Chapter 21

Compounds from Natural Sources for New Diagnostics and Drugs against Biofilm Infections

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Additional information is available at the end of the chapter

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Abstract

Biofilm infections represent a new medical challenge that drives towards the discovery of new diagnostics and new drugs specifically designed for this purpose. All living organisms offer a huge source of compounds which represent the biochemical substrate of the biological competition on the Earth and can be used to this aim. We describe an innovative diagnostic tool to early diagnose medical device infections sustained by Staphylococci; then we list new compounds that modulate bacterial phenotype and reduce virulence without affecting bacterial viability so as to avoid the emergence of genetic resistances. These compounds are all derived from natural sources: prokaryotes, plants, and human body. From prokaryotes we studied new compounds extracted from different environmental bacterial species, including Antarctic species growing in extreme environments. We describe also the anti-biofilm properties of extracts obtained from plants well known since centuries in folk medicine. The humoral immune response is the source of the last anti-biofilm compound: transferrin (Tf), a protein derived from human plasma involved in inflammation and natural immunity. All these compounds can be used as scaffolds for the design of new drugs active on the sessile form of pathogens prevalent in human biofilm infections.

Keywords: human, diseases, infection, therapy, diagnosis

1. Introduction

Since 1929, the discovery of penicillin radically changed the history of human infection diseases, opening the path to the discovery of a wide array of new antibacterial compounds. After a few decades, the appearance of genotypic resistances undermined the dream of definitive human victory on infectious diseases. In the 1970s, some unexplainable cases of drug resistance sustained

by strains prone to conventional antibiotics were attributed to biofilm. At the beginning, sessile phenotype has been related only to medical devices infections, but as long as knowledge on biofilm behavior increased, the majority of chronic human infections have been attributed to sessile phenotype. It has been demonstrated that sessile bacteria resist to antibiotics due to a variety of causes including, a reduced penetration of drugs in the deep layers of biofilm, a favored diffusion of resistance genes, a drift of bacterial metabolism towards anaerobiosis causing a reduced cellular division, and a dramatic reduction of susceptibility to drugs. Efforts have been devoted to restore sensitivity of sessile bacteria to antibiotics and to identify new compounds to treat biofilm. In this chapter, we describe our efforts in this field. Since the early 1990s, our lab dedicated many efforts to identify new diagnostics and new therapeutical strategies to counteract biofilm infections in humans. We shall first describe a diagnostic tool based on enzyme-linked immunosorbent assay (ELISA) technique to early identify Staphylococcal colonization of medical devices, then we shall list an array of different compounds active on bacterial biofilms.

2. An ELISA assays for early diagnosis of biofilm infections

Specimen culturing is the gold standard for diagnosing bacterial infections, but growing bacteria from a biofilm is not reliable. Other testing modalities, such as polymerase chain reaction Polymerase chain reaction (PCR) and serology assays are nonspecific for biofilm infections and include the risk of contamination during sampling. In recent years, many attempts have been performed to create new for early, noninvasive diagnosis. A main feature of infections on implanted medical devices is the absence or paucity of local signs and general symptoms of infection/inflammation. The first signs are due to dysfunction of the device itself (loosening of orthopedic prostheses, cardiac valve regurgitation) and device-related damage to the surrounding tissues. Prompt detection of biofilm infection in the initial asymptomatic phases can allow earlier medical/surgical treatment. Highly virulent organisms (e.g., *Staphylococcus aureus* and gram-negative bacilli) induce early appearance of symptoms. Less virulent bacteria (e.g., coagulase-negative staphylococci) usually induce low-grade, indolent infections that remain clinically silent for years. Laboratory tests suggesting inflammation and possible infection by a bacterial pathogen (i.e., C-reactive protein, Complete blood counts, erythrocyte sedimentation rate) do not demonstrate the presence of bacteria and can mislead. An interesting alternative is based on the use of immunodiagnostics detecting antibodies directed against antigens that are specific for the sessile form of bacterial species, especially those that are traditionally considered saprophytes. Since staphylococci are highly prevalent in biofilm infections on medical devices, we developed a simple and reliable immunodiagnostic assay devoted to the diagnosis of staphylococcal biofilm infections [1] based on enzyme-linked immunosorbent assay (ELISA), a simple, rapid, and repeatable technology, that do not require removal of the implanted device. Our ELISA test allows to detect antigen-bound immunoglobulins with peroxidase-conjugated antibodies against human immunoglobulin G (IgG) or immunoglobulin M (IgM), and expresses antibody titers as units of optical density. The assay was tested in patients with late-onset infections involving synthetic vascular grafts

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(LO-SVGI). Antibodies (IgG and IgM) against staphylococcal slime polysaccharide antigens (SSPA) were titrated in sera collected from 38 patients with active LO-SVGIs caused by different staphylococcal species (group A). For control purposes, assays were performed on sera from 10 patients with active LO-SVGIs caused by bacteria other than staphylococcus (group B); 16 healthy patients with histories of staphylococcal LO-SVGI that had been eradicated 6–72 months earlier by means of graft replacement (group C); 17 healthy patients with synthetic vascular grafts and no evidence of current or past graft infections (group D); and 58 healthy subjects with no implanted medical devices or prostheses of any type (group E). Infections were classified as late onset only when first manifestations occurred 2 years or more after implantation of the vascular graft. All infections (ongoing and past) were microbiologically confirmed based on cultures of the explanted graft. The results of this study are summarized in **Table 1** and **Figure 1**.

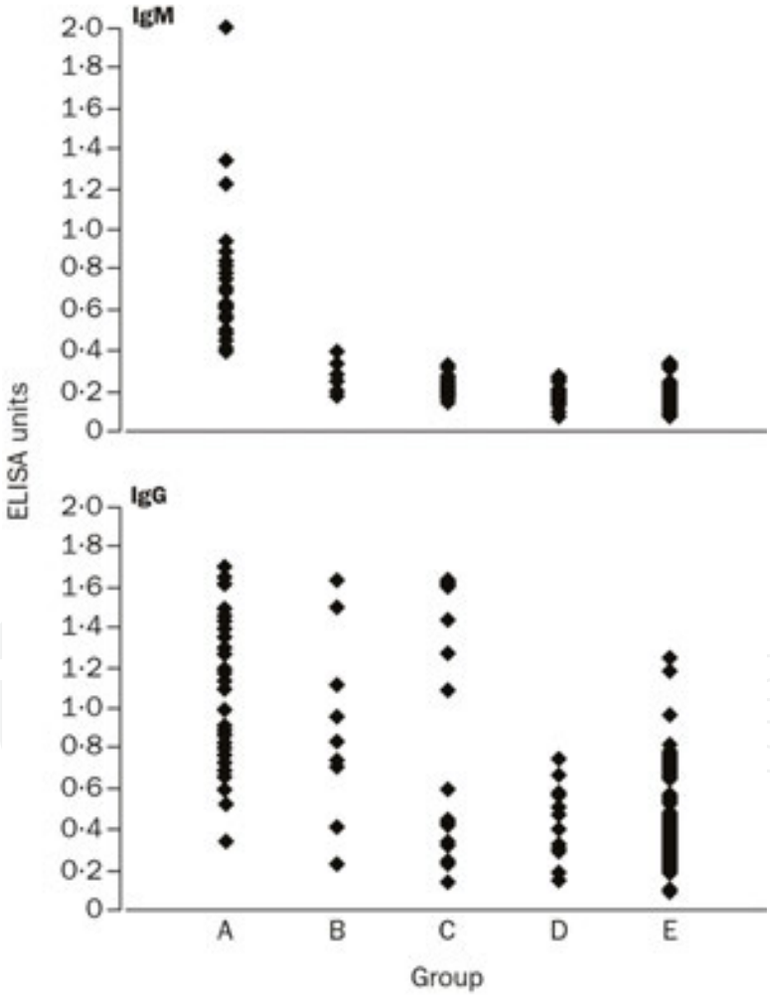

Figure 1. IgM and IgG antibody titres against SSPA. Group A = individuals with staphylococcal LO-SVGI; group B = individuals with non-staphylococcal LO-SVGI; group C = individuals with previous history of staphylococcal LO-SVGI, with successful graft replacement; group D = individuals with synthetic vascular graft implanted 14–78 months before study entry and no previous history of graft infections; group E = individuals free of prosthetic devices.

	Group					
	A (n = 38)	**B (n = 10)**	**C (n = 16)**	**D (n = 17)**	**E (n = 58)**	**B+C+D+E (n = 101)**
IgM titre (mean, SD) (EU)	0.69 (0.37)	0.28 (0.07)	0.26 (0.06)	0.18 (0.05)	0.17 (0.06)	0.19 (0.08)
IgG titre (mean, SD) (EU)	1.13 (0.36)	0.86 (0.46)	0.82 (0.57)	0.46 (0.27)	0.55 (0.26)	0.60 (0.37)
Positive tests for an IgM titre (number, %) (EU)						
≥0.35	38 (100%)	2 (20%)	0	0	0	2 (2%)
≥0.40	37 (97%)	0	0	0	0	0

Group A = patients with an ongoing LO-SVGI caused by *Staphylococcus epidermidis* (n = 6), *S. aureus* (n = 2), coagulase-negative staphylococci other than *S. epidermidis* (n = 24), and mixed infection by one more staphylococcal species and *enterococci, Pseudomonas aeruginosa*, or *Escherichia coli* (n = 6); group B = patients with LO-SVGI caused by bacteria other than staphylococcus—*P. aeruginosa* (n = 2), mixed infections by gram-negative bacilli (*Pseudomonas* spp. or Enterobacteriaceae; n = 5), mixed infection by *Enterococcus* spp and *Enterobacter cloacae* (n = 1), mixed fungal-bacterial infections (*Candida albicans* and *Enterococcus* spp. or *P. aeruginosa*) (n = 2); group C = healthy patients with a history of staphylococcal LO-SVGI, followed by successful graft replacement; group D = healthy patients with a synthetic vascular graft, with no previous history of graft infections; group E = patients with no prosthetic device.

Table 1. Comparison of titres of IgM and IgG antibodies against staphylococcal slime polysaccharide antigens (SSPA) (ELISA units [EU]) in sera from patients with an ongoing staphylococcal late-onset infection of synthetic vascular graft (LO-SVGI) and in controls.

The highest titers of IgG antibody to SSPA were noted in individuals with ongoing staphylococcal LO-SVGIs (group A). However, high titers were also seen in the control groups, which precluded the use of IgG titers for diagnostic purposes. In contrast, titers of IgM antibodies against SSPA were higher in the group A patients. There was virtually no overlap between the titers of these patients and those of controls. IgM antibody of 0.4 ELISA units (EU) or more indicated ongoing staphylococcal LO-SVGIs with detection rates of 97% and no false positives. When a cutoff of 0.35 ELISA units was used, the detection rate increased to 100%, but the false-positive rate also rose to 2%. The substantial difference observed between patients with ongoing versus previous staphylococcal LO-SVGIs (group A vs. group C) suggested that levels of IgM antibody against SSPA decrease rapidly after successful graft substitution. Recurrence of graft infection was associated with the return of elevated IgM antibody titers. We concluded that SSPA ELISA positivity can be used as a marker of active staphylococcal graft infections. Anti-SSPA ELISA was also tested for the diagnosis of orthopedic joint prosthetic infections (DOJP-Is). To this aim, we compared the titers of IgM antibodies against SSPA in the sera of 90 subjects [2]. Studied population included 29 subjects with ongoing staphylococcal DOJP-Is (group A), 34 subjects with orthopedic joint prostheses implanted at least 1 year previously without infection (group B), and 27 subjects not previously operated for orthopedic implants, attending the hospital for noninfectious diseases (group C). All subjects in group A underwent surgical removal of the infected prosthesis, and staphylococcal infection had been microbiologically confirmed by intraoperative cultures. For orthopedic applications, we adopted a cutoff value of 0.35 EU. The main results, summarized in **Table 2**, show that high anti-SSPA IgM levels may provide for noninvasive detection of the immune response elicited by biofilm colonization on artificial orthopedic implants. We did

not evaluate IgG titers, because they were not associated with current infection both in the previous study on vascular grafts and in a preliminary analysis on five cases and eight controls. According to these results, we can affirm that anti-SSPA ELISA assay is effective in detecting antibodies in DOJP-Is caused by different staphylococcal species.

Group (no. of samples)	Mean (SD) IgM titre (EU)	% (no.) of positive tests for an IgM titre:	
		≥0.35 EU	≥0.40 EU
Prosthesis infection[a] (29)	0.72 (0.55)	89.7 (26)	69.0 (20)
Prosthesis, no infection[a] (34)	0.21 (0.09)[b]	8.8 (3)	5.9 (2)
No Prosthesis, no infection (27)	0.20 (0.05)[b]	0 (0)	0 (0)
All controls (61)	0.21 (0.07)[b]	4.9 (3)	3.3 (2)

[a] Prosthesis infection is defined by subjects with an ongoing prosthetic infection caused by *Staphylococcus epidermidis* (n = 15), *Staphylococcus aureus* (n = 8), coagulase-negative staphylococci other than *S. epidermidis* (n = 2), and mixed infection by one or more staphylococcal species plus enterococci, *Pseudomonas aeruginosa*, or *Escherichia coli* (n = 4).
[b] $P<0.001$ versus infected subjects (Kruskal-Wallis test).

Table 2. Comparison of titers of IgM antibodies against SSPA, expressed as ELISA units, in sera from subjects with an ongoing staphylococcal late-onset infection of orthopedic prostheses and in controls.

Periodic testing for IgM antibodies against SSPA could prove useful in the follow-up of patients with implanted vascular and orthopedic devices. The SSPA ELISA displays a strong advantage over other available methods used to diagnose biofilm-related infections, in fact it is versatile because it can detect antibodies in biofilm infections caused by different staphylococcal species. The higher diagnostic value of the IgM titers depends on the choice of the antigen: a mixture of purified polysaccharide antigens extracted from the biofilm matrix. Polysaccharide antigens are known to elicit a thymus-independent humoral response based exclusively on IgM production. This response is maintained as long as the antigenic stimulus is present, and there is no shift to IgG production. IgMs elicited by antigens of this type are synthesized by a particular subpopulation of B lymphocytes (B1): blood IgM + IgD + CD27 + cells that correspond to splenic marginal zone B cells. Because of the absence of thymic involvement and IgG production, these responses are regarded as expressions of innate immunity. The peculiar behavior of the immune response to polysaccharide antigens represents a diagnostic advantage since it can be used at any time to evaluate the possibility of device infection, even during the post-replacement follow-up. The interest in the development of alternative anti-infective approaches for the prevention and treatment of staphylococcal infections has increased in recent years [3–5]. But our group has been working on the search of new compounds active as anti-biofilm drugs since the early 1990s [6].

2.1. New compounds from prokaryotes for the therapy of biofilm infections.

Our first attempt, for the search of new anti-biofilm drugs, was based on the clinical observation that the administration of proteolytic enzymes can enhance therapeutic outcomes in the treatment of contact lens and endo-ocular prosthetic devices infections. The hypothesis was

that proteolytic treatment could damage the proteic structure of biofilm matrix reverting bacteria to a condition of susceptibility to antibiotics. In our first study [6] four different proteases were tested on 10 bacterial strains (5 *Staphylococcus epidermidis* and 5 *Pseudomonas aeruginosa*) with ofloxacin (dilution range, 200–0.1 mcg/ml): clostridiopeptidase A, fibrinolysin, streptokinase, and serratiopeptidase. Experiments were been performed in both planktonic and sessile form, the last one based on the colonization of polystyrene beads maintained in constant flow culture. Results showed a strong anti-biofilm activity of serratiopeptidase as demonstrated by bioluminescence count of adherent bacterial cells. A further confirmation was obtained by scanning electron microscope (SEM) image of the surface of polystyrene beads incubated for 5 days in a culture of *P. aeruginosa* containing ofloxacin alone (**Figure 2**) or ofloxacin and serratiopeptidase (**Figure 3**).

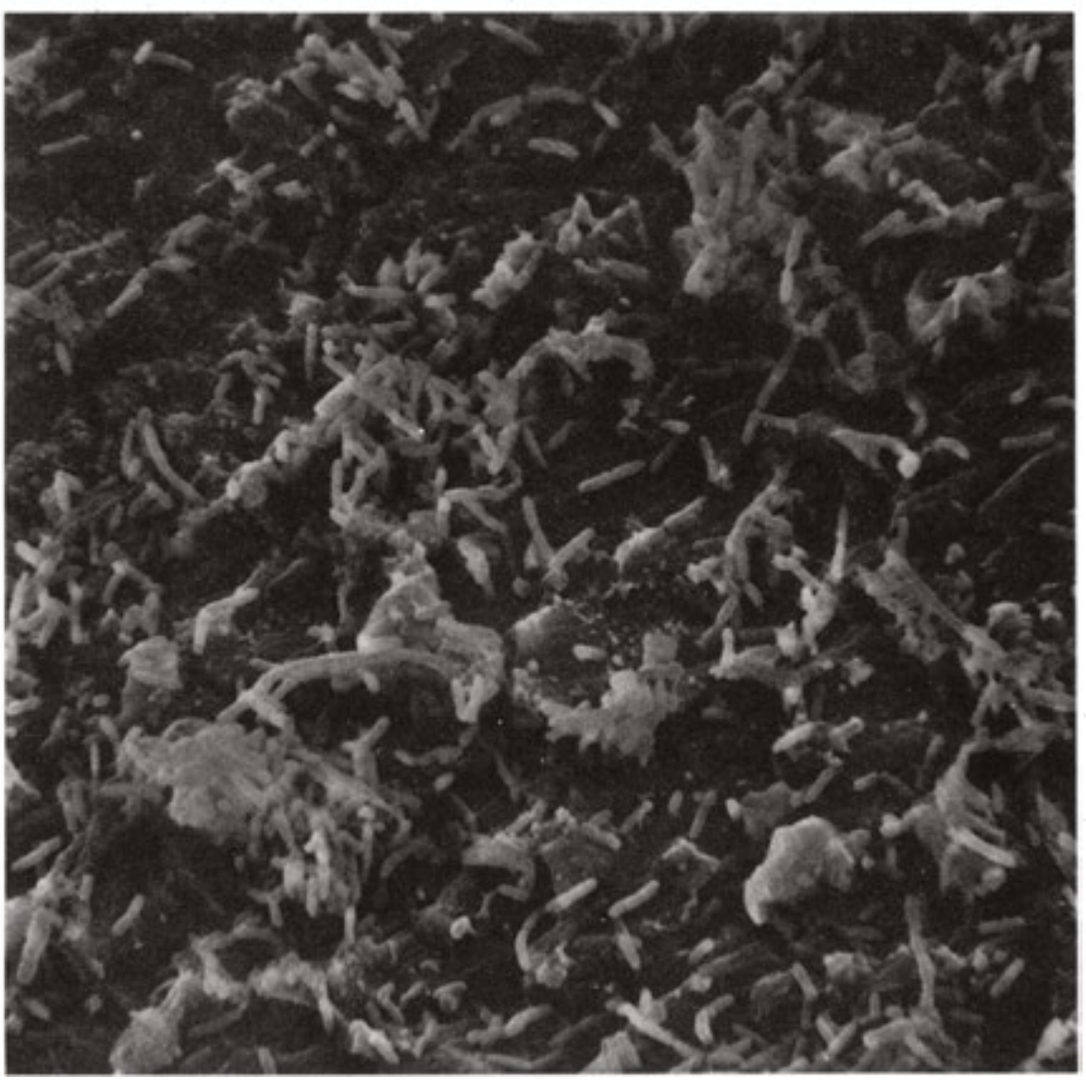

Figure 2. SEM image of a polystyrene bead incubated for 5 days at 37°C in a culture of *P. aeruginosa* in TSB with ofloxacin at a concentration equal to MIC under planktonic growth condition (0.78 mcg/ml) at a flow rate of 120 ml/h. Magnification × 1550.

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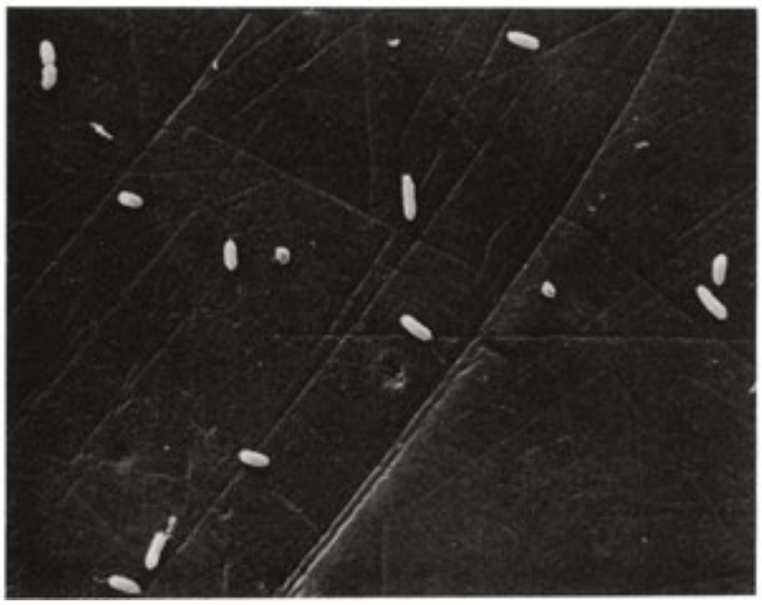

Figure 3. SEM image of a polystyrene bead incubated for 5 days at 37°C in a culture of *P. aeruginosa* in TSB with ofloxacin at a concentration equal to MIC under planktonic growth condition (0.78 mcg/ml) and serratiopeptidase at a concentration of 10 U/ml at a flow rate of 120 ml/h. Magnification × 2220.

Serratiopeptidase is a metalloprotease (containing zinc) cloned from *S. marcescens*, showing a strong proteolytic activity and widely used in therapy for decades for its anti-inflammatory properties and for its ability to enhance the penetration of antibiotics in the site of infection. We further studied serratiopeptidase (SPEP) trying to understand its mechanism of action on a molecular level. To this aim, our group examined the effect of two families of proteases on *S. aureus* and *S. epidermidis* strains. In particular, we used three serine proteases (proteinase-K, PK; trypsin, TRY; chymotrypsin, CHY) and two metalloproteases (SPEP; carboxypeptidase-A, CpA) in biofilm formation assays and in human cell invasion processes (invasion only for *S. aureus*) [4]. The study was intended also at obtaining a broader knowledge on the possible use of proteases as anti-adhesive molecules whose use could be proposed in combination therapy with antibiotics. SPEP seems the most promising molecule to be developed as a novel anti-virulence tool. Its action selectively affects a discrete number of proteins clearly involved in fundamental mechanisms associated with bacterial virulence, such as adhesion, invasion, and biofilm formation and would thus hinder staphylococcal virulence properties. Adhesion of bacterial cells and formation of biofilm are finely tuned in staphylococci by an interplay of adhesins including sialoprotein binding proteins (SdrC), fibrinogen binding proteins (FnBP-A/B, Embp, and ClfA), biofilm-associated protein (Aap and Bap), extracellular matrix binding proteins (SasG), autolysins (Alt and AtlE), proteins involved in polysaccharide intracellular adhesin (PIA) synthesis (IcaADBC), and others [7]. In order to ascertain the presence of genes that code for proteins that modulate adhesion and biofilm formation the studied strains were studied by PCR.

We found no relationship between bacterial gene profile and proteases activity. Cell surface protein samples from treated and untreated cultures of all staphylococcal strains were

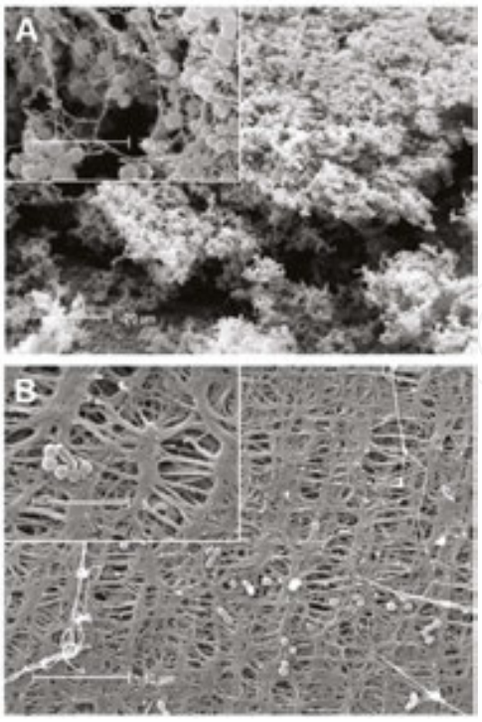

Figure 4. Scanning electron microscopy analysis of *S. aureus* 6538P grown overnight in BHI in presence of a PFTE filter as a substrate for sessile growth in the absence (A), or in the presence (B) of SPEP. In the magnification box of panel A, the extracellular matrix is visible and biofilm-embedded bacteria cover the filter surface. In panel B, the PTFE filter background is visible and only single cells are present.

simultaneously analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The ability of each protease to interfere with *S. aureus* capacity to adhere and invade human cells was tested by antibiotic protection assay on HeLa cell line. We found that only SPEP and CpA had a comparable action on all tested strains; on the contrary all tested serine proteases showed different behavior on each bacterial strain suggesting a nonspecific and indiscriminate effect of these proteases. For this reason, we focused on SPEP and CpA action on surface proteins. SPEP treatment resulted in an effective and broad-spectrum reduction of biofilm formation and its action appears to be more selective, sequence-specific [8, 9] and proportional to biofilm production. Antibiotic protection assay performed on HeLa cells showed that SPEP treatment strongly impaired *S. aureus* invasion efficiency. This is similar to what found by our group for *L. monocytogenes* [10], thus confirming the broad spectrum of this protease also against virulence properties different from biofilm formation. The action of CpA results in an increase of biofilm accumulation, while the action of SPEP impairs surface adhesins/autolysins and probably impairs also the adhesive moieties of the altruistic suicidal cells. The altruistic suicide mechanism was found to be responsible for the lysis of a bacterial subfraction in *S. aureus* biofilm [11]. SPEP and CpA are able to act both on surface adhesins and on the lysed cellular debris derived from the suicidal subpopulation. It is important to underline that SPEP neither

influences bacterial viability when used at the concentrations adopted in this work and at higher concentrations, nor displays a cytotoxic effect on eukaryotic cell lines. Based on these results, confirmed by the images obtained in SEM [12] (**Figure 4**), we focused on the molecular aspects of SPEP action on *S. aureus*, a bacterial pathogen often associated with nosocomial and community-acquired infections, capable to express a multiplicity of virulence factors secreted or associated to bacterial cell surface; these factors include bacterial products that mediate adhesion to the surface of host cells and to damaged tissues. It has been ascertained that in *S. aureus* surface proteins play a fundamental role in virulence properties, including biofilm formation.

A successful strategy to hinder bacterial infection should not affect processes essential for bacterial survival in order to avoid the rapid appearance of escape mutants. A smarter approach should target the main virulence factors of *S. aureus* and avoid interferences on the viability of bacteria.

	Biofilm formation	
	Control	SPEP-treated
6538P	1.64 ± 0.15	0.086 ± 0.015
25923	1.68 ± 0.23	0.63 ± 0.12
12598	0.35 ± 0.05	0.21 ± 0.03
BAA1556	2.15 ± 0.08	0.47 ± 0.04

Based on the 590 nm OD absorbance produced by *S. aureus* strains. Data represent the mean ± SD of three independent experiments.

Table 3. Effect of SPEP treatment on staphylococcal biofilm formation.

Two steps lead to biofilm formation are adherence to a surface by bacterial cells and progressive growth of cell clusters in multilayers. The study of the factors that gather cells into a biofilm has evidenced the existence of strains producing either polysaccharide intercellular adhesion/poly-N-acetylglucosamine (PIA/PNAG) or a protein-dependent biofilm, even if in staphylococci the best-known biofilm mechanism depends on the production of PIA/PNAG, an extracellular polysaccharide adhesin [13, 14]. It has been shown that, besides their best-known role in the eukaryotic invasion process, fibronectin-binding proteins (FnBPs) play a relevant role in biofilm-associated foreign-body infections. Some proteins are multifunctional factors involved in metabolic pathways, in adhesion to extracellular matrix and invasion of host cells, such serine-aspartate repeat-containing protein D (SdrD), Elongation factor-Tu (EF-Tu), Elongation factor-G (EF-G), Atl, SsA2, and second immunoglobulin-binding protein (Sbi). In order to understand the molecular mechanism of SPEP action on *S. aureus* we confirmed its action on biofilm growth and studied the proteomic patterns of treated and untreated bacterial cells. Experiments were performed on four *S. aureus* strains: ATCC 6538P (DSMZ 346), reference strain for antimicrobial testing; ATCC 25923 (DSMZ 1104); ATCC 12598 (DSMZ 20372); ATCC BAA1556 (FPR3757 strain) is an USA 300 strain [15]. The biofilm-forming ability

was tested by quantitative assay. They showed different capabilities to form biofilm: three strains were strong biofilm producers with biofilm amount higher than 1.6 OD at 590 nm, while ATCC 12598 is a medium biofilm producer. Biofilm quantification was performed in Static biofilm assay according to Christensen [16] and in dynamic condition (BioFlux 2000 microfluidics system) which allows the acquisition of microscopic images over time. Results of SPEP effect on biofilm formation of four *S. aureus* strains are summarized in **Table 3**. We found also that SPEP is also extremely effective in the dispersal of *S. aureus* preformed biofilm suggesting that SPEP affects also mature biofilm.

Experiments in BioFlux system, performed on MSSA 6538P and MRSA BAA1556, showed that SPEP clearly impairs biofilm formation as already seen in static system. The SPEP action on the proteome of tested strains was assessed on surface proteins extracted according to the method of Tabouret [17]. Extracted proteins were separated on SDS-PAGE. Many specific protein bands detected in the untreated *S. aureus* protein profiles either disappeared or drastically diminished in intensity after SPEP incubation. This effect was clearly visible for all bacterial strains analyzed, and peptide mixtures obtained by in situ digestion were analyzed by MALDI-TOF Mass Spectrometry; when necessary, peptide mixtures were analyzed by LCMS/MS using a 4000Q-Trap coupled to an 1100 nano HPLC system. Analysis of the results showed the disappearance of specific proteins after SPEP treatment, including some surface proteins that mediate adhesion and invasion in eukaryotic cells, such as SsA2, SdrD, Atl, and Sbi, homolog to SpA. Notably, treatment with serratiopeptidase influenced the expression of cytoplasmic proteins involved in carbohydrate metabolism (in particular, proteins of the glycolytic pathway) and in energy production. EF-G, EF-Tu, and the dihydrolipoyl transacetylase, an enzyme component of the multienzyme pyruvate dehydrogenase complex [18], are further examples of factors participating in energetic metabolic pathways that are also involved in adhesion and invasion in eukaryotic cells. Investigation of the surface subproteome of *Listeria monocytogenes* studied previously, revealed a remarkably high number of proteins with a function in the cytoplasmic compartment. Many reports suggest that anchorless proteins of the bacterial surface promote bacterial adhesion and invasion of eukaryotic cells [19]. Proteins may perform different functions that depend on other proteins they can transiently associate with. As a consequence, proteins involved in bacterial metabolism are not only involved in energy production, playing an alternative role on bacterial cell surface, but also they may facilitate efficient invasion of eukaryotic cells. Some proteins of the adhesion family, alkyltransferase-like (ATL) protein, Sbi, Elongation Factor-Tu, Elongation Factor-G, and Serine-aspartate repeat-containing protein D, deserve particular attention. Atl disappeared after SPEP treatment, confirming that SPEP modulates adhesins and autolysins in *S. aureus*. Recently, a novel mechanism involved in staphylococcal internalization by host cells, which is mediated by the major autolysin/adhesins Atl in *S. aureus* has been described [20]. A microbial surface components recognizing adhesive matrix molecule (MSCRAMMS) member acting as a homologous to SpA is Sbi; this multifunctional protein binds host complement components Factor H and C3, IgG and b2-glycoprotein I and hinders innate immune recognition. Sbi inhibits both complement activation and lyses of rabbit erythrocytes mediated by the alternative pathway of human serum [21]. SdrD, a MSCRAMM family surface protein, plays an important role in *S. aureus* adhesion and patho-

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genesis. The crystal structure of the domains of this protein has been elucidated and the ligand-binding site of SdrD was characterized. EF-Tu and EF-G, belonging to the so-called 'moonlight' proteins, perform different functions unrelated to splice variants, gene fusions, and generation of proteolytic fragments. Elongation Factor-Tu is considered as a main factor associated to cell wall in most bacterial species; among them *S. aureus* [18], *M. leprae* [23], *L. johnsonii* [22], M. pneumoniae, where it mediates fibronectin binding together with the pyruvate dehydrogenase E1 subunit [24]. In *L. monocytogenes*, EF-Tu was identified together with EF-G. Recently, EF-Tu has been identified as a surface protein possessing the characteristics of an adhesion factor and showing the capacity to induce a proinflammatory response. We used a methicillin-sensible *S. aureus* strain (6538P) and a methicillin-resistant strain (BAA1556) to assess serratiopeptidase influence on their ability to adhere and invade eukaryotic cells. Adhesion and invasion efficiency after serratiopeptidase treatment is shown in **Table 4**. The test was performed according to the antibiotic protection assay. According to our experimental data serratiopeptidase does not affect adhesion efficiency of 6538P while, as regards BAA1556, the adhesion efficiency was partially upset. About the invasion, expressed as the percent of the adhered bacteria which invaded HeLa cells, our data showed that about 50% of the BAA1556 adhering to HeLa cells invaded them. Even if methicillin-sensible strains are scarcely invasive, SPEP induced a 200-fold reduction of its invasion efficiency; furthermore, methicillin-resistant strain (BAA1556) showed a drastic reduction of invasion efficiency (3000-fold) after treatment with serratiopeptidase.

	Untreated		**SPEP-treated**	
	Adhesion [a]	**Invasion** [b]	**Adhesion** [a]	**Invasion** [b]
6538P	2.75 ± 0.45	0.7 × 10–2 ± 0.1 × 10–2	2.80 ± 1.80	0.3 × 10–4 ± 0.0 × 10–4
BAA1556	4.94 ± 0.97	2.5 ± 0.79	2.82 ± 1.46	0.7 × 10–3 ± 0.1 × 10–3

[a] Adhesion is expressed as the percentage of the initial inoculum of bacteria that adhered to HeLa cells 1 h post-infection at 37°C.
[b] Invasion efficiency is expressed as the percentage of adhered bacteria that were gentamicin-resistant 1 h post-infection. Data represent the mean ± SD of three independent experiments.

Table 4. Adhesion and invasion capabilities of SPEP-treated and untreated *S. aureus*.

It is not yet clear if serratiopeptidase action is mediated by its proteolytic function or to the activation of specific signal transduction pathways that regulate expression of proteins.

In the last years, our group started working also on compounds obtained from marine bacteria. Marine bacteria from Antarctica represent an untapped reservoir of biodiversity and produce several compounds which may be of potential biotechnological interest; culture supernatants derived from most of them have been shown to exhibit anti-biofilm activity against both gram-positive and gram-negative bacteria, including *Acinetobacter, S. aureus, Salmonella typhimurium, Shigella sonnei, Listeria monocytogenes*, and several *Bacillus* species [25].

Marine bacteria belonging to the genus *Pseudoalteromonas* produce compounds of biotechnological interest, including anti-biofilm molecules [26]. Starting from these considerations, we tested on different staphylococcal strains the anti-biofilm activity of cell-free supernatant of Antarctic marine bacterium *P. haloplanktis* TAC125 grown in planktonic and sessile form [27, 28]. Our results show that *S. epidermidis* biofilm growth is inhibited only by *P. haloplanktis* TAC125 supernatant grown in static condition. A possible explanation for this result can be found in the peculiar biofilm microenvironment that lead to production of specific metabolites or polymers due to metabolic rewiring of sessile bacteria [29] that could inhibit growth of other microorganisms. In accordance with this hypothesis, many anti-biofilm compounds were identified from cultured biofilms [25]. It is interesting to note that previously characterized anti-biofilm compounds have often a broad-spectrum biofilm inhibition activity [25, 26, 30] while the *P. haloplanktis* TAC125 anti-biofilm molecule seemed to be species-specific. *P. haloplanktis* TAC125 supernatant was, in fact, inactive against biofilm of *S. aureus* and *P. aeruginosa*. The majority of the anti-biofilm compounds described in literature so far also exert an antibacterial activity. *P. haloplanktis* TAC125 supernatant does not show antibacterial activity against planktonic forms, while its action is directed against sessile forms possibly mediated by mechanisms other than growth inhibition. Only few natural molecules display this mode of action. Three hypothetical modes of action could be proposed. (i) anti-biofilm compounds could be surfactants acting on physical characteristics of biotic and abiotic surfaces; (ii) they could act by competitive inhibition of multivalent carbohydrate-protein interactions [31]. Thus, the anti-biofilm compound might block lectins or sugar-binding proteins present on the surface of bacteria, or block tip adhesins of fimbriae and pili [32]; (iii) *P. haloplanktis* TAC125 anti-biofilm compound might act as a signaling molecule that modulates the gene expression of recipient bacteria [33]. Indeed, bacterial communication is one of the regulatory mechanisms suggested to be involved in biofilm formation [34]. The results of the initial attachment assay indicated that the cultured supernatant-inhibited biofilm formation by contrasting the initial attachment of bacterial cells to the surface. *P. haloplanktis* TAC125 anti-biofilm compound affects mature biofilm but its action requires a long time. This observation suggests a mechanism of action mediated by a signaling system that down-regulates adhesive properties of biofilm matrix and bacterial cell surface rather than by surfactant activity that, on the contrary, would rapidly carry on its action. Different systems have been described so far for bacterial communication. A well-characterized system (acting for intra- and interspecies communication) is based on the autoinducer-2 (AI-2) produced by *luxS* gene, as a signaling molecule [35]. *LuxS* has also been identified in *S. epidermidis* [36], but *P. haloplanktis* TAC125 genome analysis revealed that the Antarctic bacterium is devoid of *luxS* gene [37] suggesting the anti-biofilm activity could be due to a not identified signaling molecule. The anti-biofilm effects of *P. haloplanktis* exoproducts could be due to a novel molecule or the synergistic actions of different molecules. The *P. haloplanktis* TAC125 anti-biofilm molecule was active against several *S. epidermidis* strains, among others it was effective on the clinical isolate O-47 which is a naturally occurring *agr* mutant [33], but it was inactive on *S. epidermidis* XX-17 *ica* mutant [38].

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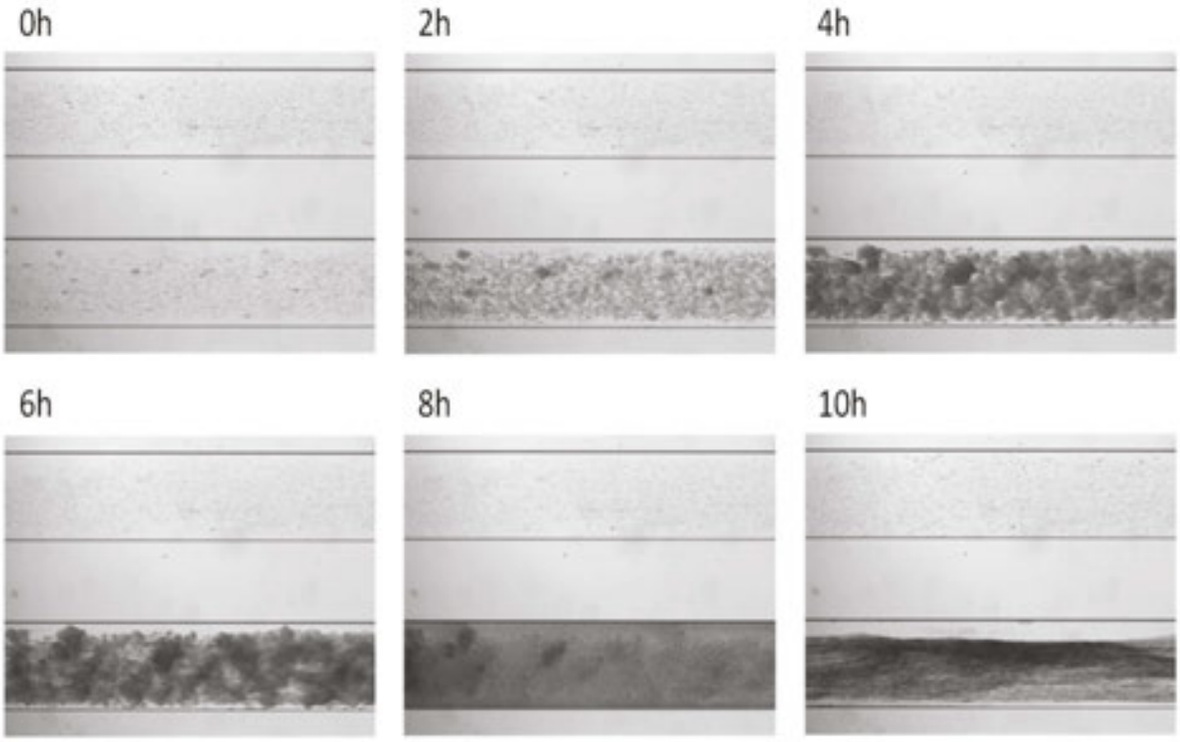

Figure 5. Biofilm formation of *S. epidermidis* O-47 in a BioFlux system. Each image contains two channels: top channel was SN-treated sample and bottom channel was the control one. Bright-field microscopic images were collected at 1-min intervals. The images presented were taken from the complete set of 720 images (see supplementary video bioflux for a video compilation of these images) taken at 40 × magnification.

The efficacy of *P. haloplanktis* supernatant was also confirmed on *S. epidermidis* biofilm formation in dynamic condition using BioFlux system (**Figure 5**). Our results demonstrated that in dynamic condition *P. haloplanktis* TAC125 anti-biofilm was effective against *S. epidermidis* biofilm formation. The use of *P. haloplanktis* TAC125 anti-biofilm molecule/s in combination therapy with antibiotics during persistent infection by staphylococci could be proposed. Furthermore, we evaluated the anti-biofilm activity of supernatants derived from cultures of several cold-adapted bacteria belonging to *Pseudoalteromonas*, *Psychrobacter*, and *Psychromonas* genera. Supernatants were obtained from bacterial cultures made both in sessile and planktonic conditions. The potential anti-biofilm activity was tested on bacterial cultures of *P. aeruginosa* PAO1, three different strains of *S. aureus*, and three different strains of *S. epidermidis* species [39]. In these species, the matrix composition, the molecules involved in attachment, development, detachment phases, and the regulation of quorum-sensing systems are different [40, 41]. Several cold-adapted bacteria produce molecules that are able to interfere with *S. aureus* biofilm formation. These molecules seem to be proteinaceous. On the contrary, only few Polar strains produce anti-biofilm molecules active on *S. epidermidis* O-47 and RP62A biofilms, and none were able to interfere with *S. epidermidis* XX-17 biofilm formation. It is important to underline that XX-17 strain produces a biofilm characterized by a polysaccharide ica-independent poorly characterized so far. Moreover, the six staphylococcal strains considered here were previously investigated to assess the presence of genes coding for

various proteins involved in adhesion and biofilm formation [4]. In *P. aeruginosa* the biofilm matrix is totally different, because the bacterium produces three exopolysaccharides, the glucose-rich Pel polysaccharide [42], the mannose-rich Psl polysaccharide [42], and alginate [43]. We used *P. aeruginosa* the reference strain PAO1 since the biofilm characterization of this strain was previously reported [44]. The reported differences in biofilm features of the three pathogens could explain the different ability of cold-adapted bacteria supernatants to impair their biofilm formation. It is interesting to note that, in all reported cases, the supernatants proved to be non-biocidal and specifically directed against biofilm. All studied Polar strains were able to produce anti-biofilm molecules against *P. aeruginosa* biofilm. Furthermore in almost all cases, the anti-biofilm molecules seemed to have the same chemical-physical features (were not heat-labile and seem to have a non-protein nature). These results could suggest that the molecule responsible for the anti-biofilm activity is the same for all cold-adapted strains, in particular could be polysaccharides or a small molecule acting as quorum sensing inhibitor. The ability of cold-adapted marine bacteria to produce several anti-biofilm molecules could suggest that the capacity to prevent the biofilm and colonization by bacterial competitors is a selective advantage in this extreme environment.

2.2. New compounds from Eukaryotes for the therapy of biofilm infections.

2.2.1. Compounds from plants.

Considering that plants have already yielded compounds with inhibiting activities against gram-positive bacteria [45], and that the use of medicinal and herbal remedies to treat infectious diseases is common in many countries [46], we have also attempted the discovery of new leads from plants. We explored several plant extracts searching for specific antibacterial activity from fractionated pools [47]. We considered extracts from *Krameria, Aesculus hippocastanum*, and *Chelidonium majus* plants; these plants have proved to possess a plethora of active principles in diverse pathologies. From active fractions, we purified and identified antimicrobial compounds. We identified and purified a dihydroxybenzofuran (DHBF) derivative that could be used as a possible active molecule from fractions of compounds extracted from the *Krameria lappacea* (Dombey) Burdet (para or Brazilian rhatany) and tested in microbiological assays. Our results demonstrated that extracts obtained from *C. majus* are the most active in a screening study [48], it has been shown that crude extracts *C. majus* exhibited antibacterial activity against *S. aureus* ATCC 25923 [49]. From the preliminary microbiological assays probing fractions of compounds extracted from *Aesculus hippocastanum* and *C. majus*, we identified and successively isolated the proanthocyanidin (proAc) from the former plant and the cheliritrin (CH) and the sanguinarin (SA) alkaloids from the latter plant to study their antimicrobial activity. All compounds are described in **Figure 6**. We tested bacteriostatic and bactericidal action on the planktonic form and quantified the efficacy on inhibition of biofilm formation and growth on *S. aureus* and *S. epidermidis*. We also presented proteomic evidence of the alteration of bacterial surface proteome. Both DHBF and SA had a similar marked bactericidal effect. Interestingly, both DHBF and SA were able to inhibit biofilm accumulation in *S. aureus* at concentrations between 1.4 to six-fold lower than those corresponding to MIC/MBC, **Figure 7** and **Table 5**. The inhibition data were interpreted using a

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Hill-type equation [50], **Figure 7**, where EC50 (the effective concentration at which 50% inhibition of biofilm formation is observed) is obtained with a fitting procedure. Experiments performed with sub-MIC concentration demonstrate that studied compounds had not antimicrobial activity at sub-MIC concentration. Therefore, we speculated that these compounds can inhibit biofilm formation but do not kill bacterial cells in the planktonic form. Their action should be rather based on the prevention of *S. aureus* transition to the sessile phenotype. On mature biofilm both DHBF and SA were not effective. Both SA and DHBF exhibited an inhibitory activity of de novo biofilm formation in *S. epidermidis* at a concentration about two-fold lower than the MIC and MBC range. SA also showed an inhibitory activity on the mature biofilm, DHBF showed no such activity at all. CH and proAc did not possess bactericidal activity. On the contrary, planktonic growth of both *S. aureus* and *S. epidermidis* was inhibited by CH (MIC 16–32 μM), while proAc did not show bacteriostatic activity (**Table 5**). However, biofilm growth of *S. aureus* and *S. epidermidis* was inhibited by both compounds. The proAc seemed to be the best inhibitor performing similarly on both strains, CH inhibited biofilm formation better in *S. epidermidis* than in *S. aureus*. While CH and proAc were good inhibitors of the de novo biofilm formation they displayed no meaningful inhibitory activity on the mature biofilm.

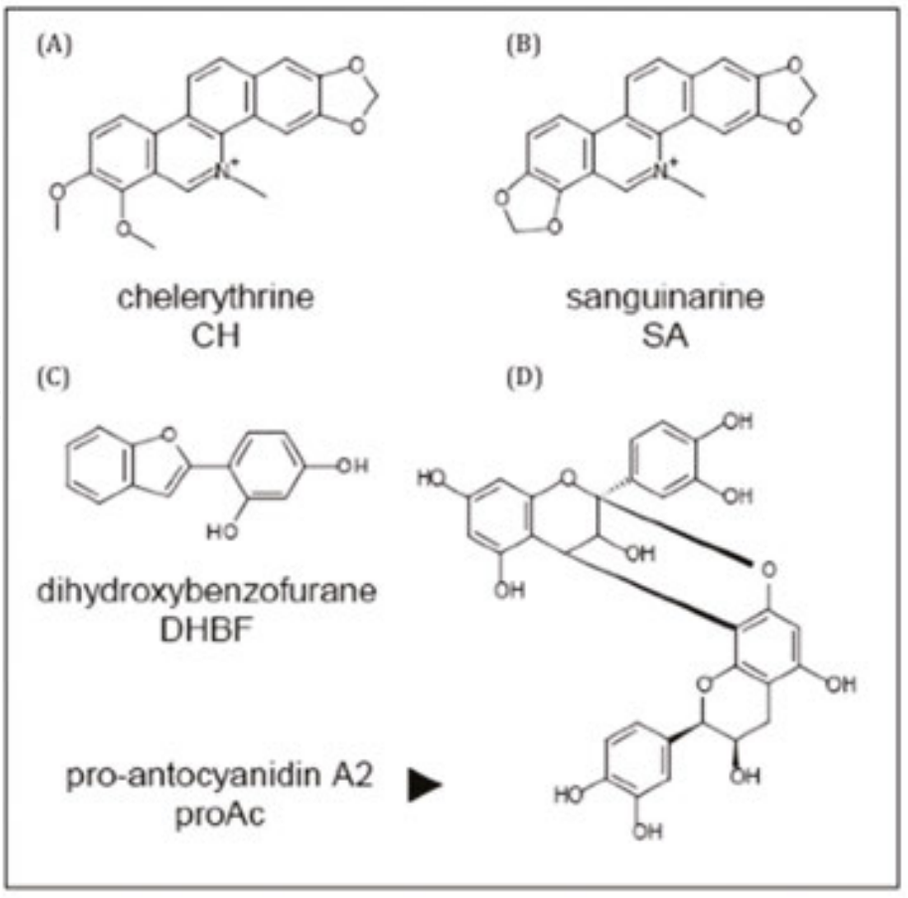

Figure 6. Structural formulas of the inhibitors used in this study, the positive charges in CH and SA are neutralized by chloride ion.

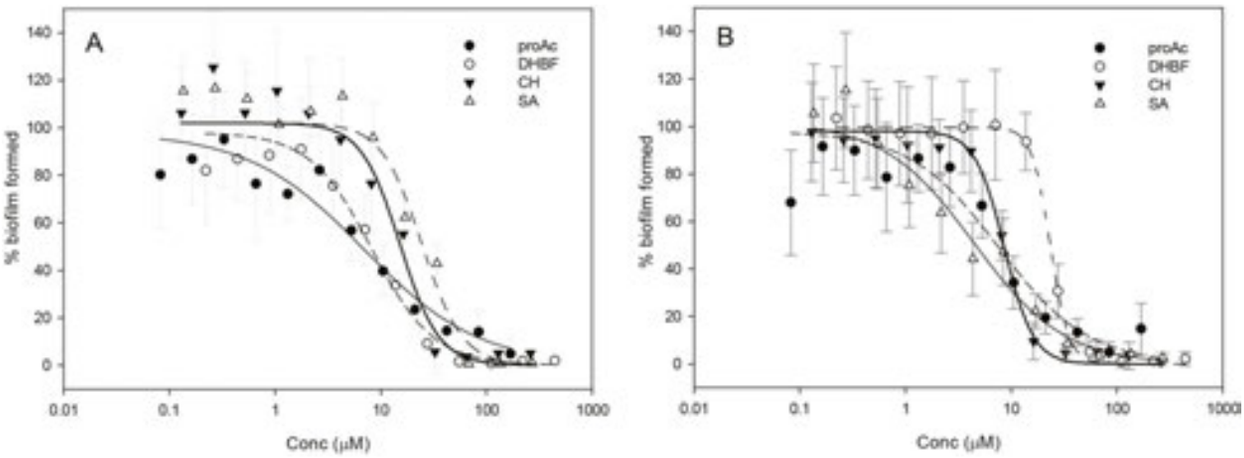

Figure 7. Inhibitory action of proAc, DHBF, CH, and SA, exerted on the ex-novo biofilm formation on *S. aureus* 6538P (panel A) and on *S. epidermidis* RP62A (panel B). Standard deviations from four replicates are represented as error bars in dark gray. The fitting curves shown on the curves were obtained with a single-step inhibition model and fitted with the equation "f={%biofilm formation / [1 + (C/EC50)^slope]}"; where C is the actual concentration of the inhibitor.

MW	Compound	*S. aureus* 6538P				*S. epidermidis* RP62A			
		M.I.C. [μM] [a]	M.B.C. [μM] [a]	EC50 (μM) [b]	slope	M.I.C. [μM] [a]	M.B.C. [μM] [a]	EC50 (μM)	slope
383.8	**CH**	16.3; 32.6	>260	15.2 ± 2.3	2.4 ± 0.7	16.3; 32.6	>260	8.6 ± 0.4	3.1 ± 0.5
367.8	**SA**	34.0; 68.0	34.0; 68.0	24.5 ± 3.6	2.3 ± 0.7	8.5; 17.0	34.0; 68.0	4.4 ± 1.3	1.0 ± 0.2
226.2	**DHBF**	55.3; 110.6	55.3; 110.6	8.2 ± 1.2	1.5 ± 0.3	55.3; 110.6	55.3; 110.6	23.5 ± 0.6	4.9 ± 0.6
592.6	**proAC**	>168.7	>168.7	6.9 ± 2.4	0.8 ± 0.2	>168.7	>168.7	7.6 ± 2.7	1.0 ± 0.3

[a] MIC and MBC were determined using CLSI guidelines.
[b] EC50 is referred to biofilm formation.

Table 5. Antibacterial activity of natural compounds.

According to our proteomics data, we observed that SA, CH, and proAc down-regulate proteins involved in *S. aureus* pathways. Notably the vast majority of down-regulated cell surface proteins were cytoplasmic. This suggests that SA, CH, and proAc can enter in the bacterial cells and possibly affect intracellular processes. In conclusion, we showed that two of these compounds possessed interesting potential to become active principles of new drugs. In particular, both proAc and CH were molecules which fulfill the requirements for inhibition of de novo biofilm formation without bactericidal activity.

2.2.2. Compounds from human body.

Studies performed on various fish species and in swine which led to the identification of innate immuno factors important to be selected for resistance to gram-negative infections, have pointed the attention on Tf as a candidate gene for disease resistance [51, 52]. Therefore, Tf is considered as a relevant safeguard for human body, facing infections sustained by bacteria. Tf is a glycoprotein; its molecular structure shows the presence of two lobes, each binding one iron III ion. Apo-Tf is the denomination of its iron-depleted form; holo-Tf is the iron-loaded form; both forms are collectively named Tfs. Tf has been shown to exert both a bacteriostatic and bactericidal effect in vitro on a variety of microbial pathogens [53, 54]. The antimicrobial activity of transferrin is conventionally related to the iron-depleted form but some studies demonstrated that the mechanism of its antibacterial activity could not be referred only to iron deprivation [55, 56].

We investigated the effect of human apo-Tf and holo-Tf on biofilm formation by *S. aureus* (CA-MRSA USA300 type (ST8-IV) and ATCC 6538P strain) and *S. epidermidis* (A clinical isolate and ATCC 35984 strain). Our aim was to determine whether Tfs were able to interfere with microbial adherence of *S. aureus* and *S. epidermidis* to abiotic surfaces and to eukaryotic host cell [47]. A strong reduction in biofilm formation with both Tfs was obtained albeit at very different concentrations. In particular, the reduction in biofilm formation was higher with apo-Tf rather than obtained with holo-Tf. The *S. aureus* biofilm formation was 50% inhibited at about 50 μg/ml and 65 μg/ml, respectively for 6538P and USA300. The *S. epidermidis* biofilm was inhibited at higher concentrations, namely at 95 μg/ml and 250 μg/ml for RP62A and O-47, respectively. The holo-Tf also inhibited biofilm formation in all the strains, however at very high concentrations. A 50% reduction of biofilm formation by *S. aureus* strains (6538P and USA300) was obtained with concentrations of holo-Tf, respectively of 550 μg/ml and 600 μg/ml. *S. epidermidis* is a good biofilm former hence higher concentrations of holo-Tf are necessary to obtain a 50% reduction (2.0 mg/ml for RP62A and 2.5 mg/ml for O-47). As regards, the activity of holo-Tf on bacterial adhesion on eukaryotic cells our results show a 30% reduction for 6538P adhesion and an increase of adhesion for USA300. Tfs inhibited invasion, although with different efficacy. Similarly to the interference with adhesion process, holo-Tf exerts a stronger inhibition on bacterial invasion than Apo-Tf. Our result demonstrates that Tfs can be proposed as anti-infective compounds in *S. aureus* infections thank to their capability to reduce virulence of bacterial strains depending on adhesion, biofilm formation, and invasion. Our data suggest that holo-Tf plays a major role, as demonstrated on *S. aureus* pathogen strain CA-MRSA USA300 type (ST8-IV) responsible for severe community-associated staphylococcal disease, especially in the USA and in Europe.

3. Conclusions

More than four billion years ago, bacteria appeared on the earth and rapidly evolved in different species which spread and colonized nearly all ecological niches. Since that time prokaryotes and then eukaryotes (plants and animals, unicellular and multicellular) struggle

to maintain a perpetual condition of equilibrium between cohabitants. This equilibrium is obtained by the selection of organisms that can coexist and by the elimination of those who exert a negative influence on others. From the point of view of bacteria, this means that a bacterial cell has to defend its position in a niche; in fact in a prokaryotic community, all cells communicate by means of small molecules in order to allow only the presence of commensals, while multicellular organisms distinguish and select saprophytes from pathogens in order to allow the residency of the first ones and to counteract or even kill the last ones. In this struggle for survival, all living organisms including prokaryotes and all eukaryotes (plants and animals) produce compounds that counteract undesired bacteria by killing them or by modulating their virulence. Molecules that modulate bacterial virulence can be considered as a huge source of molecules to be studied as lead compounds for the development of new antibacterial drugs. We gave some examples derived from our experience in this field, by the description of compounds obtained from prokaryotes, from plants and from humans that can interfere with bacterial phenotype in order to reduce virulence. They represent thus promising scaffolds to use for further development of antibacterial drugs, which may overcome the insurgence of resistance.

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