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Posted Date: 16 April 2026

doi: 10.20944/preprints202604.1158.v1

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Antimicrobial Potential of the Marine Bacterium *Marinomonas mediterranea* Secretome Against Clinically Relevant Bacterial and Fungi Pathogens

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Abstract

The rise of antimicrobial resistance is reducing the effectiveness of current therapies and highlights the need for new antimicrobial agents. Marine environments, rich in microorganisms adapted to extreme conditions, represent a promising reservoir of bioactive compounds. The marine bacterium *Marinomonas mediterranea* secretes, among others, lysine oxidase (LodA) and glycine oxidase (GoxA), enzymes that generate hydrogen peroxide and exhibit antibacterial activity. Based on this mechanism, we hypothesized that modifying specific experimental conditions could enhance the antimicrobial potential of the secreted enzymes and additional compounds, broadening their activity spectrum. In this study, culture conditions were optimized to promote the production of inhibitory molecules, and the antimicrobial activity of the *M. mediterranea* secretome was assessed against clinically relevant microorganisms. The concentrated secretome obtained from growth in Marine Broth exhibited antimicrobial activity against the Gram-negative and Gram-positive bacteria analyzed and several pathogenic fungi, an effect attributable to some proteins present in it. Among them, LodA and GoxA, previously associated with bacterial growth inhibition, also appear to contribute to antifungal activity. Moreover, valyl-tRNA synthetase emerged as a potential new antimicrobial factor. These findings highlight the biotechnological potential of marine microorganisms and support further exploration of their secretomes for the development of new antimicrobial agents.

Keywords: *Marinomonas mediterranea;* secretome; antimicrobial proteins; lysine oxidase (LodA); glycine oxidase (GoxA); valyl-tRNA synthetase; marine bioactive compounds; marine bioprospecting

1. Introduction

Antimicrobial drugs, including antibiotics and antifungals, are indispensable tools for the prevention and treatment of infections caused by bacteria and fungi. The appearance of new infectious diseases, together with the return of previously controlled ones and the steady rise in resistance to current therapies, has turned antimicrobial resistance into a major global health challenge [1–3]. Antimicrobial resistance compromises the effectiveness of conventional treatments, increases mortality associated with resistant infections, and poses a serious threat to global health security [4,5].

The widespread and often inappropriate use of antibiotics and antifungals in clinical, veterinary, and agricultural settings has accelerated the spread of multidrug-resistant strains. As a result,

 doi:10.20944/preprints202604.1158.v1

treatment options are becoming increasingly limited, and the socioeconomic impact of persistent or untreatable infections continues to grow [5–8]. According to the World Health Organization (WHO) Global Antibiotic Resistance Surveillance Report 2025, approximately one in six bacterial infections worldwide is resistant to antibiotics [3].

Among the most clinically relevant pathogens are those collectively referred to as ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.), which account for a substantial proportion of nosocomial infections and are notorious for their ability to evade multiple antimicrobial agents [2]. In parallel, opportunistic fungal infections represent a growing threat, particularly among immunocompromised patients. Species such as *Candida spp.* and *Aspergillus spp.* cause invasive infections associated with high mortality rates [9]. The limited availability of effective antifungal therapies, together with the emergence of resistant strains, underscores the urgent need to identify new bioactive compounds with therapeutic potential. WHO reports indicate that the current pace of antimicrobial development is not keeping up with the spread of multidrug-resistant pathogens, reinforcing the need to explore alternative sources of antimicrobial agents, including those derived from marine ecosystems [3].

Marine ecosystems are increasingly recognized as a rich source of bioactive compounds with antimicrobial activity. In contrast to terrestrial microorganisms, marine bacteria produce structurally and functionally unique compounds, shaped by their adaptation to highly competitive oligotrophic environments and extreme conditions of pressure, temperature, salinity, oxygen availability, light and pH. These environmental pressures have shaped microorganisms capable of producing a wide variety of bioactive substances, such as antibiotics, antifungals, antimicrobial peptides, bacteriocins, pigments or enzymes, among others [10–13]. To date, more than 23,000 marine bioactive compounds with biotechnological and pharmacological relevance have been identified [7]. Representative examples include MC21-A, isolated from *Pseudoalteromonas phenolica* sp. nov. O-BC30^T, which exhibits activity against *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* (MRSA), and other Gram-positive bacteria [14], as well as pelagiomycin A, produced by *Pelagiobacter variabilis*, with activity against both Gram-positive and Gram-negative bacteria [15]. Additional examples of antimicrobial compounds of marine origin, together with their biological sources and reported activities, are summarized in Table 1, highlighting the marine environment as a largely untapped reservoir of bioactive molecules with therapeutic potential.

Table 1. Representative antimicrobial compounds isolated from marine microorganisms and their reported target organisms.

MICROORGANISM	ISOLATION SOURCE	COMPOUND(S)	REPORTED ACTIVITY	REF.
Nocardiopsis dassonvillei HR10-5	Marine sediment	Nocapyrones E-G	*Bacillus subtilis*	[16]
Bacillus sp. 09ID194	Marine sediment	Macrolactin W	*B. subtilis, S. aureus, E. coli, P. aeruginosa*	[17]
Streptomyces microflavus	Marine sediment	Chromomycins	*E. faecium, E. faecalis, Salmonella enterica,* methicillin-resistant *S. aureus,* methicillin-sensitive *S. aureus*	[18]
Paenibacillus profundus Sl79	Marine sediment	Linear glyceride-derived heptapeptide	*S. aureus, Staphylococcus epidermidis, B. subtilis, E. faecium*	[19]
Streptomyces sp. CNH-189	Marine sediment	Meroclorins A	Methicillin-resistant *S. aureus, Clostridium difficile*	[20]
Streptomyces sp. CMB-M0244	Marine sediment	Molemycin A	*S. aureus, S. epidermidis, B. subtilis, E. coli, P. aeruginosa, Mycobacterium bovis*	[21]
Streptomyces VITAK1	Marine sediment	Coumarin CDTM	*S. aureus, Bacillus cereus, Salmonella paratyphi, K. pneumoniae, P. aeruginosa, Proteus vulgaris, E. coli*	[22]
Bacillus licheniformis 09IDYM23	Marine sediment	Ieodoglucamide C, Ieodoglycolipid	*S. aureus, B. subtilis, B. cereus, Salmonella typhi, E. coli, P. aeruginosa, Candida albicans*	[23]
Pseudoalteromonas phenolica O-BC30^T	Seawater	MC21-A	*Enterococcus serolicida, E. faecium, E. faecalis, Streptococcus* spp., methicillin-resistant *S. aureus*	[14]

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Streptomyces xinghaiensis NRRL B24674^T	Seawater	Xinghaiamine A	*A. baumannii, P. aeruginosa, E. coli, S. aureus, B. subtilis*	[24]
Pseudomonas bromoutilis	Seawater	Pentabromopseudilin	*S. aureus, Diplococcus pneumoniae, Streptococcus pyogenes*	[25]
Pseudomonas sp. UJ-6	Seawater	1-acetyl-β-carboline	Methicillin-resistant *S. aureus*	[26]
Streptomyces seoulensis	Seawater	Streptoseomycin	*Helicobacter pylori, Lactobacillus acidophilus,Bifidobacterium bifidum, Eubacterium brachy, Propionibacterium acnes, S. aureus, Micrococcus luteus, B. subtilis*	[27]
Micromonospora sp. TP-A0316	Seawater	Arisostatins A, B	*M. luteus, S. aureus, B. subtilis*	[28]
Bacillus amyloliquefaciens MTCC 10456	Seawater	Bacilysin	*Malassezia furfur, Malassezia globosa*	[29]
Pseudomonas putida	Seawater	9,10-dihydrophenanthrene-2-carboxylic acid	*C. albicans*	[30]
Pseudonocardia sp. SCSIO 01299	Seawater	Pseudonocardians A-C	*S. aureus, E. faecalis, Bacillus thuringiensis*	[31]
Aneurinibacillus aneurinilyticus SBP-11	Seawater	Aneurinifactin	*K. pneumoniae, E. coli, S. aureus, P. aeruginosa, B. subtilis, Vibrio cholerae*	[32]
Brevibacillus laterosporus PNG276	Seawater	Tauramamide	*Enterococcus* sp.	[33]
Streptomyces sp. M-207	Coral (*Lophelia pertusa*)	Lobophorin K	*P. aeruginosa, A. baumannii, K. pneumoniae, E. coli,* methicillin-sensitive *S. aureus*	[34]
Nocardia sp. ALAA 2000	Macroalga (*Laurencia spectabilis*)	Chrysophanol-8-methyl ether, Asphodelin, Juticidin B, Ayamycin	Broad-spectrum bacteria and fungi	[35]
Pelagiobacter variabilis	Macroalga (*Pocockiella variegata*)	Pelagiomycin A	*S. aureus, Enteroccoccus hirae, B. subtilis, K. pneumoniae, E. coli, P. aeruginosa, Salmonella choleraesuis, P. vulgaris, Shigella sonnei*	[15]
Marinomonas mediterranea	Seagrass (*Posidonia oceanica*)	Lysine oxidase, Glycine oxidase, R-type bacteriocin	Gram-positive and Gram-negative bacteria	[36–41]

The marine bacterium *M. mediterranea*, a member of the microbiota associated with the seagrass *Posidonia oceanica* [41], is characterized by its ability to synthesize two enzymes with antimicrobial properties. *Marinomonas mediterranea* produces oxidases, formerly referred to as marinocin, and an R-type bacteriocin, both of which display antibacterial activity against some Gram-positive and Gram-negative bacteria [36–39]. The antibacterial activity of both enzymes is attributed to the release of hydrogen peroxide (H_2O_2) during their catalytic reactions, an effect inhibited in the presence of catalase [36,37,40]. These oxidases catalyze oxidative deamination reactions: lysine oxidase converts L-lysine into 6-semialdehyde-2-aminoadipic acid, ammonia, and H_2O_2 [36], while glycine oxidase converts L-glycine into glyoxylate, ammonium, and H_2O_2 [37]. The hydrogen peroxide generated plays an essential bactericidal role by inducing oxidative damage in exposed cells [42,43]. In addition to these oxidase-based systems, *M. mediterranea* has also been reported to produce an R-type bacteriocin with targeted activity against closely related *Marinomonas* species [39], although this phage-derived structure is not known to be secreted under standard laboratory conditions. Despite the recognized antibacterial potential of *M. mediterranea*, its spectrum of activity may be broader. Modifying experimental conditions and using different culture media could lead to the production of bioactive compounds with enhanced inhibitory capacity. To date, its inhibitory potential against clinically relevant bacteria such as *Klebsiella pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa*, as well as the fungi *Candidozyma auris, Aspergillus fumigatus, Scedosporium boydii,* and *Lomentospora prolificans* has not been evaluated yet.

In this work, we set out to evaluate the antimicrobial activity of MMB-1^T strain *M. mediterranea* against clinically relevant bacterial and fungal pathogens and to characterize the metabolites involved, with a particular focus on the bioactive components present in its secretome. To this end, culture conditions were optimized to promote the production of inhibitory compounds, and the

 doi:10.20944/preprints202604.1158.v1

antimicrobial activity of the secreted fraction was evaluated against a panel of pathogenic microorganisms of clinical interest. In addition, proteomic analyses were conducted to identify secreted proteins potentially responsible for the observed antimicrobial effects. By exploring both antibacterial and antifungal activities, this work seeks to expand current knowledge of the antimicrobial capabilities of *M. mediterranea* and to assess its secretome as a promising source of novel bioactive compounds with potential therapeutic applications.

2. Results

2.1. Detection of Antimicrobial Activity in the Secretome of M. mediterranea Obtained from Different Culture Media and Optimization of the Detection Method

Growth inhibition halos were detected for most reference microorganisms under specific conditions. In contrast, no antifungal activity was observed against *Candidozyma auris* (previously known as *Candida auris)* or *Aspergillus fumigatus* under any tested condition, and these species were excluded from subsequent analyses. With the exception of *Staphylococcus aureus*, secretome obtained from half-diluted Marine Broth (dMB) (whether concentrated or unconcentrated), showed no inhibitory activity. In Bushnell Haas Broth supplemented with glucose and NaCl (BHB), antimicrobial activity was detected only in concentrated secretomes and was restricted to *Enterococcus faecalis*, *S. aureus*, and *Acinetobacter baumannii*. For the remaining strains (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Scedosporium boydii,* and *Lomentospora prolificans*), inhibitory activity was detected exclusively in secretomes obtained from the growth of *M. mediterranea* in Marine Broth (MB), regardless of concentration.

The mean diameters of the inhibition halos produced by the compounds present in concentrated MB secretomes (1.57 ± 0.08 cm) were significantly larger (ANOVA, Dunnett's T3, $p < 0.05$) than those observed under other condition in which inhibitory activity was detected (Figure 1). These results identified MB as the most suitable medium for producing secretomes with strong antimicrobial activity.

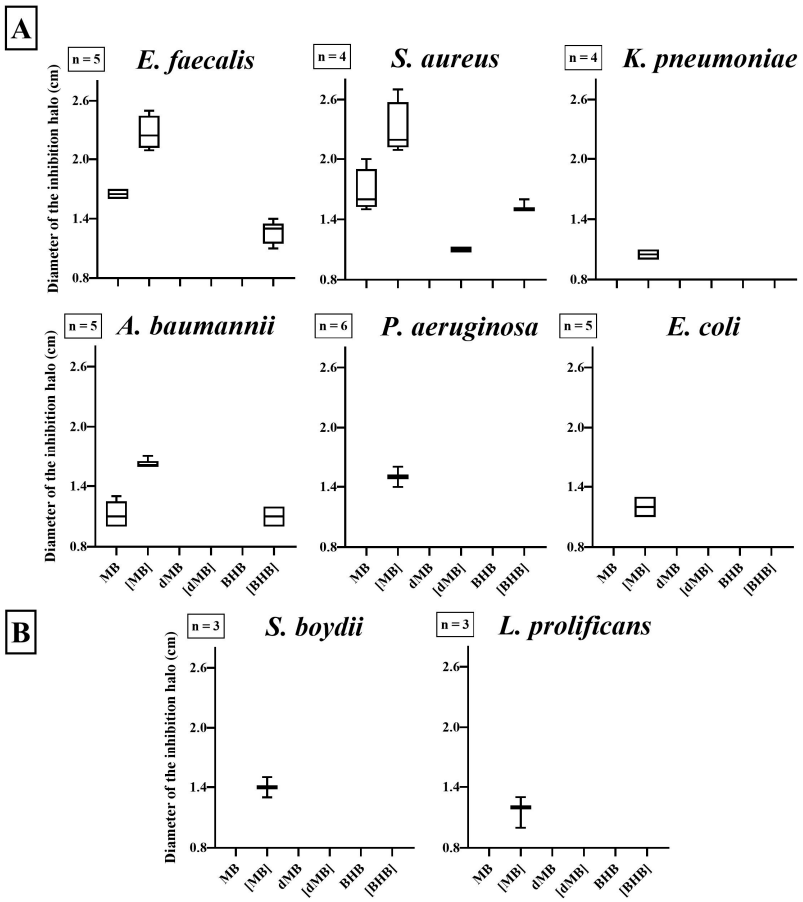

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Figure 1. Mean diameter (+SE) of inhibition zones generated by the antimicrobial activity of the [concentrated] and unconcentrated secretome of M. mediterranea grown in three culture media: Marine Broth (MB), half-diluted Marine Broth (dMB), and Bushnell Haas Broth (BHB) supplemented with glucose and NaCl. Antimicrobial activity is shown against clinically relevant bacteria (A) and fungi (B). Mean values were calculated from the number of replicates indicated in the figure (n = x).

Based on these findings, MB was selected as the optimal medium for antimicrobial assays. The effect of secretome concentration on inhibitory activity was evaluated using 0- to 14-fold concentrated samples. A progressive increase in inhibition halo diameter was observed with increasing concentration, with the most pronounced changes between 7- and 9.5-fold concentrations, depending on the microorganism (Figure 2). To balance efficiency and experimental practicality, MB secretomes were concentrated between 7- and 9.5-fold for subsequent assays.

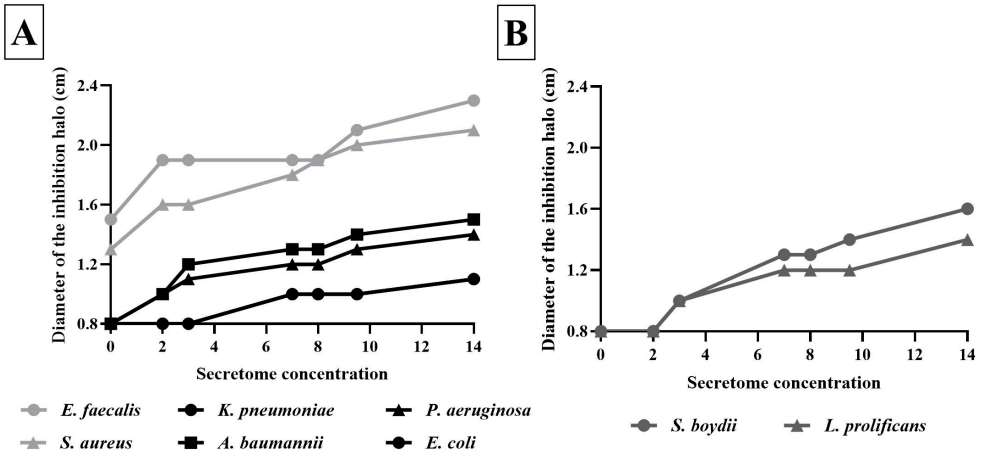

Figure 2. Diameters of inhibition halos generated by the antimicrobial activity of the *M. mediterranea* secretome grown in Marine Broth across a concentration gradient (0- to 14-fold). Panels show activity against clinically relevant bacteria (A) and fungi (B).

2.2. Inhibitory Capacity of the Optimal Secretome of M. mediterranea Against Clinically Relevant Microorganisms and the Impact of Catalase on Its Antimicrobial Activity

Using the selected conditions (MB medium, 7- to 9.5-fold concentrated secretomes), antimicrobial activity was evaluated against clinically relevant bacteria and fungi. The largest inhibition halos were observed for *S. aureus* (2.38 ± 0.10 cm) and *E. faecalis* (2.21 ± 0.10 cm), whereas smaller halos were detected for Gram-negative bacteria (*P.aeruginosa* 1.58 ± 0.05 cm; *A. baumannii* 1.56 ± 0.03 cm; *E. coli* 1.33 ± 0.07 cm; *K. pneumoniae* 1.20 ± 0.06 cm) and filamentous fungi (*S. boydii* 1.57 ± 0.07 cm; *L. prolificans* 1.36 ± 0.08 cm) (Kruskal-Wallis, $p < 0.05$).

Grouping microorganisms by cell wall type revealed significantly larger inhibition halos against Gram-positive bacteria (2.29 ± 0.07 cm) compared to Gram-negative bacteria (1.43 ± 0.03 cm) and fungi (1.46 ± 0.06 cm) with no differences between Gram-negative bacteria and fungi.

The effect of catalase, which degrades H_2O_2, on antimicrobial activity, was then assessed. In all conditions where antimicrobial activity was detected (see Results, Section 2.1), the addition of catalase completely abolished the inhibition previously observed (Figure 3). These findings demonstrate that H_2O_2 plays a major role in the antimicrobial activity of the secretome.

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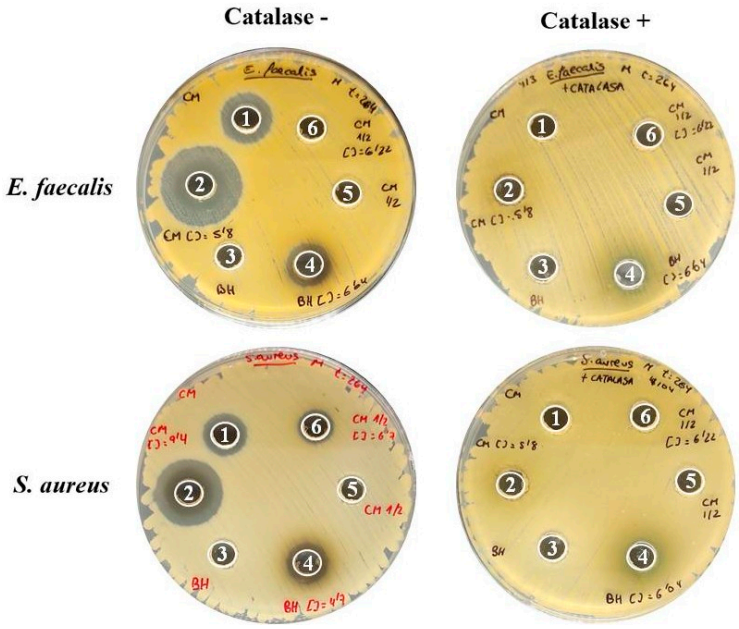

Figure 3. Representative example of the impact of catalase on the antimicrobial activity of the Marinomonas mediterranea secretome grown in three culture media: Marine Broth (1,2), half-diluted Marine Broth (3,4), and Bushnell Haas Broth supplemented with glucose and NaCl (5,6), tested against Enterococcus faecalis and Staphylococcus aureus. Odd numbers indicate non-concentrated secretomes, whereas even numbers correspond to concentrated secretomes. In the plate treated with catalase, the darkening around the Bushnell Haas Broth well is due to the addition of the secretome itself and not to antimicrobial activity.

2.3. Detection and Quantification of the Growth Inhibitory Capacity of Fractions Obtained by High-Performance Liquid Chromatography (HPLC) from the Secretome of M. mediterranea

Proteins from the MB secretome were fractionated by HPLC (F1 to F5) based on size and molecular weight. Antibacterial activity was detected in F1 (2487.8 - 284.9 kDa) and F2 (284.9 - 32.6 kDa), while antifungal activity was restricted to F1. Subfractions F1.3 (586.7 - 284.9 kDa) and F2.1 (284.9 - 138.4 kDa) retained antibacterial activity, whereas antifungal activity was observed only in subfraction F1.3 (Figure 4). The mean inhibition halo of subfraction F1.3 (1.72 ± 0.08 cm) was significantly larger than that of F2.1 (1.18 ± 0.11 cm) and comparable to the complete secretome (1.90 ± 0.08 cm) (ANOVA, Bonferroni, $p < 0.05$).

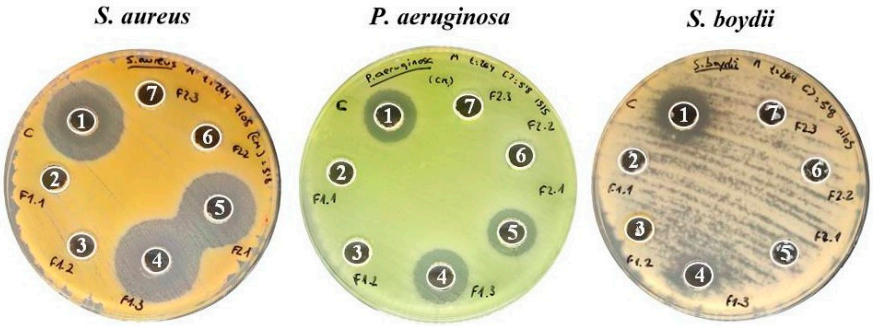

Figure 4. Representative examples of the antimicrobial activity of inhibitory compounds present in subfractions of the Marinomonas mediterranea concentrated secretome cultured in Marine Broth and separated by High-Performance Liquid Chromatography. Antimicrobial activity of the following subfractions is shown: Control (1), F1.1 (2), F1.2 (3), F1.3 (4), F2.1 (5), F2.2 (6), and F2.3 (7), tested against Staphylococcus aureus, Pseudomonas aeruginosa, and Scedosporium boydii.

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In both, the total secretome and subfraction F1.3, the mean inhibition halos against the two Gram-positive bacteria were larger than those against the Gram-negative bacteria and fungi (ANOVA, Bonferroni, $p < 0.05$) (Figure 5). Regarding subfraction F2.1, the inhibition halo against *E. faecalis* was larger than that against the other bacteria (ANOVA, Bonferroni, $p < 0.05$), and no inhibition was observed against the two fungi.

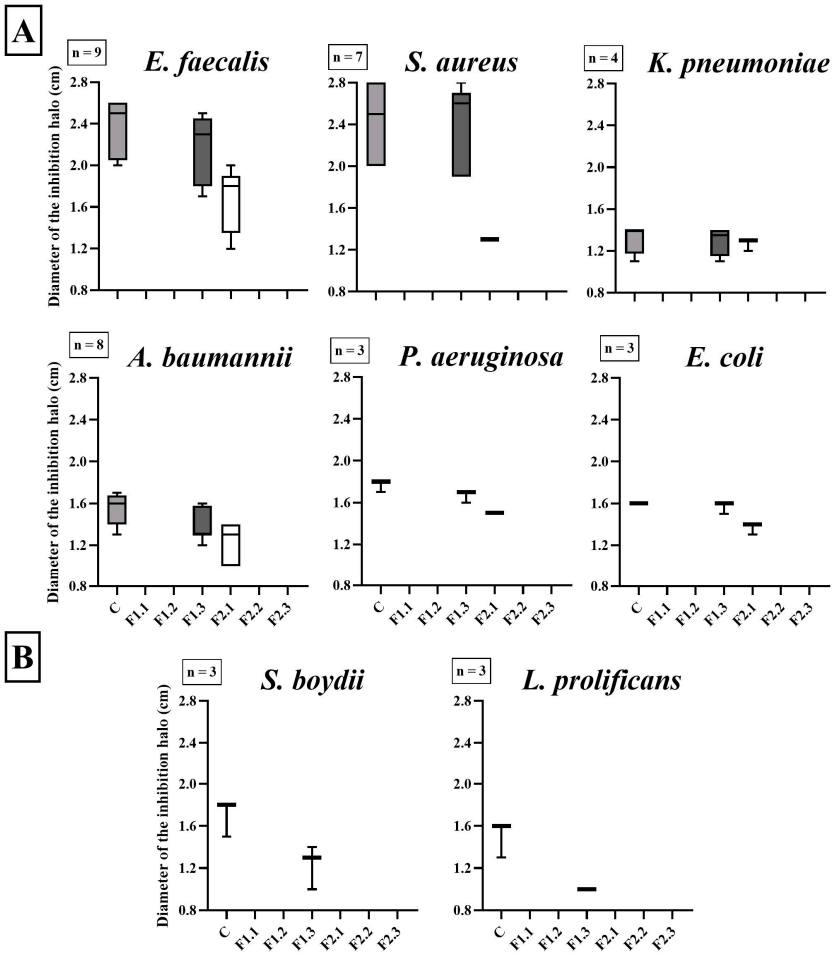

Figure 5. Antimicrobial activity of the complete secretome (C, Control) and subfractions F1.1-F1.3 and F2.1-F2.3 obtained from the concentrated secretome of M. mediterranea cultured in Marine Broth against clinically relevant microorganisms: (A) bacteria; (B) fungi. Mean values were calculated from the number of replicates indicated in the figure (n = x).

2.4. Proteomic Analysis

Proteins in subfractions F1.3 and F2.1 were identified using Liquid Chromatography-rhigh resolution mass spectrometry (LC-HRMS). After filtering non-relevant entries, 181 proteins were classified into three groups: shared proteins (68), proteins exclusive to F1.3 (89), and exclusive to F2.1 (24).

Functional annotation revealed 16 biological processes plus unassigned proteins. Some proteins were classified in more than one process. These results include both the proteins found exclusively in each of the two subfractions and those shared between them (Figura 6).

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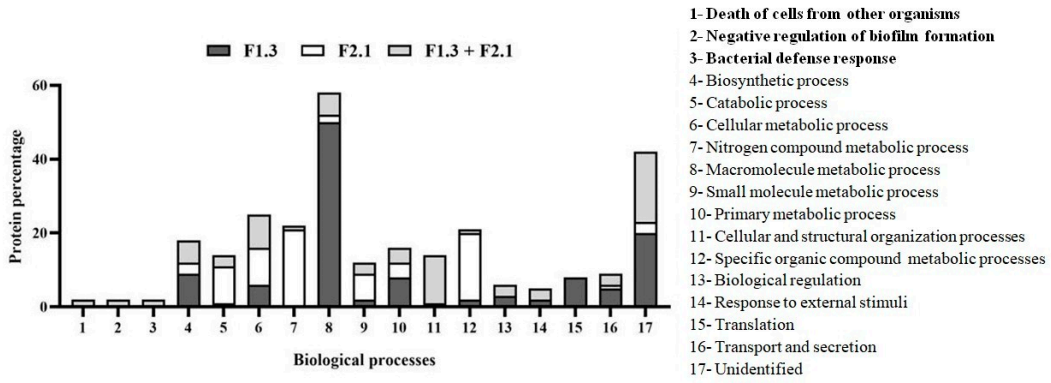

Figure 6. Percentage of proteins involved in each of the biological processes (1-17), distributed among proteins present in subfraction F1.3 (gray), subfraction F2.1 (white), and proteins shared by both fractions (light gray). The three processes highlighted in bold correspond to biological functions associated with antimicrobial activity.

Proteins exclusively in F1.3 were predominantly associated with metabolic processes, translation and response to internal and external stimuli. Proteins exclusively in F2.1 were mostly linked to catabolic processes and general metabolism. In contrast, proteins shared by both subfractions were enriched in processes related to antagonistic interactions, including cell death, negative regulation of biofilm formation, and bacterial defense responses (Figura 7). Among proteins potentially responsible for antimicrobial activity, lysine oxidase (LodA), glycine oxidase (GoxA), and valyl-tRNA synthetase (ValRS) were identified.

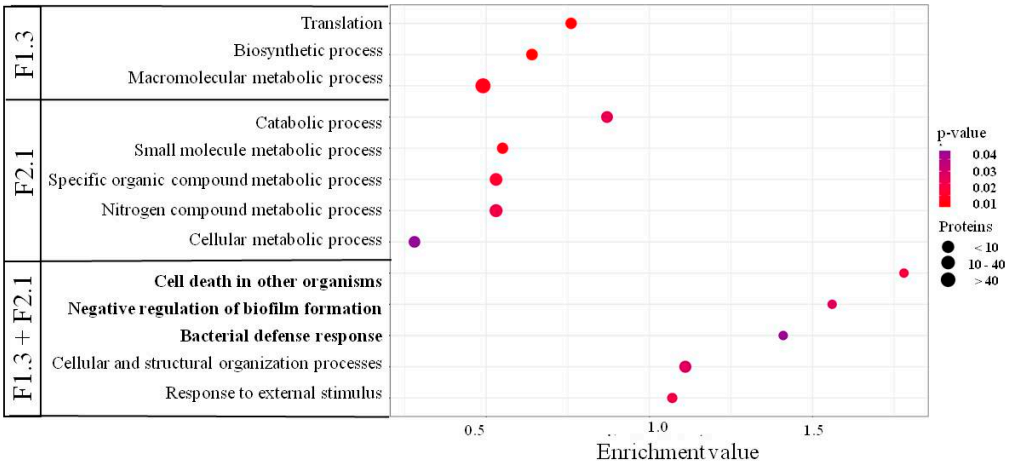

Figure 7. Bubble chart showing the most enriched biological processes. The distribution of points reflects variability in enrichment values and in the number of proteins involved. Colors represent statistical significance (Benjamini-Hochberg correction), with redder colors indicating greater significance. Point size corresponds to the number of proteins associated with each process, with larger points indicating higher representation (SRplot). The three processes highlighted in bold correspond to biological functions associated with antimicrobial activity, each involving three proteins.

2.5. Protein Profiling of Selected Subfractions by SDS-PAGE and Proteomic Analysis of High-Abundance Bands

Once a general analysis of the extracts had been completed and the proteins with potential antimicrobial activity had been identified, a one-dimensional electrophoresis (SDS-PAGE) was performed to gain a deeper and more quantitative understanding of the proteins present in each subfraction. This approach made it possible to examine the size of the most abundant proteins and to obtain a more detailed quantitative profile of the secretome. In subfractions F1.3 and F2.1, two

 doi:10.20944/preprints202604.1158.v1

denatured protein bands with the same molecular weight were detected: approximately 200 kDa (A) and 95 kDa (B), as shown in Figure 8. F1.3 subfraction also displayed an additional band of distinct size intense band of approximately 72 kDa (C) was observed (Figure 8).

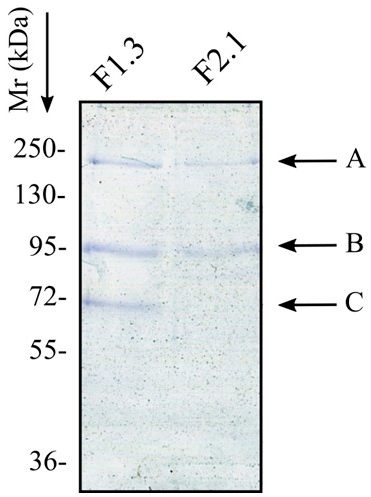

Figure 8. SDS-PAGE (8%) protein profile of M. mediterranea secretome subfractions F1.3 and F2.1. The first lane corresponds to subfraction F1.3, whereas the second lane corresponds to subfraction F2.1. Arrows indicate the most abundant protein bands that were manually exised for subsequent indentification by LC-MS/MS. Molecular weight standards (PageRuler Plus) are indicated in kDa on the left.

The proteins corresponding to the most abundant bands (A, B, and C) were identified by LC-MS/MS. The proteins that showed high false discovery rates (FDR) in each of the bands were analyzed. In band A, six proteins were identified, primarily associated with cellular metabolism, stimulus responses, and antagonistic activity, with LodA standing out due to its potential involvement in antimicrobial activity. Band B contained nine proteins that shared these functional categories but also included proteins linked to broader regulatory mechanisms. Among the proteins associated with antagonistic activity were LodA, GoxA, and ValRS. Band C contained six proteins with functional profiles similar to those in band A, with LodA as the predominant protein. The theoretical molecular masses of LodA, GoxA, and ValRS were 80.8, 76.2, and 75.4 kDa, respectively, consistent with the estimated range of the excited bands.

3. Discussion

The marine bacterium *Marinomonas mediterranea* has previously been reported to produce inhibitory compounds active against a broad range of Gram-positive (*Staphylococcus aureus*, *Bacillus* sp., *Streptomyces antibioticus*, *Streptococcus agalactiae* and *Enterococcus faecalis*) and Gram-negative bacteria (*E. coli*, *Vibrio cholerae*, *V. parahemolyticus*, *V. harveyi* and *Pseudomonas* sp.) [38,40]. Until now, however, antifungal activity had not been documented (no effect on the growth of *Candida utilis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Penicillium chrysogenum*) [38]. In this study, we observed for the first that bioactive compounds secreted by *M. mediterranea* inhibit clinically important fungal species (*S. boydii* and *L. prolificans*), in addition to displaying antibacterial activity against additional bacterial pathogens that had not been previously tested (*K. pneumoniae, A. baumannii* and *P. aeruginosa*).

Among the known antibacterial compounds produced by this bacterium are lysine oxidase (LodA) [36] and glycine oxidase (GoxA) [37], which are secreted during the stationary or senescence phase [38,41,44]. These oxidases account for the H_2O_2-mediated antibacterial activity. This study aimed to identify the antimicrobial compounds present in the secretome of the MMB-1^T strain (*M. mediterranea* CECT 4803^T) and to determine whether these bioactive components exhibit a broader spectrum of activity than previously reported or represent novel antimicrobial agents. To address

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this, we evaluated the secretome under different culture media (Marine Broth, MB; half-diluted Marine Broth, dMB; and Bushnell Haas Broth supplemented with glucose and NaCl, BHB), concentrations and processing conditions to identify those that best revealed antimicrobial activity. MB consistently yielded the strongest activity, likely because its higher amino acid content promotes bacterial growth and increases the availability of L-lysine and L-glycine, which are required for LodA and GoxA activity [41,42,44,45]. This contrasts with previous reports in which minimal media were optimal [38], highlighting the importance of both nutrient composition and culture duration in modulating secretome activity.

Based on our results, concentrating the secretome 7- to 9.5-fold was sufficient to achieve maximal inhibitory activity under MB conditions, and higher concentration levels (14-fold) did not improve inhibition; therefore, for reasons of methodological sustainability, secretomes were concentrated within this range. Activity was generally stronger against Gram-positive bacteria than against Gram-negative bacteria or fungi. This variability likely reflects differences in membrane composition (particularly unsaturated fatty acids in membrane lipids [46]) and antioxidant defenses, including catalase and superoxide dismutase, which mitigate H_2O_2 effects [47–49]. Indeed, the addition of catalase abolished antimicrobial activity, confirming that H_2O_2 generated by LodA and GoxA is a major contributor to inhibition against both bacteria and fungi. These findings confirm the antibacterial activity of *M. mediterranea* compounds and reveal, for the first time, their antifungal potential. The lack of detectable activity against *C. auris* and *A. fumigatus* suggests that these species may possess strong specific resistance mechanisms, possibly linked to stronger oxidative stress defenses or distinct cell wall and membrane properties [48–50].

HPLC fractionation showed that antimicrobial activity was restricted to specific molecular-weight ranges (F1.3 (586.7 - 284.9 kDa) and F2.1 (284.9 - 138.4 kDa)). Subfraction F1.3 exhibited both antibacterial and antifungal activity, whereas F2.1 lacked detectable antifungal effect. Mass spectrometry confirmed the presence of LodA and GoxA in both active subfractions. The weaker activity of F2.1 may simply reflect a lower abundance of active proteins compared with F1.3. In addition, ValRS was identified as a potential novel antimicrobial protein, supporting previous genomic predictions [51], although its specific activity requires further investigation. The absence of detectable R-type bacteriocin activity likely reflects that these phage-derived structures are not expressed constitutively and often require induction by stress or DNA-damaging agents, conditions that were not reproduced under these laboratory settings. In *M. mediterranea*, R-type bacteriocin release has been observed only after mitomycin-induced prophage activation [39], which further supports the idea that its expression may remain silent during standard cultivation.

Bioinformatic analysis of proteins in active subfractions revealed enrichment in biological processes related to metabolism, translation and antimicrobial responses, including cell death in other organisms, negative regulation of biofilm formation, and bacterial defense. Proteins shared between subfractions were mainly linked to antagonistic interactions, suggesting a central role in microbial inhibition.

The proteomic analysis of the most abundant protein bands in the secretome of *M. mediterranea* revealed a consistent presence of proteins associated with metabolic functions, external stimulus response, and antimicrobial activity, with LodA emerging as a recurrent and predominant component across all bands. The identification of LodA and GoxA, with theoretical molecular masses consistent with previously reported values (80.9 kDa for LodA [40] and 76.2 kDa for GoxA [37]), together with ValRS, whose predicted molecular mass is 75.4 kDa, was particularly notable in band B, where these proteins are associated with antimicrobial defense responses. These findings suggest that the antimicrobial activity of the secretome may result from the combined action of well-characterized enzymes and additional proteins with potential bioactive roles, supporting the possibility that *M. mediterranea* exhibits a broader antimicrobial spectrum than previously recognized.

Overall, the results highlight the strong antimicrobial potential of *M. mediterranea* secreted proteins, especially when the bacterium is grown in amino acid-rich media. The same proteins

 doi:10.20944/preprints202604.1158.v1

responsible for antibacterial effects also contributed to antifungal activity, expanding the spectrum of inhibitory compounds produced by this marine bacterium. Compared to many marine-derived antimicrobials that require extensive purification, the activity of the *M. mediterranea* secretome highlights its potential as a readily exploitable antimicrobial system. The inhibitory activity observed against pathogens such as *A. baumannii, P. aeruginosa* or *S. boydii* is particularly relevant, given their high intrinsic resistance to many conventional antimicrobials.

This study underscores the biotechnological potential of marine-derived antimicrobials and supports further exploration of marine bacterial secretomes as alternative therapeutic agents against multidrug-resistant pathogens. Future work should address the specific activity, interactions, and minimum inhibitory concentrations of these proteins, as well as clarify the contribution of valyl-tRNA synthetase to the observed antimicrobial effects. Although this enzyme was detected in active subfractions, it remains to be determined whether valyl-tRNA synthetase exerts direct growth-inhibitory effects or acts indirectly by modulating the oxidative antimicrobial system. Altogether, this work broadens the antimicrobial spectrum attributed to *M. mediterranea* and reinforces the idea that marine bacterial secretomes, especially those enriched in oxidative enzymes, represent a promising and still underexplored source of new antimicrobial strategies.

4. Materials and Methods

4.1. Maintenance and Cultivation of Bacteria and Fungi

To assess antibacterial activity, six clinically relevant bacterial strains from the Spanish Type Culture Collection (CECT) were used as reference microorganisms: *Enterococcus faecalis* CECT 481 (Gram-positive), *Staphylococcus aureus* CECT 240 (Gram-positive), *Klebsiella pneumoniae* CECT 143T (Gram-negative), *Acinetobacter baumannii* CECT 9111 (Gram-negative), *Pseudomonas aeruginosa* CECT 108 (Gram-negative), and *Escherichia coli* CECT 101 (Gram-negative).

For antifungal activity, the following reference strains were employed: *Scedosporium boydii* CBS 116995 (Centraalbureau voor Schimmelcultures), *Lomentospora prolificans* CECT 20842, *Candidozyma auris* CECT 13225, and *Aspergillus fumigatus* Af293.

All cultures were stored at 4 ºC in slant agar tubes until use: Marine Agar for *M. mediterranea*, Nutrient Agar for bacterial strains, and Sabouraud Agar for fungal strains. For antimicrobial activity assays, fresh cultures were prepared 24 - 48 h prior to testing in their respective media. Reference bacteria and fungi were incubated at 37 ºC, whereas *M. mediterranea* was grown at 25 ºC. All cultures were kept in darkness and without agitation.

4.2. Secretome Preparation from Marinomonas Mediterranea Grown in Different Culture Media

To obtain the secretome, understood as the set of proteins released into the extracellular medium, *M. mediterranea* was cultured in three different media: Marine Broth (MB), half-diluted Marine Broth (dMB), and the minimal medium Bushnell Haas Broth (BHB) supplemented with glucose (2 g/L) as a carbon source and NaCl (20 g/L) to achieve optimal salinity.

M. mediterranea was inoculated into 250 mL flasks containing MB, dMB or BHB at an initial density of 10^6 cells/mL and incubated at 25 ºC with shaking (90 rpm) in darkness for 260 - 300 h (stationary/senescence phase). Subsequently, cultures were centrifuged at 6000 rpm for 20 min, and the supernatants were filtered through 0.2 µm polycarbonate filters (47 mm diameter, Whatman) to remove bacterial cells.

The resulting sterile secretome solution was divided into two portions: half of the total volume was concentrated by ultrafiltration (4000 rpm) using a membrane with a molecular weight cut-off of 5000 MWCO (Vivaspin 20, Sartorius), while the remaining half was kept unconcentrated. Concentrated secretomes were collected at different concentration factors (×2 to ×14) to determine the levels at which inhibition of clinically relevant microorganisms was strongest (Figure 9).

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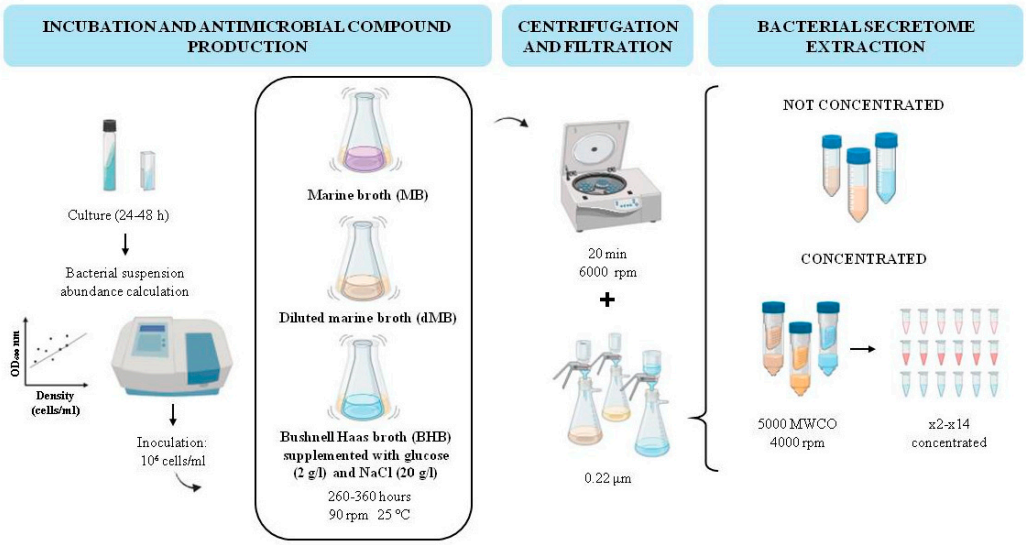

Figure 9. Experimental scheme for the preparation of the secretome of *Marinomonas mediterranea*. Image created with BioRender.

4.3. Evaluation of the Antimicrobial Activity of Secretomes of M. mediterranea Grown in Different Culture Media

The antimicrobial activity of concentrated and unconcentrated secretomes obtained from cultures in MB, dMB and BHB was assessed using the well-diffusion method [52].

Reference microorganisms were grown 24 - 48 h before testing in the corresponding media described above. Active cultures were inoculated onto Petri dishes containing Mueller-Hinton agar (MH), the standard medium recommended for antimicrobial susceptibility testing [53].

To ensure uniform growth, bacterial strains were seeded onto MH using sterile swabs dipped in suspensions adjusted to an optical density of 0.5 at 600 nm (OD_{600} = 0.5). Fungal strains were seeded directly onto MH from recent cultures using sterile swabs (Figure 9).

After the inoculum was absorbed, wells of 0.8 cm in diameter were made using the base of sterile pipette tips. Into each well, 150 µL of the sterile secretome solution corresponding to each medium, either concentrated or unconcentrated, was added. Between three and nine replicates of the procedure were performed. Petri dishes were incubated at 37 ºC for 24 - 48 h with the lid facing upward. After 24 h, an additional 150 µL of sterile secretome was added to wells in plates seeded with fungal strains. Antimicrobial activity was quantified by measuring the diameter of the inhibition halos (Figure 10).

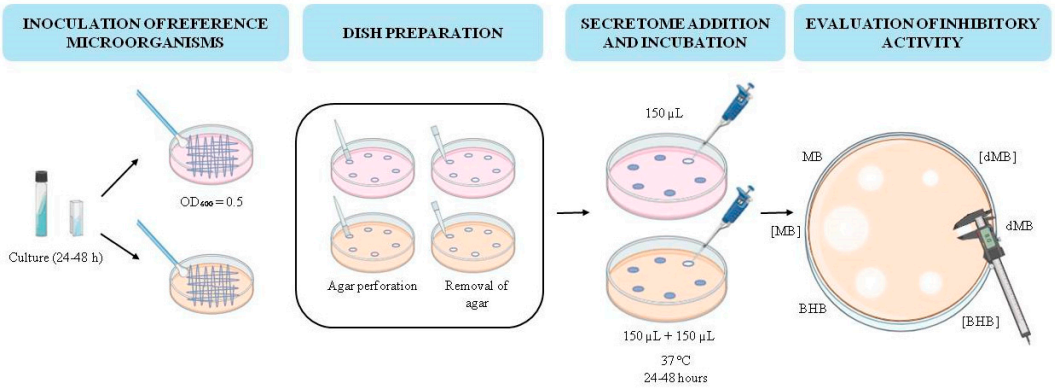

Figure 10. Experimental scheme for the evaluation of inhibitory activity of (unconcentrated or [concentrated]) secretomes obtained from Marinomonas mediterranea cultured in different media: Marine Broth (MB); hald-diluted Marine Broth (dMB) and Bushnell Haas Broth supplemented with glucose and NaCl (BHB) Reference

 doi:10.20944/preprints202604.1158.v1

bacteria were seeded at an inoculum density of OD600 = 0.5, while fungi were inoculated directly from recent cultures. Image created with BioRender.

4.4. Detection of Antimicrobial Activity Associated with Hydrogen Peroxide (H_2O_2) Production

To determine whether antimicrobial activity was linked to H_2O_2 production, 10 µL of a catalase solution (0.1 mg/mL; Sigma-Aldrich E3289) was added to wells containing concentrated secretome from each culture medium [40]. If an inhibition halo appeared in the absence of catalase but not after catalase treatment, the inhibitory effect was attributed to H_2O_2 production.

4.5. High-Performance Liquid Chromatography (HPLC) Fractionation of the Sterile Secretome of Marinomonas Mediterranea

To identify proteins with antimicrobial properties, a conventional effect-directed analysis (EDA) approach was adapted from a previous work [53]. This technique allowed simplification of the complexity of the secretome, which contains diverse proteins, including those with potential antimicrobial activity. Traditionally, EDA has been applied to identify toxic compounds in environmental samples. In the conventional process, an environmental sample is first analyzed using toxicity bioassays. If toxicity is detected, the sample is fractionated according to different physicochemical properties to reduce complexity. Each fraction is subsequently subjected to the same bioassays, and toxic fractions are chemically analyzed.

In this study, the methodology was adapted to identify proteins with antimicrobial activity in the sterile exudates of *M. mediterranea*. Only the medium that exhibited the highest antimicrobial activity was subjected to fractionation and secretome characterization.

Once the antimicrobial activity of the secretomes had been confirmed using the well-diffusion method, fractionation of proteins by size range was performed from the concentrated solution using HPLC. A size-exclusion column (Yarra™ SEC-2000, 300 x 7.8 mm, 3 µm) was employed, coupled to a diode array detector and an automated fraction collector (Agilent 1260 Infinity II, Santa Clara, CA, USA). Elution was carried out under isocratic flow conditions with a buffer solution containing 100 mM phosphate and 150 mM NaCl at pH 6.8, at a flow rate of 1 mL/min for 18 min [55,56].

From the concentrated secretome solution, five fractions with different molecular size ranges were obtained (F1: 2487.8 - 284.9 kDa; F2: 284.9 - 32.6 kDa; F3: 32.6 - 3.7 kDa; F4: 3.74 - 0.43 kDa; F5: 0.43 - 0.05 kDa). Each fraction was subsequently evaluated for antimicrobial activity against the reference microorganisms following the procedure described above (see Section 4.3).

For comparative analysis, both the obtained fractions and the initial concentrated secretome solution were tested, the latter serving as a control. Since the secretome was diluted in the mobile phase during fractionation, each fraction was reconcentrated to its original concentration prior to testing. To assess whether the fractionation procedure resulted in any loss of activity, the antimicrobial activity of a blank (distilled water) and a combined fraction (mixture of all fractions) was also analyzed.

Further fractionation was performed on those fractions that exhibited inhibitory activity, yielding subfractions with smaller molecular size ranges than the initial ones (F1.1: 2487.8 - 1208.1 kDa; F1.2: 1208.1 - 586.7 kDa; F1.3: 586.7 - 284.9 kDa; F2.1: 284.9 - 138.4 kDa; F2.2: 138.4 - 67.2 kDa; F2.3: 67.2 - 32.6 kDa). This procedure allowed a higher resolution in the separation of proteins. As in the previous case, the antimicrobial activity of these subfractions was evaluated against the reference microorganisms, and 3 to 8 replicates of the procedure were performed.

4.6. Proteomic Analysis: Identification of Proteins Present in the M. mediterranea Secretome Subfractions by Liquid Chromatography-Rhigh Resolution Mass Spectrometry (LC-HRMS)

Once the subfractions with antimicrobial activity had been identified, they were characterized by ultra-high-performance liquid chromatography (EASY-nLC 1200, Thermo Scientific) coupled to

 doi:10.20944/preprints202604.1158.v1

LC-HRMS (Orbitrap Exploris 480, Thermo Scientific) to identify proteins or peptides responsible for antimicrobial activity.

For proteomic analysis, 200 µL of each subfraction was precipitated using the 2D Clean-Up kit (Cytiva). The protein pellet was solubilized in Laemmli sample buffer and loaded onto a 4 - 20% Tris-glycine gel (Bio-Rad). After brief separation, the gel was stained with GelCode Blue Safe Protein Stain (Thermo Scientific), and selected bands were manually excised for in-gel digestion with modifications [57]. Proteins were reduced with DTT, alkylated with iodoacetamide, and digested with trypsin (Roche Diagnostics). Peptides were extracted with NH_4HCO_3/acetonitrile and trifluoroacetic acid/acetonitrile, dried in a SpeedVac (Thermo Fisher Scientific), and desalted using Empore C18 Stage Tips (CDS Analytical).

Mass spectrometric analysis was performed using an EASY-nLC 1200 system coupled to an Exploris 480 mass spectrometer (Thermo Scientific). Peptides were separated on an Acclaim PepMap column and analyzed in positive ion mode with higher-energy C-trap dissociation. Data acquisition was performed using Xcalibur software (Thermo Scientific).

Data were processed with Proteome Discoverer 2.2 (Thermo Scientific) and searched against the UniProtKB database of *M. mediterranea*. Mass tolerances were set at 10 ppm for precursors and 0.02 Da for fragments, allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation and N-terminal acetylation were considered variable modifications. A false discovery rate of 1% was applied for both peptides and proteins.

4.7. Bioinformatic Analysis of Subfractions of Interest

Bioinformatic analysis of the compounds obtained from the *M. mediterranea* secretome included a general description, identification of the most enriched proteins, and evaluation of proteins of interest with potential antimicrobial activity. InterPro and STRING databases were used.

Proteins identified by LC-HRMS were filtered to reduce complexity by removing contaminants, single-peptide identifications, and proteins with score 0. Filtered extracts were grouped according to proteins common to all fractions or specific to individual extracts. Each group was analyzed separately, considering InterPro GO terms with a significance threshold of $p < 0.05$ (Benjamini-Hochberg correction).

Biological processes associated with each protein were identified using STRING (accessed September 2024), based on detected proteins compared with expected proteins for each process. Comparative analyses and enrichment plots were generated using the SRplot web platform (www.bioinformatics.com.cn).

4.8. Analysis of the Size of the most Abundant Proteins in the M. mediterranea Secretome by Electrophoresis and Identification of the Bands of Interest by LC-HRMS

To characterize the most abundant proteins in the *M. mediterranea* secretome, a one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 8% acrylamide gels. A total of 60 µL of secretome subfractions were loaded per lane, and proteins were separated at 200 V, 100 W and 70 mA for 45 min using a Mini-Protean II system (Bio-Rad). A molecular weight marker (PageRuler Plus, Thermo Fisher Scientific) was included for size estimation. Gels were stained with Coomassie Brilliant Blue R to visualize protein bands of interest and digitalized with ImageScanner III (GE Healthcare, Freiburg, Germany) [58].

For protein identification, samples were run again under the same electrophoretic conditions and stained with Coomassie Brilliant Blue G-250 to ensure compatibility with downstream analysis. Selected bands were excised manually, destained, and stored at -20 ºC until processing. The most abundant proteins in each band were subsequently identified by LC-HRMS [58].

Selected SDS-PAGE bands were subjected to in-gel digestion according to the procedure described in [55], with minor modifications. Gel pieces were reduced with 10 mM DTT and alkylated with 25 mM iodoacetamide, then digested overnight at 37 ºC with proteomics-grade trypsin. Peptides

 doi:10.20944/preprints202604.1158.v1

were extracted with ammonium bicarbonate/acetonitrile and 0.1% TFA/acetonitrile, pooled, dried, and desalted using C18 StageTips.

Peptide mixtures were analyzed by nanoLC-HRMS using an EASY-nLC 1200 system coupled to an Exploris 240 mass spectrometer (Thermo Scientific). Peptides were separated on an Acclaim PepMap RSLC analytical column using a 62-min acetonitrile gradient at 300 nL min^{-1}. Full MS scans were acquired at 120,000 resolution, and HCD MS/MS spectra at 15,000 resolution. Data acquisition was performed in positive ion mode using Xcalibur software.

Raw data were processed with Proteome Discoverer 2.2 and searched against a *Marinomonas mediterranea* UniProtKB database (2024_03). Searches allowed two missed cleavages, with 10 ppm precursor and 0.02 Da fragment tolerances. Carbamidomethyl-Cys was set as a fixed modification, and Met oxidation and N-terminal acetylation as variable modifications. Peptide and protein FDR thresholds were set at 1%.

4.9. Statistical Analysis of Antimicrobial Activity

Comparisons between the mean diameters of inhibition halos for the evaluation of antimicrobial activity against bacteria and fungi were performed using SPSS Statistics 29.0.2.0. Statistically significant differences were assessed between inhibition halos produced by inhibitory compounds present in the different culture media, both concentrated and unconcentrated extracts. In addition, secretomes with higher production of bioactive compounds were compared by evaluating inhibition halos generated against different reference microorganisms and against groups of microorganisms classified according to cell wall structure.

A coefficient of variation of 20% was used as the criterion for reproducibility. For comparisons involving more than one mean of independent samples, analysis of variance (ANOVA) was applied to normally distributed data, followed by Bonferroni post hoc tests (for equal variances) or Dunnett's T3 test (for unequal variances). For non-normally distributed data, the non-parametric Kruskal-Wallis test was used. Differences were considered statistically significant at $p < 0.05$.

5. Conclusions

This study demonstrates the antimicrobial potential of compounds secreted by *Marinomonas mediterranea*, identifying nutrient- and amino acid-rich Marine Broth as effective condition for their production. Under these conditions, the resulting secretome showed stronger inhibitory activity against Gram-positive bacteria and variable activity against Gram-negative bacteria and clinically relevant fungi, primarily associated with the presence of oxidative enzymes capable of generating hydrogen peroxide.

Proteomic analyses confirmed the involvement of lysine oxidase LodA and the glycine oxidase GoxA, and additional secreted proteins, including valyl-tRNA synthetase, were identified as potential contributors to the observed antimicrobial effects.

These findings highlight marine bacterial secretomes as promising and still underexplored sources of bioactive compounds with activity against multidrug-resistant pathogens. Future research should focus on defining the specific contribution and mechanism of action of individual proteins, determining their minimum inhibitory concentrations, and clarifying the potential role of valyl-tRNA synthetase within antimicrobial system, thereby advancing the development of novel marine-derived antimicrobial strategies.

Author Contributions: Conceptualization, I.A. and Z.B.; methodology, O.S., A.H.F., A.Z.E., M.S. and O.R.; validation, N.E, A.R., I.A and Z.B.; formal analysis, O.S., I.A and Z.B.; investigation, O.S., I.A and Z.B.; resources, N.E, A.R., I.A and Z.B.; data curation, O.S., I.A and Z.B.; writing-original draft preparation, O.S.; writing-review, editing and supervision, I.A. and Z.B.; project administration and funding acquisition, I.A., Z.B. and N.E. All authors have read and agreed to the published version of the manuscript.

 doi:10.20944/preprints202604.1158.v1

Funding: This research was supported by the MIMAS Consolidated Research Group, recognized and funded by the Basque Government (IT1657-22), and by the Research Group grants from the University of the Basque Country (GIU17/43). Additional support was provided by the Basque Government through the Nitrify (00004-INA2022-33) and Nitribac (00004-2101022023) projects. This study also forms part of the TED2021-132109B-C21 research project, funded by MCIN/AEI/10.13039/501100011033, Spain, and the European Union NextGenerationEU/PRTR.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this article. Additional information is available from the corresponding author upon reasonable request.

Acknowledgments: Mass spectrometry analysis was performed in the Proteomics Core Facility-SGIKER at the University of the Basque Country.

Conflicts of Interest: The authors have declared no conflict of interest.

Abbreviations

BHB	Bushnell Haas Broth supplemented with glucose and NaCl
CBS	Centraalbureau voor Schimmelcultures
CECT	Spanish Type Culture Collection
dMB	Half-diluted Marine Broth
EDA	Effect-directed analysis
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.),
GoxA	Glycine oxidase
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid Chromatography
LC-HRMS	Liquid Chromatography-rhigh resolution mass spectrometry
LodA	Lysine oxidase
MB	Marine Broth
MH	Mueller Hinton medium
MRSA	Methicillin-resistant *Staphylococcus aureus*
MWCO	Molecular weight cut-off
OD	Optical density
SDS-PAGE	One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WHO	World Health Organization

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