CE from *Bacillus* sp. PS62, through the serial dilution assay. All the tested concentrations (0.25, 0.5, 1, 2, 4, 8, 16 mg/mL) of *Bacillus* sp. PS62 extracts showed inhibitory activity against the target pathogen *P. damselae* subsp. *piscicida*. The inhibitory activity was also exhibited at the lowest concentrations (0.25 and 0.5 mg/mL), even if with a decrease in diameter of the inhibition halo.

3.5. Cytotoxicity Assay

No cytotoxic effects were evidenced for the tested bacterial extracts in terms of haemolytic activity.

4. Discussion

Several species of interest to aquaculture are constantly threatened by microbial infections and diseases, which cause several damages in a field of great importance at industrial level. The use of commercial antibiotics for the treatment of diseases in farmed fishes has been underway for a long time, with consequent onset of bacterial resistance to drugs in both humans and fishes, and environmental impact by the non-absorbed fractions of antibiotics [3]. Moreover, the use of antibiotic reduces the confidence level of potential clients in the high quality of aquaculture products, thus increasing the numerous difficulties for a field that is still supporting competition with the wild resource and facing the reluctance of customers. Sonia et al. [36] suggested that bacteria which developed resistance to antibiotics have to be fronted with novel and safer antibiotics and recognized in the natural products of marine origin a possible candidate [37].

The marine environment is the last frontier in the search for natural antibiotics, by virtue of the high biodiversity which offer a good probability of success. Marine plant and benthic organisms, with particular regard to seaweeds [38], have been studied as a source of several metabolites, such as polysaccharides, polyunsaturated fatty acids, phenolic compounds, and carotenoids, with a wide

spectrum of activities, i.e., antiviral, antiprotozoal, antifungal, and antibacterial properties [39]. On the contrary, literature on marine bacterial metabolites active against pathogens involved in aquaculture diseases is really scant. Moreover, it is known that many bioactive compounds isolated from marine plants and animals actually derive from bacteria associated with them in symbiotic relationships. The main aim of the present study consisted in the investigation of marine bacterial strains of different origins against the most dangerous pathogens in aquaculture. Strains of Mediterranean origin, some of which are associated with benthic organisms, have been used because of their simplicity of cultivation in vitro, the speed of growth, and their potentiality reported in biopolymer production [19,20,23]. On the other hand, strains of Arctic origin were used as strong specialised microorganisms, able to produce specific cellular constituents [40] or high concentration of inhibitory enzymes [41] with peculiar chemical properties and effectiveness.

One of the most used assays to select bacterial candidates able to produce bioactive molecules with antibacterial activity is the agar diffusion method, through which the pathogen agents are exposed to extracellular products released by bacterial producers. Despite the great reproducibility of the test and the low cost, it could be not used to investigate possible in vivo effects [42]. The preliminary screenings are very useful for a rapid selection of bacteria with bioactivity against pathogens, but it is necessary to perform more than one test to obtain consistent and clear results. The pre-screening procedures allowed it to be demonstrated that among all microorganisms considered, it is possible to find bacterial strains with optimal potentiality for the inhibition of pathogens mainly occurring in aquaculture. In the preliminary phases of this study, the antibacterial activity was mainly exhibited against *A. hydrophyla* and *P. damselae* subsp. *piscicida*. The bacterial species affiliated to *Aeromonas* and *Vibrio* genera are well known causes of different pathogenic states of fishes and shellfishes [26,43]. *P. damselae* subspecies are emerging pathogens in aquaculture systems and cause fish photobacteriosis [44], as for *P. damselae* subsp. *piscicida* reported as responsible for fish photobacteriosis [5]. Interestingly, the Mediterranean isolate *Bacillus* sp. PS62 produced an inhibition halo (37 ± 0.0; data not shown) against *P. damselae* subsp. *piscicida*, equal in diameter to that produced from the positive control, i.e., tetracycline. Similarly, the polar strains *Pseudomonas* sp. C1S1 and *Microbacterium* sp. C1S13A produced a zone of inhibition (10 ± 0.0 mm) really near to that obtained from tetracycline (13 ± 0.0 mm) against *A. hydrophila* (data not shown). When compared with strains of Mediterranean origin, very few Arctic isolates showed antibacterial activity, but in contrast they exhibited a broader activity against more than one pathogen. It is noteworthy, however, that the absence of response in the preliminary assays, when this occurred, could be dependent on different factors, such as the incubation temperature and the composition of culture media. Indeed, the temperature of 15 °C was chosen as optimal growth temperature for polar strains, but the production of bioactive molecules could be stimulated by stressful environmental factors, as such as suboptimal temperatures.

The inhibitory activity may derive from the synthesis and diffusion of antibiotic molecules or from the fast use of nutrient components in the culture medium by the tested strain. For this reason, here the antibacterial activity of cell-free supernatants (presumably containing the bioactive molecules) of the most promising strains was investigated. The production of secondary metabolites with antimicrobial activity could be strongly affected by cultural conditions and media composition, which could improve the crude extract yield [45]. Due to the weak activity observed for CFS from TSB bacterial cultures, it was attempted to test the addition of glucose, sucrose and glycerol as additional carbon sources to enhance the production of compounds with antibacterial activity. The strategy resulted in being promising, as the addition of sugars led to a global increase in inhibitory activity, i.e., from inhibition halos with a diameter of less than 8 mm (TSB without sugar addition) to inhibition halos with an average diameter equal or greater than 10 mm. Anyway, only glucose and sucrose resulted in being a suitable carbon source for the enhancement of antibacterial activity, whereas the addition of glycerol did not provide remarkable results.

Strains belonging to the genera *Bacillus* [46], *Pseudoalteromonas* [47], *Pseudomonas* [48], *Vibrio* [49], *Aeromonas* [50], *Alteromonas* [51], *Marinomonas* [16], and *Photobacterium* [52] are known to produce

compounds with antimicrobial activity. The phylogenetic affiliation of the Mediterranean strain PS62 confirms the interest of members belonging to the *Bacillus* genus as among most promising candidates in this field. Moreover, the origin of the strain *Bacillus* sp. PS62 highlighted again the great potential of benthic organism-associated bacteria in the production of bioactive molecules [19,23]. Several studies reporting the activity of *Bacillus* isolates are available in literature. Vaseeharan and Ramasamy [53] reported the use of *Bacillus subtilis* BT23 as probiotic in the treatment of infectious diseases caused by the pathogen *V. harveyi* in the farming of shrimps (*Penaeus monodon*), both in vivo and in vitro. Balcàzar and Rojas-Luna [54] reported the probiotic effects of *Bacillus subtilis* UTM126 isolated from shrimp (*Litopenaeus vannamei*) culture tanks against the same pathogen. Here, the CEs obtained from most promising strains confirmed the inhibitory activity observed with CFSc, as in the case of *Bacillus* sp. PS62, whose extract at a concentration of 0.25 mg/m was enough to produce an inhibition of about the 30% of *P. damselae* subsp. *piscicida* growth. Differently, inhibitory activity against bacterial pathogens was not observed for *Tenacibaculum* sp. W14 and *Salinibacterium* sp. C2W9 extracts. This finding suggests that a refinement of the extraction procedure is probably necessary to isolate properly the active fractions from the supernatants. Indeed, the extraction protocols could be based on the use of different mixtures of solvents which act differently depending on their polarity, with consequent different efficiency. However, further investigations are needed to evaluate the composition of the active molecules and to elucidate their chemical structure.

In addition to the promising results obtained by strains affiliated to just known genera, here interesting antimicrobial activity was detected also for genera scantly reported in this field, i.e., the isolate W14, affiliated to the genus *Tenacibaculum*. Several cold-adapted microbial species, such as *Pseudoalteromonas* [45,55–57], *Alteromonas* [58], *Pseudomonas* [59] and *Vibrio* [60] have been reported to possess antimicrobial activity, but reports are scant on the activity against pathogens of relevance in aquaculture, or exhibited by *Salinibacterium* members, which resulted here in being active against *P. damselae* subsp. *piscicida* (strain C2W9).

5. Conclusions

In view of the critical situation in which the aquaculture section is involved, it is important to take every possible route that can provide valid solutions. This study provides insights about the potential of extracts of marine bacterial origin that, after appropriate optimization and large-scale production analysis, could be employed for their beneficial effects in the treatment of bacterial diseases. Further studies are necessary to provide information about the mechanisms that control the observed inhibitory activity. The results from the present study suggest promising perspective in the use of bacterial metabolites for the treatment of fish diseases. Further analyses will be performed to investigate their possible use as dietary supplements, and to improve the chemical structure of these molecules and their interaction at the basis of the antagonistic effect.

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Abbreviations

CSM	Cross-streaking method
OLA	Overlay Assay
CFS	Cell-free supernatant
CFS	Tenfold concentrated supernatantc
CE	Crude Extract

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