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REVIEW ARTICLE

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Bioactive compounds: a goldmine for defining new strategies against pathogenic bacterial biofilms?

Arianna Pompilio[a], Marco Scocchi[b], Maria Luisa Mangoni[c], Samira Shirooie[d], Annalisa Serio[e], Ygor Ferreira Garcia da Costa[f], Maria Silvana Alves[f], Gökçe Şeker Karatoprak[g], Ipek Süntar[h], Haroon Khan[i] and Giovanni Di Bonaventura[a]

[a]Department of Medical, Oral and Biotechnological Sciences, and Center for Advanced Studies and Technology (CAST), "G. d'Annunzio" University of Chieti-Pescara, Chieti, Italy; [b]Department of Life Sciences, University of Trieste, Trieste, Italy; [c]Department of Biochemical Sciences, Sapienza University of Rome, Laboratory affiliated to Pasteur Italia-Fondazione Cenci Bolognetti, Rome, Italy; [d]Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran; [e]Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy; [f]Laboratory of Cellular and Molecular Bioactivity, Pharmaceutical Research Center, Faculty of Pharmacy, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil; [g]Department of Pharmacognosy, Faculty of Pharmacy, Erciyes University, Talas, Kayseri, Turkey; [h]Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Etiler, Ankara, Turkey; [i]Department of Pharmacy, Abdul Wali Khan University, Mardan, Pakistan

ABSTRACT

Most human infectious diseases are caused by microorganisms growing as biofilms. These three-dimensional self-organized communities are embedded in a dense matrix allowing microorganisms to persistently inhabit abiotic and biotic surfaces due to increased resistance to both antibiotics and effectors of the immune system. Consequently, there is an urgent need for novel strategies to control biofilm-associated infections. Natural products offer a vast array of chemical structures and possess a wide variety of biological properties; therefore, they have been and continue to be exploited in the search for potential biofilm inhibitors with a specific or multi-locus mechanism of action. This review provides an updated discussion of the major bioactive compounds isolated from several natural sources – such as plants, lichens, algae, microorganisms, animals, and humans – with the potential to inhibit biofilm formation and/or to disperse established biofilms by bacterial pathogens. Despite the very large number of bioactive products, their exact mechanism of action often remains to be clarified and, in some cases, the identity of the active molecule is still unknown. This knowledge gap should be filled thus allowing development of these products not only as novel drugs to combat bacterial biofilms, but also as antibiotic adjuvants to restore the therapeutic efficacy of current antibiotics.

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1. Introduction

Through evolutionary changes, microorganisms have developed survival strategies thus adapting to hostile environments. One such adaptation is existence as biofilms, a collective of multicellular microorganisms adhering to a surface and enclosed in an extracellular matrix (Ćirić et al. 2019). Biofilms can be considered a significant threat to global health, and food and pharmaceutical industries. Reports from the National Institutes of Health indicate that about 80% of human infections are caused by pathogenic biofilms including nosocomial pneumonia cases, surgical and burn wound infections, catheter-related infections, etc (Dongari-Bagtzoglou 2008; Jakobsen et al. 2017). More to the point, biofilm cells possess a higher capacity for adaptive resistance to antibiotics and disinfectants than their planktonic counterparts. Antibiotic resistance is a major obstacle in treating biofilm-related infections (Lu et al. 2019). Therefore, new strategies other than antibiotics should be developed to counteract the biofilm formation.

Several researchers have investigated natural products to find agents to prevent and control biofilms (Song et al. 2018). Indeed, there is a long history of human use of natural products and their secondary

CONTACT Giovanni Di Bonaventura gdibonaventura@unich.it Department of Medical, Oral and Biotechnological Sciences, and Center for Advanced Studies and Technology (CAST), "G. d'Annunzio" University of Chieti-Pescara, Via dei Vestini 31, Chieti 66100, Italy; Marco Scocchi mscocchi@units.it Department of Life Sciences, University of Trieste, Trieste, Italy

metabolites as drugs, in food and in cosmetics. In this context, natural products can offer a broad range of applications for the control of bacterial infections. Many studies have shown that various natural products have antimicrobial activity as well as inhibit biofilm formation (Lau and Plotkin 2013; Karbasizade et al. 2017; Ćirić et al. 2019). Therefore, they could be promising for the discovery of potential components as antibiofilm agents.

Here, we reviewed bioactive molecules – mostly from natural sources – that have the potential to improve the treatment of biofilm-associated infections.

2. Biofilm formation, structure and physiology

The term biofilm, coined by Costerton et al. (1978), refers to a cluster of single or diverse species of microbial cells (bacteria or fungi) attached to a biotic or abiotic surface. This community is encased in a self-produced extracellular polymeric substance (EPS) which constitutes 90% of the total organic material and consisting of proteins, exopolysaccharides, lipids, minerals, and extracellular DNA (Costerton et al. 1995; Donlan 2002; Flemming and Wingender 2010; Kostakioti et al. 2013). EPS provides mechanical stability to the biofilm structure, supporting its survival in hostile environmental conditions, such as limited nutrient availability, and exposure to both antimicrobial agents and immunological defense systems (Coughlan et al. 2016; Flemming et al. 2020).

Biofilm development is a multi-step process (Stoodley et al. 2002; Nishitani et al. 2015). It starts with a reversible attachment of planktonic cells to a surface, followed by the formation of the so-called "microcolony", because of simultaneous bacterial aggregation, clonal growth, and stable cell-cell interactions. Once attached, the cells secrete EPS and microcolony growth leads to a mature biofilm. This stage is irreversible and promotes coordinated agglomeration along with differentiation of cells based on their metabolic state (Chmielewski and Frank 2003). The cells are embedded within a thick and stable complex biomolecular layer acquiring the typical "mushroom-like" structure with water channels that allow the diffusion of nutrients, oxygen, and signalling molecules (Lehner et al. 2005; Roy et al. 2018). The final step consists of the detachment and dispersal, via active or mechanical processes (Stoodley et al. 2002; Nishitani et al. 2015), of single or clustered cells able to colonize surrounding sites either in planktonic form or by forming new sessile communities (Landini et al. 2010).

Within a biofilm, microorganisms can communicate with each other through quorum sensing (QS). This relies upon a system that includes diffusible chemical signalling molecules called autoinducers: mainly *n*-acyl-homoserine lactones in Gram-negative, autoinducing peptides in Gram-positive, and autoinducer-2 in both Gram-positive and Gram-negative bacteria. In response to cell-population density or environmental inductions, QS regulates the metabolic activity of planktonic cells via specific signal transduction pathways. In this way, QS reprograms nutrient utilisation and alters the virulence and the tolerance of the individual cells to harsh environmental conditions (Costerton et al. 1995).

An important feature of biofilm is its physiological heterogeneity. Mature biofilms exhibit a complex 3D-structure with numerous microenvironments that differ in terms of osmolarity, nutritional supply, oxygen, chemicals, and cell density (Fux et al. 2005). The variety of phenotypes deriving from this heterogeneity allows different groups of cells (e.g. biofilm matrix producers or persister cells) to perform specialized tasks within the biofilm for the benefit of the cellular community (van Gestel et al. 2015). Three distinctive microenvironments can be identified inside a mature biofilm: (i) an outer surface layer surrounding the biofilm with a high concentration of substrates, nutrients, and oxygen; (ii) a central zone where cells depend exclusively on fermentation for survival, rich in substrates but poor in oxygen; and (iii) an inner zone, close to the adhesion surface, mainly consisting of metabolically quiescent cells and deficient in substrates and oxygen (Stewart and Franklin 2008). Cells on the surface of the biofilm grow fast, while cell growth becomes increasingly restricted in the innermost layers. The first two zones are rich in matrix-producing cells, while the inner part can host a divergent subpopulation of persister cells, which represent an impediment to the development of targeted, effective, and strategic antimicrobial therapies. Exposure to antibiotics kills the susceptible cells allowing the persister cells to repopulate the biofilms, thus rendering the antimicrobial strategy ineffective and leading to chronic infections (Lewis 2007).

3. Inherent resistance of biofilm to conventional antibiotics

According to the World Health Organization (World Health Organization 2017), antibiotic resistance is one of the most complex and serious global health challenges of the 21st century. Biofilm-forming bacteria are related to this phenomenon because they exhibit

increased resistance to antibiotics, disinfectants, and host immunity (Høiby et al. 2010; McDougald et al. 2011; Tenke et al. 2012). Antibiotic resistance of biofilms is easily reproduced *in vitro*, thus indicating that host factors are not required to express this biofilm trait (Stewart 2002). Even in individuals with competent innate and adaptive immune responses, biofilm-based infections are extremely difficult to cure (Stewart and Costerton 2001). Bacteria incorporated into biofilms are intrinsically more resistant to antibiotic treatments than non-adherent planktonic (free-living) cells of the same strain, as a subset of biofilm bacterial cells can survive in presence of high concentrations of bactericidal antibiotics up to 1,000-fold the Minimum Inhibitory Concentration (MIC) (Lebeaux et al. 2014; Verderosa et al. 2019). Initially, this ability of pathogenic biofilms to survive treatment due to their lower susceptibility to antibiotics was named "recalcitrance" (Lebeaux et al. 2014; Hall and Mah 2017). However, this concept has recently been updated as being a reversible and noninherited subset of biofilm bacteria able to survive in the presence of antibiotics. This disappears when the biofilm is disrupted, and bacteria return to a planktonic state (Lebeaux et al. 2014). The recalcitrance of biofilm makes it extremely difficult to successfully treat and eradicate (Verderosa et al. 2019). The resistance mechanisms of biofilm-forming bacteria are modulated by both intracellular and intercellular communication and regulation of gene expression in a coordinated manner, where QS signalling systems play an essential role (Singh et al. 2017). Therefore, the multicellularity of biofilm bacterial communities is substantially involved in antibiotic resistance (Sharma et al., 2019). The multifactorial phenomenon of biofilm recalcitrance is based on a mixture of resistance and tolerance events and involves different mechanisms, depending on the class of antibiotic used (Mah and O'Toole 2001; Lebeaux et al. 2014): (i) reduced/delayed antibiotic diffusion/penetration through EPS; (ii) expression of biofilm-specific genetic mechanisms; (iii) drug indifference and an altered microenvironment; and (iv) presence of persister cells. In the past, the lack of diffusion/penetration of the antibiotic through the EPS was considered to be responsible of biofilm recalcitrance (Lebeaux et al. 2014). In this case, the mechanical and physicochemical properties of the biofilm matrix explained by its EPS content may act as a physical or chemically reactive barrier, due to the presence of numerous anionic and cationic molecules such as proteins, glycoproteins, and glycolipids that can bind to charged antibiotics, reducing, or delaying diffusion/penetration of these substances (Tenke et al. 2012; Lebeaux et al. 2014). However, although the literature describes the importance of this mechanism, it is not convincing enough to fully explain the increased resistance of biofilms (Lebeaux et al. 2014; Hall and Mah 2017). From a genetic point of view, horizontal gene transfer can also occur through the transfer of plasmids between cells in a biofilm via conjugation (Hall and Mah 2017; Sharma et al., 2019). In fact, biofilms offer appropriate conditions for conjugation such as high cell density, increased genetic competence and accumulation of genetic elements or uptake of resistance genes (Sharma et al., 2019). Moreover, conjugation is probably more efficient in biofilm-forming bacteria cells than planktonic ones because of the environment of the biofilm (i.e. sessile nature and spatial proximity of cells) (Hall and Mah 2017). Some genetic resistance mechanisms, such as expression of periplasmic glucans and efflux pumps within a specie or among different species, can be activated during the biofilm lifestyle (Lebeaux et al. 2014). Another resistance mechanism is related to drug indifference and an altered microenvironment, and deep biofilm layers correspond to a particular physicochemical microenvironment due to different gradients of nutrients, waste, pH, oxygen, and metabolic by-products through the extracellular matrix (Lebeaux et al. 2014). For example, some cells deeply located inside the biofilm structure experience nutrient limitation, and for this reason are in a slow growing or starved state. These nutrient-depleted zones can lead to a stationary phase-like dormancy which can affect antibiotic efficacy and, consequently, the general antibiotic resistance (Taraszkiewicz et al. 2013). Another important mechanism that should be considered is the formation of persister (dormant) cells within the biofilm, which are controlled by the growth phases of the bacterial communities. These cells proliferate rapidly and survive even in the presence of lethal concentrations of antibiotics (Lebeaux et al. 2014; Singh et al. 2017).

4. Role and impact of biofilm formation in healthcare

Biofilms play a crucial role in our healthcare system. Due to the biofilm's inherent recalcitrance to antibiotics and ability to evade the immune responses (Ciofu et al. 2017; Jamal et al. 2018), it is estimated that its formation accounts for most chronic microbial infections in humans, including both device-related infections and those established on biotic surfaces (Del Pozo 2018). Consequently, the development of biofilms poses a clinical challenge to healthcare professionals (Vestby et al. 2020), with implications for aetiological diagnosis,

treatment, and clinical outcome (Ciofu et al. 2017; Khatoon et al. 2018; Sharma et al., 2019).

Biofilms cause infections associated with various indwelling medical devices, mainly those described for dental and orthopaedic prosthetics (Lamagni et al. 2015; Khatoon et al. 2018), along with central venous catheters, prosthetic heart valves, prosthetic joints, and urinary catheters (Agarwal et al. 2010; Rabin et al. 2015; Del Pozo 2018). The formation of a biofilm on indwelling medical devices can be exceptionally challenging as eradication of infection often requires complete surgical removal or the replacement of the infected foreign body (Khatoon et al. 2018; Chomsky-Higgins and Kahn 2019; Bernhardt et al. 2020).

Biofilms are found on almost every tissue of the human body, causing persistent infections such as otitis media and sinusitis, lung infections in cystic fibrosis (CF) patients, wound infections as well as diabetic foot ulcers, urinary tract infections, and periodontitis (Del Pozo 2018).

Most clinically relevant microorganisms can form a biofilm, either as single species or as consortia of microbes from multiple species (Wolcott et al. 2013; Pompilio et al. 2015). The capability to form biofilm has been demonstrated in both Gram-positive (e.g. *Staphylococcus epidermidis, Staphylococcus aureus*) and Gram-negative (e.g. *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*) bacteria (Hall-Stoodley et al. 2004; Agarwal et al. 2010; Rabin et al. 2015).

Biofilm-associated infections have a major impact on public health as they are associated with higher healthcare costs due to prolonged hospitalisation, administration of antimicrobial therapies for long periods (Høiby et al. 2010), and management of infected prostheses (Lamagni et al. 2015; Khatoon et al. 2018).

Therefore, alternative therapeutic approaches are required to efficiently address the morbidity and mortality associated with biofilm infections worldwide.

5. Bioactive molecules with antibiofilm activity

In recent decades, a variety of bioactive compounds have been discovered from nature. Here, we report the antibiofilm properties of the most representative compounds, dividing them according to their natural source. Bioactive molecules from plants, lichens and algae, microorganisms, animal sources, including humans have been reviewed.

5.1. Compounds extracted from plants

Several studies pointed out the antibiofilm activity of natural products extracted from plants (Signoretto et al. 2012; Lu et al. 2019; Walsh et al. 2019), many of which are derived from edible vegetables (berries, fruits, the genus *Allium*, the genus *Capsicum* and other spices and aromatic plants such as cumin and coriander, mushrooms, tea, wine, and coffee). Their antibiofilm activity – mainly due to the abundant presence of phenolic compounds, flavonoids, certain oligosaccharides, and lipids including terpenes and fatty acids – is generally concentration-dependent and occurs via various mechanisms of action. The most promising compounds are those active at very low concentrations, also able to disrupt the preformed biofilm, and whose antibiofilm activity does not rely on cells inactivation. Some examples can be found in Table 1.

The most important feature of these bioactive compounds is that usually they exert antibiofilm activity at sub-lethal concentrations, thus not imposing any selective pressure on cells, and avoiding promoting the development of resistant populations. For example, a reducing effect of the cranberry oligosaccharide-containing fraction Cran1b-F2 on the formation of biofilm by uropathogenic *E. coli* has been demonstrated for at least 48 h without inhibiting growth, thus confirming that the effect was not due to a delay in cells growth (Sun et al. 2015). In another study, sub-inhibitory concentrations of caprylic acid, a medium chain saturated fatty acid found in palm and coconut oil, were effective in preventing biofilm formation by multidrug-resistant (MDR) *K. pneumoniae* (Gupta et al. 2020). Coating catheter with caprylic acid was effective in preventing biofilm formation thus highlighting the potential for preventing device related *Klebsiella* biofilm infections (Gupta et al. 2020).

The activity at low concentrations, which is often dose-dependent, suggests that the antibiofilm effect depends on mechanisms of action other than inhibition of cell growth. A variety of natural products such as pepper, garlic, allicin from onions, curcumin from turmeric, ginger, cinnamon, cumin, and compounds derived from grapes have been demonstrated to hamper and even inhibit biofilm formation by interfering with the QS signalling (Sybiya Vasantha Packiavathy et al. 2012; Kalia et al. 2015; Kim et al. 2015; Von Borowski et al. 2019). Moreover, by suppressing the expression of QS-regulated genes, particularly those related to virulence, bacteria also exhibit reduced motility (particularly swarming and swimming), adhesion, virulence, and pigment production (Kumar et al. 2020).

Table 1. Some representative compounds from plants with antibiofilm activity and related mechanisms.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Oligosaccharide-containing fractions	*Vaccinium macrocarpon* (cranberry)	*E. coli* (uropathogenic)	Biofilm formation reduction: 50% at 1.25 mg/mL (cranf1b-F2) Concentration-dependent effect between 10 and 0.625 mg/mL	Not reported	(Sun et al. 2015)
Caprylic acid	Coconut and palm oil	*K. pneumoniae* (MDR strains)	Biofilm formation reduction: 65% at 0.125xMIC[b] 83% at 0.25xMIC Mature 72 h-old biofilm reduction by 86% at 15xMIC	↓ EPS[c] formation	(Gupta et al. 2020)
Phenolic compounds Tannins Amino acids	*Capsicum baccatum* var. *pendulum* seeds (aqueous extract)	*S. epidermidis* *P. aeruginosa*	Biofilm formation reduction: 80% at 0.004 mg/mL (*S. epidermidis*) 60% at 0.004 mg/mL (*P. aeruginosa*)	Cell membrane damage ↓ EPS formation	(Von Borowski et al. 2019)
Phenolic acids Proanthocyanidins Antocyanins Organic acids	*Vaccinium macrocarpon* (cranberry)	Multi-species biofilm model (*S. oralis*, *V. parvula*, *A. naeslundii*, *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*)	Biofilm formation reduction: from 75% (*F. nucleatum*) to 98% (*S. oralis*) at 0.2 mg/mL	Glucosyl-transferase and fructosyl-transferase inhibition	(Sánchez et al. 2020)
Phloretin	Apples Strawberries	*E. coli* O157:H7	Biofilm formation reduction onto human colonic epithelial cells: 89% at 25 μg/mL 93% at 50 μg/mL	↓ bacterial adhesion ↓ expression of curli genes (*csgA*, *csgB*) ↓ fimbria formation QS[d] inhibition	(Lee et al. 2011)
Catechins	Green tea	*P. gingivalis* *E. coli* *S. mutans* *E. corrodens*	Biofilm formation reduction: 80% at 1 mM 60% at 0.75 mM	Bacterial membrane damage QS inhibition	(Matsunaga et al. 2010; Gopal et al. 2016)
Quercetin	Apples Berries Vegetables	*S. pneumoniae*	Biofilm formation reduction: 30% at ≥12.5 μM	↓ LasI, LasR, RhlI and RhlR QS systems	(Wang et al. 2018)
Berberine	European barberry Oregon grape Tree turmeric	*E. faecalis* *S. epidermidis*	Biofilm formation reduction: >50% at ≥60 μg/mL (*E. faecalis*) >50% at 30 μg/mL (*S. epidermidis*)	Binding to amyloid EPS-associated proteins, DNA, and cell membrane ↓ sortase A and enterococcal surface protein (*spa*) expression	(Wang et al. 2009; Chen et al. 2016)
Carvacrol	Oregano oil	MRSA S. Typhimurium *C. violaceum*	Biofilm formation reduction: 0.50–1 mM (*S. aureus*) 0.75–1.25 mM (S. Typhimurium) 0.1–0.3 mM (*C. violaceum*) No significant effect *vs.* preformed biofilms	↓ expression of *cvil* (N-acyl-L-homoserine lactone synthase) ↓ chitinase and violacein production	(Burt et al. 2014)

(*continued*)

Table 1. Continued.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Saponins	Legumes *Allium* sp.	*S. agalactiae* *S. aureus*	Biofilm formation reduction: >40% at 0.02 mg/mL	↓ expression of biofilm-related genes (*srtA, fbsC, neuA,* and *cpsE*) Cell membrane lysis	(Dong et al. 2020; Pu et al. 2020; Shang et al. 2020)
Bioactive peptides (containing Val, Leu, Ala, Arg, and Trp residues)	*Vicia faba* proteins hydrolisates	*P. aeruginosa*	Biofilm formation reduction: 75% at 0.5 mg/mL	Interaction between hydrophobic amino acids and fatty acyl chains of lipids ↓ cell surface hydrophobicity ↓ adhesion	(Karkouch et al. 2017)
Sulphur compounds	*Allium ursinum* leaves extract	*K. pneumoniae* *C. albicans* Mixed biofilm	Mature biofilm reduction: 70% (*K. pneumoniae*) 60% (*C. albicans*) Mature mixed biofilm reduction: 70% at 0.075 mg/mL	Not reported	(Galdiero et al. 2020)
Beta-glucans	*Eleusine coracana* (Finger millet)	*E. faecalis* *L. fusiformis* *P. vulgaris* *S. sonnei*	Mature biofilm reduction: 100% at 0.1 mg/mL (MIC: 0.07 mg/mL)	Cell membrane rupture Biofilm architecture disintegration	(Divya et al. 2020)
Catechin Ellagic acid Quercetin Malvidine-3 glucoside	*Eugenia brasiliensis* Lam. extract	*L. acidophilus* *S. aureus*	Mature biofilm reduction: 50% at 0.625 mg/mL (*L. acidophilus*) No activity against *S. aureus*	Not reported	(Goldoni Lazarini et al. 2018)
Fatty oils Proteins Tannins Oxalate	*Coriander sativum* L. extract	*E. coli* *S. aureus*	Biofilm formation reduction at 0.1 mg/mL: 40–59% (*E. coli*) 42–72% (*S. aureus*)	QS inhibition ↓ motility (swarming)	(Molina et al. 2020)
Curcumin Curcumin nanoparticles	*Curcuma longa*	*P. aeruginosa*	Biofilm formation reduction onto mouse pulmonary epithelial cells: 30% (curcumin) 50% (curcumin nanoparticles)	↓ adhesion	(Xue et al. 2020)
Quinic acid	*Lonicerae Japonicae Flos*	*P. aeruginosa*	Biofilm reduction in a catheter infection mouse model 90% at 1xMIC 80% at 1/4xMIC	QS inhibition ↓ EPS production	(Lu et al. 2021a)

[a]*E. coli, Escherichia coli; K. pneumoniae, Klebsiella pneumoniae;* MDR, multidrug-resistant; *S. epidermidis, Staphylococcus epidermidis; P. aeruginosa, Pseudomonas aeruginosa*; *S. oralis, Streptococcus oralis; V. parvula, Veillonella parvula, A. naeslundii, Actinomyces naeslundii; P. gingivalis, Porphyromonas gingivalis; A. actinomycetemcomitans, Aggregatibacter actinomycetemcomitans; F. nucleatum, Fusobacterium nucleatum; S. mutans, Streptococcus mutans; E. corrodens, Eikenella corrodens; S. pneumoniae, Streptococcus pneumoniae; E. faecalis, Enterococcus faecalis;* MRSA, methicillin-resistant *S. aureus;* S. Typhimurium, *Salmonella* Typhimurium*; C. violaceum, Chromobacter violaceum; S. agalactiae, Streptococcus agalactiae; S. aureus, Staphylococcus aureus; C. albicans, Candida albicans; L. fusiformis, Lactobacillus fusiformis; P. vulgaris, Proteus vulgaris; S. sonnei, Shigella sonnei; L. acidophilus, Lactobacillus acidophilus*.
[b]MIC, Minimum inhibitory concentration.
[c]EPS, Extracellular polymeric substance.
[d]QS, Quorum sensing.

Table 2. Some representative compounds from lichens and algae with antibiofilm activity and related mechanisms.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Physodic acid 3-hydroxy physodic acid Atranorin	Diethyl ether (DE) and chloroform-methanol-acetone (CMA) extracts of *Hypogymnia tubulosa* (Schaer.) Hav	*S. aureus* *E. faecalis*	Biofilm formation reduction: 36.8% CMA extract (*S. aureus*) 44.1% CMA extract (*E. faecalis*) 12.2% DE extract (*S. aureus*) No activity of DE extract on *E. faecalis*	QS[b] inhibition	(Özyiğitoğlu et al. 2017)
Usnic acid Atranorin Fumarprotocetraric acid	Lichens from Chilean territories	*S. aureus*	Biofilm formation reduction: usnic acid and atranorin show comparable activity at sub-MICs[c] (1/2×, 1/4×, 1/8x) atranorin is more active against MRSA Mature biofilm reduction (usnic acid *vs.* atranorin): 12–36% *vs.* 73–81% at 1xMIC 8–20% *vs.* 30–41% at 5xMIC 3% *vs.* 18% at 10xMIC	Usnic acid effect due to: ↓EPS[d] ↓ adhesion due to reduced expression of genes encoding elastin, laminin, and fibronectin	(Pompilio et al. 2013; 2016)
Usnic acid n-alkanes Fatty acid esters 5α-ergosta-7,22-dien-3β-ol Atranorin	Acetone (AC), methanol (ME), and ethyl acetate (EA) extracts of *Cladonia foliacea* and *Hypogymnia physodes*	*S. aureus* *P. mirabilis*	Biofilm formation reduction (BIC[e]), respectively for AC, ME, and EA: 0.6, 1.25, 2.5 mg/mL (*C. foliacea vs. S. aureus*) 2.5, 1.25, 2.5 mg/mL (*C. foliacea vs. P. mirabilis*) 0.31 mg/mL (*H. physodes vs. S. aureus*) 2.5 mg/mL (*H. physodes vs. P. mirabilis*)	Not reported	(Mitrovic et al. 2015)
Atranorin Chloroatranorin Caperatic acid Atraric acid Olivetonide Olivetol	Methanol, acetone, and ethyl acetate extracts of *Platismatia glauca* *Pseudevernia furfuracea*	*S. aureus* *P. mirabilis*	Biofilm formation reduction: acetone and ethyl acetate *P. glauca* extracts are more active than *P. furfuracea* (BIC: 0.63 mg/mL) Methanol *P. furfuracea* extract is more active on *S. aureus* (BIC: 1.25 mg/mL) and *P. mirabilis* (BIC: 0.63 mg/mL) compared to other types of extracts	Not reported	(Mitrovic et al. 2014)
Sulphated polysaccharides	*Fucus vesiculosus* *Macrocytis pyrifera* *Undaria pinnatifida* *Hizikia fusiforme* *Kjellmaniella crassifolia* *Laminaria japonica* *Sargassum honeri* *Undaria pinnatifida* *Capsosiphon fulvescens* *Codium fragile* *Grateloupia flilicina*	*E. faecalis* *S. mutans* *S. sobrinus*	Biofilm formation reduction by fucoidan F85: 100% at > 250 μg/mL (*S. mutans, S. sobrinus*) 91.3% at 1 mg/mL (*E. faecalis*)	Not reported	(Jun et al. 2018)

(*continued*)

Table 2. Continued.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Methanol extract: 9-octadecenoic acid 6,9,12-octadecatrienoic acid Phenylmethyl ester Phenol 2,6-bis (1,1-dimethylethyl) Hexadecanol Hexane extract: Benzenemethanol butanamide Hexanedioic acid dioctyl ester Phthalic acid derivatives Hexane surface extract: Piperidine 3-phenyl 6H-pyrazolo[1,2-a][1,2,4,5]tetrazine Piperazine-2-methyl, pentanal Pyrrolidine-2-carboxamide Benzimidazole 1-benzylindole	*Halimeda sp*	*V. harveyi*	Biofilm formation reduction: 21% (hexane extract of fresh algal sample) 29.2% (hexane extract of algae surface) 23.5% (hexane extract of dried algae) Inhibitory activity of methanol extracts is not found as significant as other extracts	↓ EPS production	(Abdullah Gadhi et al. 2018)
Carbohydrates Amino acids Proteins Phenolics Flavonoids Alkaloids Saponins Steroids	Dichloromethane, chloroform, ethyl acetate, methanol, ethanol, and petroleum ether extracts of *Chlorella vulgaris*	*P. aeruginosa* *S. aureus*	Biofilm formation reduction by methanol extract: 82.5% (*P. aeruginosa*) 88% (*S. aureus*)	↓ QS-controlled virulence factors	(Sridevi et al. 2019)
2(5H)-Furanone Epigallocatechin gallate	Green tea	*C. jejuni*	Biofilm formation reduction at 75% MBC[f]: $\geq$ 66% for both compounds Dose-dependent effect	Not reported	(Castillo et al. 2015)
γ-alkylidene-γ-lactones (1a-1g) γ-hydroxy-γ-lactams (2a-2g) γ-alkylidene-γ-lactams (3a-3g) γ-alkylidene-γ-lactams (4a-4g)	*Delisea pulchra*	*S. epidermidis* *P. aeruginosa*	Biofilm formation reduction (IC_{50}[g], μg/mL): 40->87.5 (1a-1g *vs. S. epidermidis*) 1.7->87.5 (1a-1g *vs. P. aeruginosa*) 13.3->87.5 (2a-2g *vs. S. epidermidis*) 2.2->87.5 (2a-2g *vs. P. aeruginosa*) 23->87.5 (3a-3g *vs. S. epidermidis*) 0.6->87.5 (3a-3g *vs. P. aeruginosa*) 12.2->87.5 (4a-4g *vs. S. epidermidis*) 1.4->87.5 (4a-4g *vs. P. aeruginosa*) Dose-dependent effect (*S. epidermidis, P. aeruginosa*) Dose-independent effect (*S. aureus, S. mutans*)	Not reported	(Pereira et al. 2014)
1′-unsubstituted and 1′-subsituted 3-alkyl-5-methylene-2(5H)-furanones.	*D. pulchra*	*S.* Typhimurium	Biofilm formation reduction (IC_{50}, μM): 10.7->1000 Planktonic growth was never affected	Not reported	(Steenackers et al. 2010)

(*continued*)

Table 2. Continued.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
			The length of the 3-alkyl chain and the bromination pattern of the ring structure were needed for biofilm inhibition by 1′-unsubstituted furanones The bromine at position 4 is crucial for the biofilm inhibitory activity		
Fucoidan	*Cladosiphon okamuranus*	*H. pylori* ATCC 43504	Inhibition of attachment to porcine gastric mucin at pH 2.0 and 4.0: 60% at 100 μg/mL 100% at 1 mg/mL	↓ adhesion to gastric mucin	(Shibata et al. 2003)
			Dose-dependent effect		
			Prevention of colonisation of Mongolian gerbils gastric mucosa: 20% at 0.5 g/100 ml		

[a]*S. aureus, Staphylococcus aureus; E. faecalis, Enterococcus faecalis;* MRSA, methicillin-resistant *S. aureus; P. mirabilis, Proteus mirabilis; S. mutans, Streptococcus mutans; S. sobrinus, Streptococcus sobrinus; V. harveyi, Vibrio harveyi; P. aeruginosa, Pseudomonas aeruginosa; C. jejuni, Campylobacter jejuni; S. epidermidis, Staphylococcus epidermidis; S.* Typhimurium, *Salmonella* Typhimurium. *H. pylori, Helicobacter pylori.* [b]QS, Quorum sensing. [c]MIC, Minimum inhibitory concentration. [d]EPS, Extracellular polymeric substance. [e]BIC, Biofilm inhibitory concentration (i.e. the lowest concentration without growth). [f]MBC, Minimum bactericidal concentration. [g]IC_{50}, concentration of compound needed to inhibit biofilm formation by 50%.

On the contrary, stress regulons are activated causing biofilm dispersion. It should be noted that biofilm formation is also related to stress response, explaining why low concentrations of bioactive molecules could instead promote biofilm formation, as observed for *Salmonella enterica, Listeria monocytogenes* and *E. coli* in the presence of 50 mg/mL of oven dried and lyophilized extracts of black grape, apple, and pitahaya (Zambrano et al. 2019). Other mechanisms of action include the inactivation by cranberry of key enzymes in biofilm formation and maturation, such as glucosyl- and fructosyl-transferases (Sánchez et al. 2020). In addition, the flavonoid procyanidins contained in berries could prevent the cell aggregation by altering the cell surface and decreasing bacterial hydrophobicity (Riihinen et al. 2014).

The flavonoid phloretin, a dihydrochalcone which is enriched in apple tree leaves, has inhibitory effects on biofilm formation of *E. coli* O157:H7 strain by preventing bacterial attachment and TNF-α-induced inflammatory response. Furthermore, phloretin suppressed the translation of toxin genes such as *hlyE* and stx_2, curli genes (*csgA* and *csgB*), autoinducer-2 importer genes (*lsrACDBF*) and prophage genes in *E. coli* (Lee et al. 2011).

The antioxidant flavonoid catechins present in green tea, such as epigallocatechin and epigallocatechin gallate, markedly reduced the biofilm formation of *Porphyromonas gingivalis* and *E. coli* via bacterial membrane damage (Gopal et al. 2016). In addition, both galloyl and pyrogallol type catechins inhibited the biofilm formation by the periodontopathogenic bacterium *Eikenella corrodens* probably by interfering with the AI-2-mediated QS system, although the authors did not report the underlying mechanism (Matsunaga et al. 2010). Quercetin is another potent antioxidant flavonoid abundant in many plants such as green tea and apple. It has been shown to have a significant inhibitory effect on biofilm formation by reducing the expression of *lasI*, *lasR*, *rhlI* and *rhlR* QS systems. By blocking the sialic acid expression, quercetin also has an inhibitory effect on biofilm formation of *Streptococcus pneumoniae* (Wang et al. 2018). Another compound showing antibiofilm potential is the alkaloid berberine, which is found in many plants such as Oregon grape, goldenseal, and tree turmeric. In an *in vitro* study using a single-canal tooth model, berberine combined with 1% chlorhexidine markedly reduced biofilm formation of *Enterococcus faecalis* (Chen et al. 2016). In addition, berberine affected the biofilm formation of *S. epidermidis* via binding to the

amyloid proteins associated with EPS (Wang et al. 2009).

Very common in nature is the monoterpenic phenol isomer carvacrol, which is abundant in the leaves of many plants including thyme, bergamot, and oregano. Burt et al. (2014) showed that carvacrol noticeably reduced the formation of *S. aureus, S. enterica*, and *Chromobacterium violaceum* biofilm. Furthermore, carvacrol reduced the biofilm production of methicillin-resistant strains of *S. aureus* (MRSA), and *L. monocytogenes*. The mechanisms underlying the antibiofilm activity of carvacrol include decreased production of N-acyl-L-homoserine lactone, and impairment of the bacterial QS, such as the production of chitinase and violacein in *C. violaceum* (Burt et al. 2014).

In addition, several studies have shown an antibiofilm activity of the triterpene glycoside saponins against various pathogens such as *Streptococcus agalactiae,* and *S. aureus* (Dong et al. 2020; Pu et al. 2020). In detail, tea saponin – extracted from seeds, leaves and other parts of the tea plant – downregulated the expression of genes involved in biofilm formation such as *srtA*, *fbsC*, *neuA*, and *cpsE* in *S. agalactiae* (Shang et al. 2020). Moreover, aqueous extracts of *Ziziphus joazeiro* leaves rich in saponins, inhibited *S. aureus* and *P. aeruginosa* biofilm formation (Cosmo Andrade et al. 2019).

Limonoids, one of the triterpenoid classes, are found in citrus fruits and many plants. Studies have indicated that isolimonic acid and ichangin are modulator of *luxO* expression and type III secretion system, and prevent biofilm formation in *Vibrio harveyi* (Vikram et al. 2011, 2012).

Vicilin and legumin B peptides released from hydrolysate of *Vicia faba* seed proteins showed interesting antibiofilm activity against *P. aeruginosa* with a minimal biofilm inhibitory concentration ($MBIC_{50}$) ranging from 12 to 35 μM (Karkouch et al. 2017). It has been suggested that bioactive peptides, particularly those containing hydrophobic and basic amino acids, could interact with the fatty acyl chains, reducing cell surface hydrophobicity and therefore hampering cells adhesion (Pletzer et al. 2016). Furthermore, the limitation of iron induced by the iron-chelating ability of peptide vicilin might contribute to inhibit biofilm formation of *P. aeruginosa* since iron availability was reported to be important for biofilm formation (Karkouch et al. 2017).

All compounds described above are active as inhibitors of biofilm formation. Some studies also report the disruptive action of plant compounds against the mature biofilm. For example, leaves extract from *Allium ursinum* containing various sulphur compounds caused a 70% reduction of mature biofilm of *K. pneumoniae* (Galdiero et al. 2020). Some polysaccharides can also affect and eradicate preformed biofilms. For example, beta-glucans from *Eleusine coracana* (Finger millet) at 0.1 mg/mL (MIC: 0.07 mg/mL) caused 100% mature biofilm disruption with the disintegration of the biofilm architecture of *E. faecalis, Lysinibacillus fusiformis, Proteus vulgaris* and *Shigella sonnei* (Divya et al. 2020). In another study, caprylic acid was effective against 72 h-old biofilms formed by clinical MDR *K. pneumoniae*, causing 86% of eradication when tested at 15xMIC (Gupta et al. 2020).

In some cases, cytotoxicity of plant extracts has been reported. The ethanolic extract of *Eugenia brasiliensis* L., containing polyphenols, at a concentration of 0.625 mg/mL resulted in the reduction of 50% of mature biofilm of *Lactobacillus acidophilus,* with negligible toxicity against macrophages and in larvae of wax moth *Galleria mellonella* (Goldoni Lazarini et al. 2018). Similarly, the aqueous extract of red pepper *Capsicum baccatum* significantly reduced *S. epidermidis* and *P. aeruginosa* biofilm formation, independently on microbial cells death and without any toxic effect on *G. mellonella* (Von Borowski et al. 2019). Also, *Coriander sativum* L. extract reduced biofilm formation by *E. coli* and *S. aureus* and was safe for *G. mellonella* (Molina et al. 2020).

Most of these studies have confirmed the antibiofilm activity of plant components in preventing biofilm formation *in vitro*, while the effectiveness in disrupting mature biofilm is often lower and requires further investigations. Moreover, the research should be focussed more on *in vivo* and *ex-vivo* activity. In this regard, Lee et al. (2011) observed that phloretin, an antioxidant agent which is enriched in apples, inhibited *E. coli* O157:H7 adhesion to human colon epithelial cells but not commensal *E. coli*. In another study, a micronized formulation of curcumin – aimed at improving curcumin solubility and biological activity – was shown effective in preventing adhesion and biofilm formation of *P. aeruginosa* onto mouse pulmonary epithelial cells, thus indicating the potential to improve the infection outcome in the lungs (Xue et al. 2020). Lu et al. found that the treatment with 1/4xMIC of *Lonicerae Japonicae Flos* crude extract was effective in reducing the viability of biofilm formed by *P. aeruginosa* onto a catheter in a biofilm-based infection murine model, with lower colony counting and less pathological changes (i.e. abscess, bleeding, and inflammation) (Lu et al. 2021a). Gene expression data indicated a significant downregulation of *rhlA*, *rhlR* and *rhlB* in *rhl* system in QS signalling pathway (Lu et al. 2021a).

Even if overall promising, these findings need to be confirmed by further studies to support development

of new antibiofilm strategies through use of plants-derived compounds.

5.2. Compounds extracted from lichens and algae

Lichens are fungi, photosynthetic partners of green algae or cyanobacteria, and compulsory symbiotic systems for some bacteria (Hodkinson and Lutzoni 2009; Selbmann et al. 2010). Lichens produce more than 100 secondary metabolites, including xanthones, depsides, dibenzofurans, depsidones, and terpene derivatives. Many of these metabolites have been shown to have antitumor, antiviral, or antimicrobial activities (Molnár and Farkas 2010). Specifically, the antibacterial properties have been increasingly demonstrated against many susceptible and MDR bacterial strains (Selbmann et al. 2010; Bate et al. 2020), it is observed that there is less focus on antibiofilm activities. A list of the most representative studies focussed on the antibiofilm activity of lichens and their metabolites is shown in Table 2.

Diethyl ether and chloroform-methanol-acetone extracts of the lichen *Hypogymnia tubulosa* (Schaer.) Hav. were investigated for their antibacterial and antibiofilm effects against *S. aureus* and *E. faecalis*. The chloroform-methanol-acetone extract of the Bolu sample had a lower antibacterial effect against planktonic cells but it significantly decreased the biofilm formation by strains representative of both species, compared to the control groups (Özyiğitoğlu et al. 2017).

In a study by Pompilio et al. (2013) some lichen metabolites – i.e. usnic acid, atranorin and fumarprotocetraric acid – were evaluated *in vitro* for their antibacterial and antibiofilm activities against MRSA and methicillin-sensitive *S. aureus* strains from CF patients. Usnic acid and atranorin showed comparable activity against biofilm formation, although atranorin was particularly active against MRSA. In contrast, usnic acid was significantly more active than atranorin against preformed (mature) biofilms. In another study, Pompilio et al. (2016) observed that antibacterial activity of usnic acid was related to damaged peptidoglycan synthesis, whereas the effect against biofilm was primarily due to an impairment of binding to host matrix proteins and a decrease in lipase and thermonuclease expression. Unfortunately, the liver toxicity of usnic acid reported by the US Food and Drug Administration greatly reduces its potential as antibiofilm agent (Guo et al. 2008).

The antibiofilm activity of *Cladonia foliacea* and *Hypogymnia physodes* extracts (methanol, acetone, and ethyl-acetate) was evaluated by Mitrovic et al. (2015) towards *S. aureus* and *Proteus mirabilis*. The methanol and ethyl-acetate extracts of *H. physodes* were found to be more active than those from *C. foliacea* with a Minimum Bactericidal Concentration (MBC) of 0.31 mg/mL for *S. aureus*. The antibiofilm activity for both lichens was confirmed with more pronounced results for *C. foliacea*. In another study focussed on *S. aureus* and *P. mirabilis*, the acetone and ethyl acetate extracts of *Platismatia glauca* were better than methanol extract with MBIC of 0.63 mg/mL for both species (Mitrovic et al. 2014). At the same time, the methanol extract of *Pseudevernia furfuracea* was effective with inhibitory concentrations of 1.25 mg/mL on *S. aureus* and 0.63 mg/mL on *P. mirabilis* (Mitrovic et al. 2014).

To the best of our knowledge, the antibiofilm activity of lichens and derivates were not yet evaluated in *in vivo* models.

Marine organisms, such as algae, are considered as one of the potential sources of diverse bioactive molecules for fighting the increasing emergence of antibiotic-resistant and biofilm-forming bacteria. Jun et al. (2018) investigated sulphated polysaccharides from various algae against bacteria forming dental plaque. At concentrations higher than 0.25 mg/mL, fucoidan completely suppressed *Streptococcus mutans* and *Streptococcus sobrinus* biofilm formation and planktonic cell growth, although it never eradicated biofilm.

The antibiofilm activity of the macroalga *Halimeda* sp. and its effects on EPS production have been evaluated against biofilm-forming *V. harveyi* (Abdullah Gadhi et al. 2018). The methanol extract prepared from dried algae showed the strongest activity and caused a significant decrease in EPS production by the biofilm cells, compared to other extracts. Treatment with the surface methanol extract of *Halimeda* sp. significantly inhibited biofilm development in a microtiter plate assay (Abdullah Gadhi et al. 2018).

The antibiofilm activity of several extracts obtained from the freshwater microalgae *Chlorella vulgaris* was investigated using the clinically relevant pathogens *P. aeruginosa* and *S. aureus*. The methanol extract at a concentration of 1 mg/mL significantly affected biofilm formation, with a reduction of 82.5% for *P. aeruginosa* and 88.0% for *S. aureus* (Sridevi et al. 2019).

The sulphated polysaccharides extracted from green algae *Chlamydomonas reinhardtii* were shown effective against biofilm formation by *Neisseria mucosa*, *E. coli*, *Streptococcus* sp. and *Bacillus subtilis* (Vishwakarma and Vavilala 2019). Exposure at 1 mg/mL caused 50% biofilm inhibition, achieving total inhibition at 4–8 mg/mL. Complete dissolution of preformed biofilms was also observed, probably mediated by interaction with EPS and its subsequent degradation.

Table 3. Some representative bioactive compounds from bacterial microorganisms with antibiofilm activity and related mechanisms.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
cyclobutane,2-hexyl-1,1,4-trimethyl thiophene, 2-butyl-5-ethyl; 1-heptyn-3-ol 8-[N-aziridylethylamino]-2–6,dimethyloctene-2 pyrrolo[1,2-a]pyrazine-1,4-dion,hexahydro octahydro-2H-pyrido(1,2-a)pyrimidin-2-one; 9,9-dimethyl-3,7-diazabicyclo[3.3.1]nonane 2,4-dihydroxy-6-propylbenzoic acid	Methanolic extract of *Streptomyces* MUSC125 strain from mangrove soil	MRSA	Biofilm formation reduction at 1.562 mg/mL Antibiofilm potential at 1/8xMIC[b] Dose-dependent effect	Iron chelation by 2,3-dihydroxybenzoic acid	(Mangzira Kemung et al. 2020)
Secondary metabolites	Crude extract of *Streptomyces* from marine sediment	*P. mirabilis* (uropathogenic)	Biofilm formation reduction: 63% at 15 mg/mL 26% at <10 mg/mL	↓ swimming motility QS[c] signal inhibition	(Younis et al. 2016)
Pyranonaphthoquinone polyketides	Pyranonaphthoquinone biosynthetic pathways of *Streptomyces*	*S. aureus*	Biofilm formation reduction (IC_{50}[d]) at: 39.3 μM (alnumycin A) 1.75 μM (alnumycin D) 2.76 μM (granaticin B) 3.87 μM (kalafungin) 2.50 μM (medermycin) Mature biofilm reduction (IC_{50}) at: 4.02 μM (alnumycin D) 3.72 μM (granaticin B) 27.8 μM (kalafungin) 24.6 μM (medermycin)	C-ribosyl unit in an unusual pyranose form is essential for the antibiofilm activity of the alnumycins Oxygenation pattern may have an impact on the antibiofilm activity of granaticin B	(Oja et al. 2015)
Rhodamine isothiocyanate analogues	Extract of *Vibrio alginolyticus* 12 from coral *Pocillopora damicornis*	*P. aeruginosa* PAO1	Biofilm formation reduction by 37.5% at 10 μg/mL Dose-dependent effect	QS inhibition (disruption of the *las* and/or *rhl* system throughout a rhodamine isothiocyanate analogue)	(Song et al. 2018)
N-acyl homoserine lactone-based QS analogues	Aqueous extract of *Rhizobium* sp. NAO1 from North Atlantic Ocean	*P. aeruginosa* PAO1	Biofilm formation reduction: 77.9% at 5% extract Dose-dependent effect	QS inhibition	(Chang et al. 2017)
Secondary metabolites	ESKAPE pathogens	ESKAPE pathogens	Biofilm formation reduction	QS inhibition (reduced production of cGMP secondary to the inhibition of diguanylate cyclases)	(Tiwari et al. 2018)
Carolacton	Extract of *Sorangium cellulosum* from soil	Oral bacteria (*S. mutans*, *S. oralis*, *S. gordonii*, *A. actinomycetemcomitans*)	CLSM[e] revealed massively reduced biofilm formation (*S. oralis*), bacterial killing (*S. mutans*), more voluminous structure with vertically orientated cell chains (*S. gordonii*)	Defect in cell division	(Stumpp et al. 2015)

(*continued*)

Table 3. Continued.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Cyclic dipeptides [cyclo(D-Leu-D-Arg), cyclo(L-Trp-L-Arg), cyclo(D-Trp-D-Arg)]	Ethyl acetate extract of *Achromobacter* sp. associated with *Rhabditis* nematode	Wound-associated bacteria (*B. subtilis, S. aureus, S. epidermidis, S. faecalis, E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, S. typhi, P. vulgaris, E. faecium*)	Synergistic effect of cyclic dipeptides and ampicillin *vs.* biofilm formation	Not reported	(Deepa et al. 2015)
CFS[f]	*Clostridium butyricum*	*A. baumannii* (MDR strains)	Biofilm formation reduction: 33.9% at 12.5% 43.1% at 25% 99.6% at 50% CLSM showed reduction in biofilm biomass and thickness, and its disintegration	Dose-dependent motility inhibition	(Shin and Eom 2020)
CFS	*Lacticaseibacillus rhamnosus* GG alginate–chitosan microcapsules	*E. coli*	Dose-dependent inhibition of biofilm formation Time-dependent disruption of mature biofilm	Inhibition of QS pathway transcriptional activators (*luxS*, *IsrK*, and *IsrR)* by the excretion of an AI-2 signalling molecule	(Song et al. 2019)
CFS	*Lactobacillus* strains from milk and yougurt	*P. aeruginosa* (MDR and non-MDR strains)	Biofilm formation reduction: 0–64% Only *L. fermentum* L1 and L2 caused 100% inhibition	Production of lactic acid, acetic acid and formic acid	(Shokri et al. 2018)
CFS	*Limosilactobacillus fermentum* TCUESC01 and *Lactiplantibacillus plantarum* TCUESC02 from the cocoa fermentation	*S. aureus*	Biofilm formation reduction: *L. fermentum* (more active vs. *L. plantarum*) causes inhibition at sub-MICs CLSM revealed significant reduction in biofilm thickness at sub-MICs	PIA[g] synthesis inhibition throughout *ica* operon modulation	(Melo et al. 2016)
CFS	*Ligilactibacillus salivarius* *L. fermentum*	*S. aureus*	Mature biofilm reduction: 6.3-Log by *L. salivarius* no reduction by *L. fermentum*	Cell wall lysis by peptidase M23B in *L. salivarius*	(Kang et al. 2017)

[a]MRSA, methicillin-resistant *Staphylococcus aureus*; *P. mirabilis, Proteus mirabilis; S. aureus, Staphylococcus aureus; P. aeruginosa, Pseudomonas aeruginosa;* ESKAPE (*Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, Enterobacter cloacae); S. mutans, Streptococcus mutans; S. oralis, Streptococcus oralis; S. gordonii, Streptococcus gordonii; A. actinomycetemcomitans, Aggregatibacter actinomycetemcomitans; B. subtilis, Bacillus subtilis; S. epidermidis, Staphylococcus epidermidis; S. faecalis, Streptococcus faecalis; E. coli, Escherichia coli; K. pneumoniae, Klebsiella pneumoniae; S. typhi, Salmonella typhi; P. vulgaris, Proteus vulgaris; E. faecium, Enterococcus faecium; A. baumannii, Acinetobacter baumannii*; MDR, multi-drug resistant. [b]MIC, Minimum inhibitory concentration. [c]QS, Quorum sensing; [d]IC_{50}, Concentration causing 50% inhibition in biofilm formation. [e]CLSM, Confocal laser scanning microscopy. [f]CFS, Cell-free supernatant. [g]PIA, Polysaccharides of intercellular adhesion.

Table 4. Some representative bioactive compounds from fungal microorganisms with antibiofilm activity and related mechanisms.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Diterpenoid sphaeropsidin A	*Diploidia corticola* from forest plants	MRSA MDR *P. aeruginosa*	Biofilm formation reduction: 62% at 1/4xMIC[b] (*P. aeruginosa*) 53% at 1/4xMIC (MRSA)	Not reported	(Roscetto et al. 2020)
Vulculic acid, curvulol	*Chaetosphaeronema achilleae*	*S. aureus* DSM 1104 (human pathogen)	Biofilm formation reduction: 96.8% at 8xMIC (vulculic acid) 91.9% at 4xMIC (vulculic acid) 96.2% at 4xMIC (curvulol)	Not reported	(Narmani et al. 2019)
Organic fungal extracts	*Penicillium* sp. strains from the leaf and the root rhizosphere of Baltic *Zostera marina*	*P. aeruginosa*	Biofilm formation reduction is strain- and culture medium-dependent	QS[c] inhibition	(Petersen et al. 2019)
Cytochalasans	*Hypoxylon fragiforme*	*S. aureus* DSM 1104	Biofilm formation reduction: 85% at 1/3xMIC (chaetoglobosin A) 61% at 1/8xMIC (chaetoglobosin A) 40–60% at sub-MICs (cytochalasin C; L-696,474; 19, 20-epoxycytochalasin C; phenochalasin D) 44% at 1/32xMIC (cytochalasin A) 91% at 1/32xMIC (L-696,474) 45%-91% at sub-MICs (other compounds)	Not reported	(Yuyama et al. 2018)
Organic extracts of: *Laetiporus sulphureus* *Macrolepiota fuliginos* *Macrolepiota procera*	Basidiomycotina fruiting bodies, both saprophytic and ectomycorrhizal	*P. aeruginosa* PA14 (human pathogen) *S. aureus* DSM 1104	Biofilm formation reduction (MBIC[d]) at: 1/4xMIC (*M. fuliginosa, M. procera*) 125 μg/mL (*L. sulphureus*)	Not reported	(de Carvalho et al. 2015)
Crude extracts	*Alternaria alternate* (foliar endophyte)	*P. aeruginosa* PAO1 (human pathogen)	Biofilm formation reduction: up to 65.2% at sub-MICs	↓ alginate production ↓ cell surface hydrophobicity ↓ EPS[e] biosynthesis ↓ motility (swimming, swarming) QS inhibition	(Rashmi et al. 2018)
Terreic acid	*Aspergillus terreus*	*E. coli* ATCC25922	MBIC equals to 2xMIC	Not reported	(Sharma et al. 2016)
Equisetin (from crude extracts)	*Fusarium* sp. Z10 (marine fungus)	*P. aeruginosa* PAO1	Biofilm formation reduction: 58.3% at sub-MIC (300 μM)	Inhibition of las and rhl QS systems ↓ motility (swarming)	(Zhang et al. 2018)
Thiodiketopiperazine derivatives	*Phoma* sp. GG1F1 (endophyte of *Glycyrrhiza glabra* Linn)	*S. aureus* *S. pyogenes*	Biofilm formation reduction: 28–57% at sub-MICs (*S. aureus*) 60.7–86% at sub-MICs (*S. pyogenes*)	Not reported	(Arora et al. 2016)

[a]MRSA, methicillin-resistant *Staphylococcus aureus;* MDR, multidrug-resistant*; P. aeruginosa, Pseudomonas aeruginosa; S. aureus, Staphylococcus aureus; E. coli, Escherichia coli; S. pyogenes, Streptococcus pyogenes.* [b]MIC, Minimum inhibitory concentration. [c]QS, Quorum sensing. [d]MBIC, Minimal biofilm inhibitory concentration (i.e. the lowest concentration of an antimicrobial substance at which there is no time-dependent increase in the mean number of biofilm viable cells). [e]EPS, Extracellular polymeric substance.

Particularly interesting are furanones from *Delisea pulchra* algae due to their broad spectrum of antibiofilm effect. Brominated furanone decreased biofilm formation onto polystyrene by *Campylobacter jejuni* (Castillo et al. 2015), *S. epidermidis* and *P. aeruginosa* (Pereira et al. 2014), with little or no inhibition of planktonic bacterial growth. Similarly, furanones with side chains were active towards *Salmonella* Typhimurium biofilm, probably due to the bromination patterns of the furanone ring structure and/or to the alkyl chain length at the three position on the ring (Steenackers et al. 2010).

Despite the increasing evidence for *in vitro* antibiofilm activity of algae and algae-derived compounds, only one study indicated their potential for *in vivo* use. Shibata et al. (2003) found that fucoidans from the brown alga *Cladosiphon ocamuranus*, contrarily to non-sulphated (dextran and mannan) and carboxylated polysaccharides, significantly suppressed the attachment of *Helicobacter pylori* to the gastric mucosa in Mongolian gerbils at pH 2.0.

Overall, the findings from *in vitro* studies suggest that lichens and algae might represent an important resource for providing antibiofilm compounds. However, these preliminary findings should be confirmed by further studies aimed to study the mechanisms underlying antibiofilm activity, as well as to evaluate the *in vivo* potential and the safety profile, also considering targeted delivery systems.

5.4. Microbial compounds

Several microorganisms in complex ecological niches produce bioactive compounds with antimicrobial activities to gain advantages over other microbes. Recent studies have identified several secondary metabolites, extracted from both bacteria (Table 3) and fungi (Table 4), with antibiofilm properties (de Carvalho et al. 2015; Deepa et al. 2015; Oja et al. 2015; Rybalchenko et al. 2015; Stumpp et al. 2015; Arora et al. 2016; Ben Taheur et al. 2016; Melo et al. 2016; Sharma et al. 2016; Younis et al. 2016; Chang et al. 2017; Kang et al. 2017; Krishnamoorthy et al. 2018; Rashmi et al. 2018; Shokri et al. 2018; Song et al. 2018; Tiwari et al. 2018; Wasfi et al. 2018; Yuyama et al. 2018; Zhang et al. 2018; Chen et al. 2019; Narmani et al. 2019; Petersen et al. 2019; Song et al. 2019; Mangzira Kemung et al. 2020; Roscetto et al. 2020; Shin and Eom 2020).

5.4.1. Bacterial compounds

Numerous studies support the potential of *Streptomyces* as a promising source of antibiofilm compounds. Mangzira Kemung et al. (2020) reported the anti-MRSA biofilm activity of the methanolic extract of *Streptomyces* sp. strain MUC 125 isolated from mangrove soil in Malaysia, mainly due to the iron chelating activity by 2,3-dihydroxybenzoic acid. Among the compounds detected by mass spectrometry analysis, pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro deserves further study as it was non-cytotoxic to mouse embryo fibroblast, as well as non-haemolytic. In another study (Younis et al. 2016), ethyl acetate secondary metabolites extract of a *Streptomyces* strain from Iraqi marine sediment showed potential for the development of new drugs to treat urinary tract infections. Indeed, at sub-MICs the extract inhibited biofilm formation and behaviour associated with uropathogenic *P. mirabilis* by interfering with the QS base molecule signal and by affecting swimming motility. Oja et al. (2015) observed that the presence of the ribose moiety in form of pyranose was essential for the activity of several pyrano-naphthoquinone polyketides from *Streptomyces* spp. towards the mature *S. aureus* biofilm. Furthermore, the most active polyketides – alnumycin D and granaticin B – shared the same structural features required for antibiofilm activity: glycosylated and uncharged, with a similar oxygenation pattern of the lateral naphthoquinone ring. However, granaticin B was more toxic to human adenocarcinoma cells, while alnumycin D displayed selective toxicity towards bacterial cells (Oja et al. 2015).

Other studies highlighted marine microbes as bioresources for the development of QS inhibitors (QSIs) and the identification of novel antifouling agents. Song et al. (2018) found that several bacterial strains isolated from the coral *Pocillopora damicornis* exhibited QS inhibitory activity. Particularly, the typical coral symbiotic bacterium H12-*Vibrio alginolyticus* inhibited *P. aeruginosa* PAO1 biofilm formation due to the disruption of the *las* and/or *rhl* system throughout a rhodamine isothiocyanate analogue. Previous findings showed that isothiocyanate is non-toxic to the mammalian cell line L929 (Borges et al. 2014). In another study (Chen et al. 2019), three compounds isolated from marine *Oceanobacillus* sp. XC22919 – i.e. 2-methyl-N-(2′-phenylethyl) butyramide, 3-methyl-N-(2′-phenylethyl)-butyramide and benzyl benzoate – showed clear QS inhibitory activity causing a dose-dependent reduction in biofilm formation by *P. aeruginosa*. Despite these promising findings, most QSIs are unsuitable for use in humans due to their toxicity, high reactivity, and instability.

Analogues of N-acyl homoserine lactone-based QS have been identified by chromatographic analysis from the extract of the *Rhizobium* sp. NAO1 isolated from the North Atlantic Ocean (Chang et al. 2017). These molecules were able to inhibit biofilm formation by *P. aeruginosa* PAO1 up to 77.9% through QS inhibition, in a dose-dependent manner and without affecting bacterial growth. The damaged biofilm was also significantly more susceptible to kanamycin, thus indicating the rationale for a combined therapeutic approach. Additional studies aimed at purifying and characterising *Rhizobium* sp. NAO1 extracts are necessary to elucidate the mechanisms of action responsible for the inhibitory properties.

The production of secondary metabolites with antibiofilm activity could represent another strategy adopted by pathogens to persist in the hospital setting. In this regard, Tiwari et al. (2018) observed that all ESKAPE (*Enterococcus faecium, S. aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa*, and *E. cloacae*) pathogens carried antimicrobial secondary metabolites, most of which (82.6%) had antibiofilm effect against each other. Interaction studies indicated that the production of c-di-GMP, a critical determinant for QS and biofilm formation, is affected by the inhibition of diguanylate cyclases activity. Screening of antimicrobial metabolites from ESKAPE pathogens could lead to the development of antibiotics for the control of infections caused by ESKAPE or other pathogens.

Synthetic derivatives of carolacton – a secondary metabolite isolated from the extracts of *Sorangium cellulosum*, a soil-dwelling Gram-negative bacterium belonging to myxobacteria group – were found to affect biofilms formed by oral plaque-forming bacteria (Stumpp et al. 2015). Their activity on *S. mutans* requires bacterial enzymatic hydrolysis which provides native carolacton resulting in biofilm damage *in vivo*. The primary effect towards *S. mutans*, *Streptococcus oralis* and *Streptococcus gordonii* consisted in a species-specific defect in cell wall synthesis and cell division, also observed in the Gram-negative periodontal pathogen *Aggregatibacter actinomycetemcomitans*.

In another study (Deepa et al. 2015), three cyclic dipeptides purified from the ethyl acetate extract of an *Achromobacter* sp. associated with a *Rhabditis* entomopathogenic nematode, displayed promising antibiofilm property against wound pathogenic bacteria (*B. subtilis*, *S. aureus, S. epidermidis*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *Salmonella* Typhi, *P. vulgaris*, and *E. faecium*). Particularly, the combination of peptides – cyclo(D-Leu-D-Arg), cyclo(L-Trp-L-Arg), and cyclo(D-Trp-D-Arg) – and ampicillin acted synergically against biofilm formation. Cyclic dipeptides showed no cytotoxicity towards normal fibroblasts, VERO cells, and L231 normal lung epithelial cells (Deepa et al. 2015), thus indicating their potential for therapeutic use against paradigmatic examples of biofilm-based infections, namely those involving skin and soft-tissue, and lung.

Several studies have investigated the inhibitory effect of cell-free supernatants (CFS) derived from probiotics against biofilm formation by pathogenic bacteria. CFS from *Clostridium butyricum* caused a reduction in biomass and thickness of *A. baumannii* biofilm (Shin and Eom 2020), and these effects were closely related to the inhibition of motility. In another study (Song et al. 2019), *Lactobacillus rhamnosus* GG microcapsules were found to decrease biofilm formation in *E. coli* because of the transcriptional inhibition of the *luxS* QS pathway mediated by the excretion of an AI-2 signalling molecule. Relevant antibiofilm properties have been demonstrated by *Lactobacillus fermentum* CFS as suggested by the broad spectrum of susceptible pathogens (Rybalchenko et al. 2015; Melo et al. 2016; Shokri et al. 2018). The activity against biofilm formed by MDR *P. aeruginosa* is mediated by the production of organic acids (i.e. lactic acid, acetic acid, and formic acid) (Shokri et al. 2018), whereas the modulation of the *ica* operon is critical in the case of *S. aureus* biofilm (Melo et al. 2016). Biofilm formation in *S. aureus* is also affected by *Ligilactobacillus salivarius* whose secretome contains five proteins – including a LysM-containing peptidoglycan binding protein and a protein peptidase M23B – with antibiofilm potential (Kang et al. 2017).

The activity of lactic acid bacteria against oral biofilm pathogens has also been investigated with a view to their use in the prevention and treatment of dental caries. Exposure to CFS of *Lacticaseibacillus casei, Limosilactobacillus reuteri, Lactobacillus salivarius,* and *Lactiplantibacillus plantarum* reduced both adhesion and biofilm formation by the caries-inducing *S. mutans* (Wasfi et al. 2018). *L. salivarius* was the most active in affecting adherence and biofilm formation (87% and 47% inhibition, respectively), because of reduced EPS production mediated by *vicRKX* operon genes down-expression. In another study (Ben Taheur et al. 2016), *Levilactobacillus brevis* FF2 and *Pediococcus pentosaceus* FG1 extracts inhibited 50% of biofilm respectively formed by *Streptococcus constellatus* B629 and *S. oralis*.

5.4.2. Fungal compounds

Among the terrestrial ecosystems, forests represent an enormous reservoir of pathogenic and endophytic fungi able to biosynthesize phytotoxic secondary metabolites that also possess antibiofilm properties.

Sphaeropsidin A – a diterpenoid produced by the pathogenic fungus *Diplodia corticola* of forest plants – significantly affected biofilm formation by MRSA and *P. aeruginosa* MDR strains at sub-inhibitory concentrations (Roscetto et al. 2020). However, it did not show sufficient selectivity between bacteria and human cells – e.g. keratinocytes – thus warranting further studies for defining its potential in the treatment of wound infections. In another study, vulculic acid and curvulol, polyketides from the plant-associated fungus *Chaetosphaeronema achilleae*, almost completely inhibited biofilm formation by *S. aureus* at sub-inhibitory concentrations, although they showed significant

Table 5. Some representative bioactive compounds from animal sources with antibiofilm activity.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
LL-37	Human skin	*S. aureus* *P. aeruginosa*	Biofilm formation reduction: 70% at 1/4xMIC[b] (16 μg/mL) (*P. aeruginosa*) Mature biofilm reduction: about 100% at 1/2xMIC (10 μM) (*S. aureus*)	↑ cell membrane permeability	(Overhage et al. 2008; Dean et al. 2011; Bandurska et al. 2015; Shurko et al. 2018; Ridyard and Overhage 2021)
LL-13 LL-17 17tF-W	Synthetic from LL-37	VRSA	Biofilm formation reduction: 100% at 1/4xMIC (128 μg/mL) (LL-13, LL-17) 50% at 1/2xMIC (32 μg/mL) (LL-17)	↓ adhesion ↓ *rhlA*, *rhlB* and *lasB* expression ↑ biosynthesis of type IV pilus	(Overhage et al. 2008; Shurko et al. 2018)
Indolicidin	Bovine neutrophils	*P. aeruginosa*	Biofilm formation reduction: 70% at 1/4xMIC (16 μg/mL)	Not reported	(Overhage et al. 2008)
SMAP-29	Sheep neutrophils	Cystic fibrosis strains: *S. maltophilia* *P. aeruginosa*	Biofilm formation reduction: 40–90% at 1/2xMIC (2 μM) (*P. aeruginosa*, *S. maltophilia*) Mature biofilm reduction: 90% at 4 μM (*S. maltophilia*)	↑ cell membrane permeability	(Pompilio et al. 2011; 2012)
BMAP-27 BMAP-28	Bovine neutrophils	Cystic fibrosis strains: *S. maltophilia* *P. aeruginosa* *S. aureus*	Biofilm formation reduction: 50–80% at 1/2xMIC (4–8 μM) (*S. maltophilia*) 20–60% at 1/2xMIC (2–4 μM) (*P. aeruginosa*) 40–60% at 1/2xMIC (8 μM) (BMAP-28 *vs. S. aureus*) Mature biofilm reduction: 50–90% at 1xMIC (8–16 μM) (*S. maltophilia*) 30–80% at 1xMIC (16 μM) (*S. aureus)*	↑ cell membrane permeability	(Pompilio et al. 2011; 2012)
17tF-W	Synthetic from LL-37	*S. aureus* USA 300	Eradication of biofilm in mouse-embedded catheters (after 250 μg/die for 3 days)	Not reported	(Narayana et al. 2019)
IDR-1018 (derived from C12 cathelicidin)	Bovine neutrophils	*P. aeruginosa* *E. coli* O157:H7 *A. baumannii* *B. cenocepacia*	Biofilm formation reduction ($MBIC_{50}$[c]) at: 5 μg/mL (*P. aeruginosa*) 8 μg/mL (*E. coli*) 2 μg/mL (*A. baumannii*) 2 μg/mL (*B. cenocepacia*)	Binding to ppGpp biofilm regulator	(de la Fuente-Núñez et al. 2014)
AS-10 (derived from the mouse cathelicidin CRAMP[d])	Mouse skin and neutrophils	*S. epidermidis*	Biofilm formation reduction: 80% at 10 μM	Not reported	(De Brucker et al. 2014)
Human β-defensin 3	Human lesioned psoriatic skin, lung, gingival epithelia	*S. epidermidis* MRSE *S. aureus*	Mature biofilm reduction: 90% at 8 μg/mL (2xMIC) (*S. epidermidis*) 90% at 16 μg/mL (2xMIC) (MRSE) 95% at 16 μg/mL (2xMIC) (*S. aureus*)	Membrane lysis due to pore formation Binding to DNA and RNA Muramidases activation	(Zhu et al. 2013; 2017)
α-defensin HNP-1	Human immune cells	*E. coli* (MDR, uropathogenic)	Inhibition and eradication of biofilm at: 30–250 μg/mL (1/4x to 2 × MIC)	Not reported	(Moazzezy et al. 2020)

(*continued*)

Table 5. Continued.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Lactoferrin	Various secretory fluids	*P. aeruginosa* *S. pneumoniae* Group B *Streptococcus*	Mature biofilm reduction: 80–90% at 40–80 μM (*S. pneumoniae*) Eradication of *S. pneumoniae* mature biofilm onto human pharyngeal cells Inhibition of group B *Streptococcus* adherence to human gestational membranes	Iron chelation Extracellular DNA degradation	(Kamiya et al. 2012; Angulo-Zamudio et al. 2019; Lu et al. 2021a)
Lactoferrin-derived peptides	Bovine lactoferrin hydrolisate	*P. fluorescens*	Biofilm formation reduction: 25% at 1.5 mg/mL 74% at 3 mg/mL	Cell wall modification ↓ motility (swimming, swarming)	(Quintieri et al. 2019)
Pleurocidin	*Pleuronectes americanus* (skin of the winter flounder)	*P. aeruginosa*	Biofilm formation reduction ($MBIC_{50}$): 4–16 μM at 1/4xMIC	Not reported	(Gopal et al. 2013)
Piscidins (p1, p3)	Mast cells of hybrid striped bass	*P. aeruginosa* PA01	Mature biofilm reduction: 50–90% at 18–16 μM 99% at 16 μM (piscidin p3 + Cu^{2+})	↑ cell membrane permeability (p1) ↑ cleavage of extracellular DNA (p3)	(Libardo et al. 2017)
DHA[e] EPA[f]	Herring oil	*S. aureus*	Biofilm formation reduction: >75% at 100 μg/mL >65% at 20 μg/mL	↓ α-hemolysin (*hla*) expression	(Kim et al. 2018)
Dermaseptin-PS4	*Phyllomedusa sauvagii*	*S. aureus* *P. aeruginosa* *E. coli*	MBIC[g] equals to: MIC (4 and 8 μM, respectively for *S. aureus* and *E. coli*) 2xMIC (32 μM, *P. aeruginosa*) MBEC[h] equals to: 4xMIC (*E. coli*) 8xMIC (*S. aureus*) 16xMIC (*P. aeruginosa*)	Membrane perturbation	(Chen et al. 2019)
Brevinin-1GHa	*Hylarana guentheri*	*S. aureus* *E. coli*	MBIC equals to: 2xMIC (4 μM, *S. aureus*) 8xMIC (32 μM, *E. coli*) MBEC equals to: 8xMIC (*S. aureus*) 16xMIC (*E. coli*)	Membrane perturbation	(Chen et al. 2018)
Citropin 1.1	*Litoria citropa*	*S. aureus*	MBEC equals to 8xMIC (16 μg/mL)	Not reported	(Dawgul et al. 2016)
Temporin A	*Rana temporaria*	*S. aureus*	MBEC equals to 4xMIC (16 μg/mL)	Not reported	(Dawgul et al. 2016)
Distinctin-like-peptide-PH	*Phyllomedusa hypochondrialis*	*E. coli*	MBIC equals to 4xMIC (128 μg/mL) MBEC equals to 8xMIC	Not reported	(Wu et al. 2018)
Temporin-GHc	*Hylarana guentheri*	*S. mutans*	Biofilm formation reduction: 50% at 2xMIC (25.2 μM)	↓ EPS formation (by down expression of *gtfs* genes)	(Zhong et al. 2019)
Dermaseptin DMS-Ps1	*Phyllomedusa sauvagei*	*E. coli* *S. aureus*	MBIC equals to 2xMIC (32 μg/mL for both species) MBEC equals to 8xMIC (*S. aureus*)	Not reported	(Song et al. 2020)

(*continued*)

Table 5. Continued.

Bioactive compound	Source	Target microorganism[a]	Antibiofilm activity	Mechanism	Study
Medusin PT1a	*Phyllomedusa tarsius*	*S. aureus*	MBIC equals to 4xMIC (32 μg/mL) MBEC equals to 8xMIC	Not reported	(Gao et al. 2017)
Temporin B	*Rana temporaria*	*S. aureus*	Biofilm formation reduction at 24 μM	Not reported	(Grassi et al. 2017)
Ranatuerin-2Pb	*Rana pipiens*	*S. aureus* *E. coli*	MBIC equals to MIC (8 μM for both species) MBEC equals to: 4xMIC (*S. aureus*) 8xMIC (*E. coli*)	Not reported	(Zhou et al. 2019)
Melittin	*Apis mellifera* venom	*P. aeruginosa* *E. coli* *K. pneumoniae*	Biofilm formation reduction at 1/10x and 1/100xMIC (MIC: 4–8 μM)	Not reported	(Dosler et al. 2016)
Hybrid melittin-cectropin	Synthetic from insect peptides	MRSA	Biofilm formation inhibition at 1/10xMIC (0.4 μM), alone and combined with antibiotics	Not reported	(Mataraci and Dosler 2012)
Propolis	*Apis mellifera* L.	*P. aeruginosa*	Biofilm formation reduction by 50% at 50 μg/mL	↓ motility (swimming)	(De Marco et al. 2017)
Methylglyoxal	Manuka (*Leptospermum scoparium*) honey	*S. aureus*	Biofilm reduction in an ovine model of biofilm-induced sinusitis: 85% at 1.8 mg/mL	Not reported	(Paramasivan et al. 2014)
Melanin Melanin-based chemicals and enzymes Polysaccharides Proteins Protein ASTM-1	Ink from *Sepia esculenta*	*E. faecalis* *P. aeruginosa* *P. vulgaris*	Biofilm formation reduction: 85.2% (*E. faecalis*) 78.7% (*P. aeruginosa*) 87.7% (*P. vulgaris*) Mature biofilm reduction at 0.1 mg/mL	Damage of cell membrane and wall Biofilm disrupting action	(Kumar et al. 2018)
Terpenoids ageloxime-D Manoalide	Marine sponges	*S. epidermidis*	Not reported	Not reported (not bactericidal)	(Hertiani et al. 2010)
Pyrrole-imidazoles (lead compounds)	*Agelasidae* (marine sponges)	MRSA *A. baumannii* (MDR strains)	Biofilm formation reduction: 50% at 6–25 μM (1/2x, 1/4xMIC) (MRSA) 50% at 25–50 μM (1/4xMIC) (*A. baumannii*) Mature biofilm reduction: 50% at 30–40 μM (MRSA)	Dispersal enhancement	(Forte et al. 2009; Rogers et al. 2010; Furlani et al. 2013)

[a]*S. aureus, Staphylococcus aureus; P. aeruginosa, Pseudomonas aeruginosa*; VRSA, vancomycin-resistant *S. aureus; S. maltophilia, Stenotrophomonas maltophilia; E. coli, Escherichia coli; A. baumannii, Acinetobacter baumannii; B. cenocepacia, Burkholderia cenocepacia; S. epidermidis, Staphylococcus epidermidis;* MRSE, methicillin-resistant *S. epidermidis;* MDR, multidrug-resistant; *S. pneumoniae, Streptococcus pneumoniae; P. fluorescens, Pseudomonas fluorescens; S. mutans, Streptococcus mutans; K. pneumoniae, Klebsiella pneumoniae;* MRSA, methicillin-resistant *S. aureus; E. faecalis, Enterococcus faecalis; P. vulgaris, Proteus vulgaris.* [b]MIC, Minimum inhibitory concentration. [c]$MBIC_{50}$, Minimum biofilm inhibition concentration (i.e. the lowest concentration that resulted in ≥50% inhibition of biofilm formation). [d]CRAMP, Cathelin-related antimicrobial peptide. [e]DHA, cis-4,7,10,13,16,19-docosahexaenoic acid. [f]EPA, cis-5,8,11,14,17-eicosapentaenoic acid. [g]MBIC, Minimum biofilm inhibitory concentration (i.e. the lowest concentration of an antimicrobial substance at which there is no time-dependent increase in the mean number of biofilm viable cells). [h]MBEC, Minimum biofilm eradication concentration (i.e. the lowest concentration of antibiotic required to eradicate the biofilm).

cytotoxicity against mouse fibroblast L929 cells and the cervix carcinoma cell line KB-3-1 (Narmani et al. 2019).

Petersen et al. (2019) isolated thirteen fungal strains, dominated by *Penicillium* spp., from the leaf and the root rhizosphere of the Baltic marine flowering plants *Zostera marina*. The organic fungal extracts showed strong anti-QS activity, and the majority of the *Penicillium* extracts displayed antimicrobial and antibiofilm activity against *P. aeruginosa*.

Several cytochalasans, macrocyclic alkaloids isolated from the ascomycete *Hypoxylon fragiforme* and other fungi (Yuyama et al. 2018), as well as extracts obtained from the fruiting bodies of several saprophytic fungi (*Laetiporus sulphureus, Macrolepiota fuliginosa,* and *Macrolepiota procera)* showed significant inhibition of *S. aureus* biofilm formation when tested at sub-MICs (de Carvalho et al. 2015). Further studies aimed at evaluating the selectivity of cytochalasans against mammalian cells are needed as they are known inhibitors of actin and, therefore, could be very toxic to eukaryotic cells.

Another study demonstrated the antagonistic effects of fungal crude extract of an endophytic fungus, *Alternaria alternata* colonising *Carica papaya*, against several QS-associated virulent factors (Rashmi et al. 2018). Particularly, a marked decrease in biofilm formation was observed, probably secondary to a reduction in EPS production, cell surface hydrophobicity, and swimming and swarming motilities. These activities were mediated by sulphurous acid, 2-propyl tridecyl ester and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester present in fungal crude extract (Rashmi et al. 2018).

Several bioactive compounds with antibiofilm potential were isolated from *Aspergillus terreus*. A secondary metabolite extracted from *A. terreus* DMTMGK004 significantly affected the production of EPS both in Gram-positive (*S. pneumoniae*) and Gram-negative (*K. pneumoniae, Haemophilus influenzae*) respiratory pathogens at half MIC value (Krishnamoorthy et al. 2018). Spectral data indicated in carbonyl group and phenolic hydroxyl group as the major functional groups present in the compound; furthermore, it exhibited a very low toxicity against VERO cells. Another secondary metabolite from *A. terreus*, the quinone epoxide terreic acid, inhibited the growth of biofilms generated by *E. coli* with a MBIC corresponding to 2xMIC (Sharma et al. 2016).

Secondary metabolites from sea-derived fungi are potentially a rich source of structurally novel and biologically active compounds. QS inhibitory activity by secondary metabolites of the marine fungus *Fusarium* sp. Z10 was detected using *P. aeruginosa* QSIs-*lasI* biosensor (Zhang et al. 2018). Equisetin, a member of the so-called 3-decalinoyltetramic acid family identified as the major active compound of this fungus, attenuated *P. aeruginosa* PAO1 biofilm formation by 58.3% at 300 μM through the downregulation of QS-related genes expression (Zhang et al. 2018).

By a screening of endophytes derived from *Glycyrrhiza glabra*, Arora et al. (2016) found two thiodiketopiperazine derivatives from a fungal culture of the genus *Phoma* that inhibited biofilm formation in *S. aureus* and even more strongly in *Streptococcus pyogenes* at sub-MIC values (biofilm inhibition at 1/2xMIC: up to 57.2% and 85.9%, respectively). However, in previous studies both derivatives were found to be strongly cytotoxic against various cancer lines (Carr et al. 2009; DeLorbe et al. 2013).

Microorganisms can truly be considered a never-ending goldmine for the development of new antibiofilm strategies. Indeed, the published literature indicates that an enormous variety of microbial bioactive compounds have been reported, although much more can be expected in the future as only a small fraction of microorganisms (<1%) have been explored in nature. However, so far these compounds cannot be used for therapeutic purposes due to low production yield, instable production, high-level purity, and high production costs. Effective strategies could be to use different culture techniques and efficient production methods, such as bacterial recombinant expression, and the isolation of new wild microorganisms from the natural environment whose bioactive proteins could serve as templates for synthetic production.

Despite the increasing body of *in vitro* evidence, to the best of our knowledge none of the above-cited bioactive compounds of microbial origin have been yet evaluated *in vivo* for antibiofilm potential. Efforts should be made to scale up the production of these compounds and explore their potential as antibiofilm agents using *in vivo* models.

5.5. Compounds extracted from human and animal sources

Many natural products isolated from humans and animals exhibit antibiofilm properties. Some representative bioactive compounds are listed in Table 5.

Several compounds belong to the class of host defence peptides (HDPs), also called antimicrobial peptides (Galdiero et al. 2019). They are found in both vertebrates and invertebrates where they serve as antimicrobial effectors of immunity (Wang 2014; Mahlapuu et al. 2016). In vertebrates, including humans,

HDPs function as the first line of defence against pathogens (Chung and Khanum 2017; Dostert et al. 2019), and often display immunomodulatory properties (Wang 2014).

In mammals, the major HDP families are the cathelicidins and defensins, both of which have antibiofilm properties (Duplantier and van Hoek 2013; Wang 2014). LL-37 is the only cathelicidin found in humans and probably the best studied HDP in terms of antibiofilm properties and mechanism of biofilm inhibition (Bandurska et al. 2015; Ridyard and Overhage 2021). The human cathelicidin proved to be effective against *S. aureus*, *A. baumannii* and *P. aeruginosa* biofilms (Overhage et al. 2008; Dean et al. 2011Feng et al., 2013; Ridyard and Overhage 2021). It was observed that LL-37 caused a higher reduction in viability than silver nanoparticles and rifampin (Kang et al. 2019). Moreover, the combination therapy with rifampin, silver nanoparticles and gentamicin was still less effective than LL-37 alone (Kang et al. 2019). Its shorter derivatives – i.e. LL-13 and LL-17 – also inhibited *S. aureus* biofilm production and restored susceptibility in vancomycin-resistant strains (Shurko et al. 2018). LL-37 affected preformed *P. aeruginosa* biofilms and inhibited biofilm formation even at concentrations far below those required to kill bacteria (Overhage et al. 2008), suggesting an inhibitory mechanism not dependent on killing activity. Although the exact mechanism by which LL-37 kills biofilm cells is not fully understood, it has been hypothesized the involvement of an induced bacterial SOS response, the promotion of the twitching motility, and the alteration of the Las and Rhl QS systems resulting in downregulation of genes essential for biofilm development (Overhage et al. 2008). Other authors observed that LL-37 can penetrate the biofilm and exert its bactericidal effect against the embedded bacteria (Kang et al. 2019).

Several non-human cathelicidins have been recognized as having antibiofilm activity although their mechanism of inhibition has not yet been studied in detail. The bovine cathelicidin indolicidin inhibited and dispersed preformed *P. aeruginosa* CF biofilms at subinhibitory concentrations (Overhage et al. 2008). The cathelicidins SMAP-29 (ovine), and BMAP-28 and BMAP-27 (bovine) have been shown to reduce biofilm formation of *S. maltophilia*, *P. aeruginosa* and *S. aureus* (BMAP-28 only) strains from CF patients, and to affect preformed biofilms such as tobramycin (Pompilio et al. 2011, 2012). The mechanism of inhibition appears to be the same as killing activity. Despite these potent antimicrobial properties, LL-37 and other cathelicidins have some limitations including high cost, lower activity in physiological environments, susceptibility to proteolytic degradation and, in some cases, toxicity to human cells (Ridyard and Overhage 2021). Naturally occurring cathelicidins have therefore been used as excellent templates for engineering optimized synthetic peptides with antibiofilm activity and improved properties. A series of novel short LL-37 peptides eliminated MRSA burden *in vivo* in both mouse-embedded catheters and their surrounding tissues (Narayana et al. 2019). Novel derivatives have also proved to affect biofilm through different mechanisms. The antibiofilm peptides IDR-1018, derived from a 12-mer cathelicidin peptide (de la Fuente-Núñez et al. 2014), and AS10 inspired by mouse LL-37 (De Brucker et al. 2014), affected biofilm formation by targeting biofilm-specific properties (Dostert et al. 2019). In this context, the peptide IDR-1018 was shown to target (p)ppGpp and marks this important signal in biofilm development (de la Fuente-Núñez et al. 2014). Understanding the specific mechanism of biofilm inhibition will allow the design of more specific and potent derivatives.

Mammalian α- and β-defensins also have numerous functions other than antimicrobial activity (Hazlett and Wu 2011). In one study, human β-defensin 3 inhibited biofilm formation of *S. aureus* and *S. epidermidis* (Zhu et al. 2013) and showed the potential to eradicate a mature biofilm formed by MRSA strains on orthopaedic implants (Zhu et al. 2013, 2017). Interestingly, inhibition and eradication of MDR uropathogenic *E. coli* biofilm with truncated α-defensin HNP-1 analogues was recently observed, although the mechanism underlying biofilm inhibition has not been investigated (Moazzezy et al. 2020).

The multifunctional defence protein lactoferrin, secreted in various mammalian biological fluids (Giansanti et al. 2016), showed antibiofilm activity against *P. aeruginosa*, probably due to its iron-chelating activity (Kamiya et al. 2012). Interestingly, a study focussing on pneumococcal biofilms showed that lactoferrin inhibited the colonisation of human airway cells by *S. pneumoniae* without affecting bacterial viability (Angulo-Zamudio et al. 2019). The mechanism of inhibition is likely mediated by the DNase activity of lactoferrin, able to degrade extracellular DNA thereby weakening its structure (Angulo-Zamudio et al. 2019). The activity of hydrolysates from bovine lactoferrin (bLFH) was also investigated *in vitro* against skin borne staphylococcal biofilms. Lactoferrin fragments showed better antibiofilm activity than antibacterial activity with a MBIC of 2.5 mg/mL against a MIC of 10–20 mg/mL (Quintieri et al. 2020). In addition, MBIC values were significantly lower for LFcinB or LFmpin, two peptides purified from the hydrolysate. In another study,

Quintieri and colleagues have observed that bLFH significantly reduced *Pseudomonas fluorescens* biofilm formation at 3 mg/kg. Proteomic analysis showed that bLFH suppressed the biofilm regulatory proteins such as PleD, TycC and GbrS and, at the same time, increased negative regulators of alginate biosynthesis (Quintieri et al. 2019). Interestingly, lactoferrin from human breast also exerts antimicrobial and antibiofilm activity against group B streptococci, inhibiting their adhesion to gestational membranes in humans (Lu et al. 2021b).

Several compounds with antibiofilm properties have been isolated from fish including pleurocidin (Gopal et al. 2013), an HDP found in the mucus secreted by the skin of winter flounder, and piscidins, HDPs first discovered in hybrid striped bass (Libardo et al. 2017). While piscidin-1 and piscidin-3 are homologous fish HDPs, only piscidin-1 was strongly membranolytic, while piscidin-3 eradicated bacterial biofilms through DNA-disruptive effects (Libardo et al. 2017).

HDPs are not the only compounds isolated from fish that exhibit antibacterial properties. Omega fatty acids from herring oil, particularly docosahexaenoic acid and eicosapentaenoic acid, markedly repressed *S. aureus* biofilm formation by downregulating the expression of the α-hemolysin *hla* gene and reducing α-hemolysin which is necessary for interactions of cells during biofilm formation (Kim et al. 2018).

Dermal glands of amphibian *Anura* (frogs and toads) are one of the largest resources of HDPs (Mangoni 2006) which display numerous activities including immunomodulatory, anticancer, antiviral and antibiofilm functions (Pantic et al. 2017; Marcocci et al. 2018). With respect to the latter, most studies have focussed on the ability of amphibian skin HDPs to inhibit biofilm formation by clinically relevant microbial species – encompassing *P. aeruginosa* and *S. aureus* – at concentrations generally higher than their MICs and ranging from 3 to 64 μM (Dosler and Karaaslan 2014; Dawgul et al. 2016; Chen et al. 2018; Wu et al. 2018; Chen et al. 2019; Zhong et al. 2019). In comparison, the ability to eradicate mature biofilm formed on plastic supports or medical devices (e.g. microtiter plates, catheters, prostheses, contact lenses) could be achieved at concentrations 4–6 folds higher (Luca et al. 2013; Dawgul et al. 2016; Song et al. 2020). Recent reports have underscored the capability of frog skin HDPs to prevent biofilm development by downregulating the expression of virulence genes, in this way modulating the production of biofilm EPS matrix, as well as QS and/or bacterial motility (Casciaro et al. 2019; Zhong et al. 2019). For some frog-skin derived peptides active against preformed biofilm, a membrane perturbing activity has been proposed as a plausible mechanism underlying killing of biofilm cells, like that previously described for the planktonic counterpart (Luca et al. 2013; Casciaro et al. 2020).

This promising scenario has been strengthened by their *in vivo* antimicrobial efficacy either in a mouse model of *Propionibacterium acnes*-induced inflammation (Wu et al. 2020) or *P. aeruginosa*-induced keratitis/lung infections (Kolar et al. 2015; Chen et al. 2017), and by their evidence in accelerating healing of infected skin lesions in experimental animal models of bacterial-infected wounds (Simonetti et al. 2012). In all these cases, the peptides did not elicit toxicity, thus highlighting their safety profile for clinical use (Wu et al. 2018). Furthermore, the N-terminal fragment of the frog skin antimicrobial peptide esculentin 1a was found to provide protection against lethal *P. aeruginosa* gut infection in the nematode *Caenorhabditis elegans* where bacteria are likely to form biofilms (Uccelletti et al. 2010). In another study, feleucin-K3, an α-helical cationic AMP isolated from the skin secretion of the Asian bombinid toad species *Bombina orientalis* showed better antibiofilm activity than amoxicillin in a model of catheter biofilm infection caused by MRSA (Guo et al. 2021).

Other studies indicated that HDPs represent the most abundant compounds from amphibians that possess antibiofilm properties (Humblot et al. 2009; Gao et al. 2017; Grassi et al. 2017; Zhou et al. 2019). Unlike mammals, HDPs from amphibian skin can well preserve antibacterial activity at concentrations found in their natural environment and in the presence of biological fluids. Some of these HDPs have already progressed into late-stage clinical studies for topical treatment of various types of infections. For example, pexiganan, an analogue of the peptide magainin isolated from *Xenopus* skin, has been evaluated as a cream in phase III clinical trial to cure mild infections in diabetic foot ulcers (Uccelletti et al. 2010).

Invertebrates, such as insects and molluscs, are also an important source of bioactive compounds with antibiofilm properties. The insect haemolytic peptide melittin has been found to inhibit Gram-negative attachment and biofilm formation, both alone and combined with antibiotics (Dosler et al. 2016). Melittin has also been tested in combination with the naturally occurring insect peptide cecropin. The melittin–cecropin hybrid proved to have antibiofilm activity against MRSA and showed synergistic effects when combined with antimicrobial agents (Mataraci and Dosler 2012). Propolis (bee glue), a resinous substance produced by honeybees (*Apis mellifera* L.), has shown *in vitro*

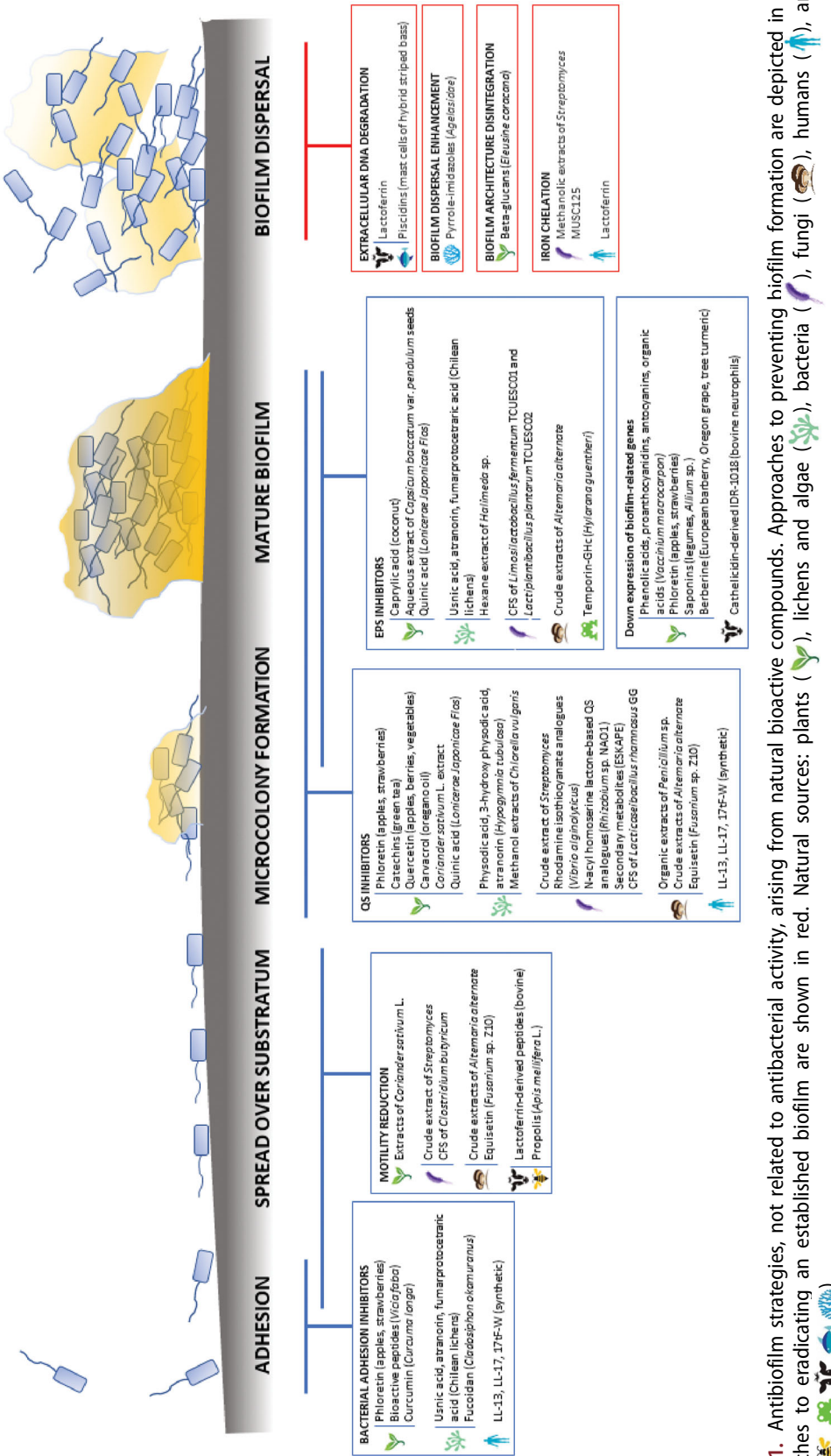

Figure 1. Antibiofilm strategies, not related to antibacterial activity, arising from natural bioactive compounds. Approaches to preventing biofilm formation are depicted in blue; approaches to eradicating an established biofilm are shown in red. Natural sources: plants ( ), lichens and algae ( ), bacteria ( ), fungi ( ), humans ( ), and animals ( ).

antibiofilm activity against *P. aeruginosa* at 50 μg/mL, reducing the biofilm mass to 60%; as well, it significantly reduced the viability of biofilm cells decreasing their swimming motility (De Marco et al. 2017). Among products deriving from bees, Manuka honey presents very interesting properties. Obtained from *Leptospermum scoparium*, it contains high amounts of flavonoids, as well as glyoxal and methylglyoxal, responsible for its antimicrobial activity (Alvarez-Suarez et al. 2014). Recently, its antibiofilm effect has been demonstrated against *P. aeruginosa* MDR strains in two *ex vivo* porcine lung models, made of bronchiolar and alveolar tissues (Roberts et al. 2019). When applied as sinus irrigation, methylglyoxal and Manuka honey were also effective against biofilm formed by *S. aureus* into sheep sinuses, being safe for the mucosa and suggesting a potential use to combat chronic rhinosinusitis (Paramasivan et al. 2014).

Encouraging results were also obtained applying chestnut honey alone or combined with phages to control *E. coli* and *P. aeruginosa* biofilms formed on porcine skin, thus paving the way for potential applications to accelerate wounds healing *in vivo* (Oliveira et al. 2018).

Sepia ink from *Sepia esculenta* contains active compounds, such as melanin and melanin-based enzymes, proteins, and glycosaminoglycans. Particularly, at a concentration of 100 μg/mL protein extract from ink revealed antibiofilm activity against *E. faecalis, P. aeruginosa,* and *P. vulgaris* with 85.3%, 78.7%, and 87.7% of biofilm inhibition, respectively. At the same concentration, it was even able to disrupt the structure of the mature biofilm (Kumar et al. 2018).

Interesting compounds with antifouling and antibiofilm properties have been described in marine sponges (Stowe et al. 2011) which protect themselves by using a plethora of secondary metabolites. Some classes – such as the terpenoids (Hertiani et al. 2010) and the pyrrole-imidazoles (Forte et al. 2009) – have been shown to modulate biofilm formation without killing the bacteria or affecting their growth. Among the terpenoids from sponges, ageloxime-D, manoalide, and two manoalide congeners were reported to be able to affect bacterial biofilms formation of *S. epidermidis*, although the mechanism of action remained unknown (Hertiani et al. 2010). In addition, marine sponges can synthesize a class of potent molecules unique to their phylum, the pyrrole-imidazole alkaloids (PIAs) (Rogers et al. 2010). PIAs have been extracted from several sponge families focussing bromopyrrole derivatives from the family *Agelasidae* (Forte et al. 2009). As natural products, PIAs inhibited biofilm formation by MRSA and *A. baumannii* MDR strains by 50% at subinhibitory micromolar concentrations. In addition, the most active compound acted synergistically with oxacillin against MRSA (Furlani et al. 2013).

6. Concluding remarks and future perspectives

One of the major current challenges in medicine is the development of control strategies in the treatment of biofilm-related infections, especially those associated with indwelling devices and those observed in the lung of CF patients. Indeed, pathogenic biofilms represent a persistent source of chronic infections due to their inherent resistance to both antibiotics and host immune response. Treatment of biofilm-related infections requires the administration of several antibiotics, usually at high doses and for prolonged periods. However, we cannot rely solely on antibiotics because their efficiency is beingly compromised by biofilm-specific structural and physiological properties, and the emergence of MDR strains. Therefore, new antibiofilm templates with novel targets and mechanisms of action are urgently needed.

In this context, natural compounds could represent a promising alternative. Indeed, the studies reviewed here suggest that numerous secondary metabolites and peptides from plants, lichens, algae, bacteria, fungi, animals and even humans are effective against biofilm formation and, in some cases, have the potential to disperse mature (established) biofilms (Forte et al. 2009; Dean et al. 2011; Pompilio et al. 2012; Furlani et al. 2013; Pompilio et al. 2013; Zhu et al. 2013; Bandurska et al. 2015; Oja et al. 2015; Dawgul et al. 2016; Gao et al. 2017; Kang et al. 2017; Libardo et al. 2017; Goldoni Lazarini et al. 2018; Kumar et al. 2018; Angulo-Zamudio et al. 2019; Chen et al. 2019; Kang et al. 2019; Song et al. 2019; Zhong et al. 2019; Zhou et al. 2019; Galdiero et al. 2020; Gupta et al. 2020).

It is interesting to note that some classes of compounds with antibiofilm properties occur in phylogenetically very distant organisms. Polyphenols, terpenoids and fatty acids displaying antibiofilm activity have been detected in many organisms, especially plants, microorganisms, and invertebrates. In contrast, vertebrates appear to antagonize biofilms with defense peptides rather than other secondary metabolites. Thanks to advances in nanotechnology, computational studies to improve peptides stability/bioavailability, and peptide synthesis technologies that reduce manufacturing costs, HDP-based formulations have acquired great potential to be developed as innovative drug candidates in either

the cosmetics or in pharmaceutical industries in the coming decades (Mahlapuu et al. 2020).

Natural products have historically been compounds of choice in the discovery of new drugs against infectious diseases, especially antibiotics. As genetically encoded products of natural selection and structurally shaped by evolution to interact with biological targets, natural products are a promising pool for the discovery of scaffolds with enormous structural diversity and various bioactivities. In addition, they act on bacteria through multiple mechanisms that are also distinct from those of antibiotics. However, several and not trivial challenges must be overcome before some natural molecules can be translated into real clinical intervention strategies, which has contributed to their unattractiveness to the pharmaceutical industry since the 1990s.

First, due to chemical stability, structural complexity, occurrence, identification and purification, the discovery of a new drug is time-consuming and laborious. Therefore, new technological developments – such as metabolomics, the use of vast databases of theoretical natural products spectra, bioinformatics, analytical chemistry, and synthetic biology – represent promising tools for drug discovery (Atanasov et al. 2021). High-throughput screening of large libraries could also speed up the development of new antibiofilm natural drugs and their introduction into clinical practice. Considering that biofilm formation is regulated by multiple, interplayed, cellular mechanisms, a "top-down" approach – i.e. whole biofilm cells-based – should be preferred to a target-based screening ("bottom-up") which requires the knowledge of already identified biofilm targets.

A second challenge is the lack of standardized antibiofilm methods, due to the uselessness of procedures applied to classical antimicrobial drugs, and the need to evaluate the results from *in vitro* biofilm assays for their predictive value for *in vivo* clinical outcome.

Third, we need to understand the exact mechanisms by which these molecules exert their effects at sub-lethal concentrations. Many bioactive molecules here described have not yet been characterized in terms of their mechanism of action (Overhage et al. 2008; Hertiani et al. 2010; Steenackers et al. 2010; Mataraci and Dosler 2012; Gopal et al. 2013; De Brucker et al. 2014; Dosler and Karaaslan 2014; Castillo et al. 2015; de Carvalho et al. 2015; Deepa et al. 2015; Mitrovic et al. 2015; Sun et al. 2015; Arora et al. 2016; Dawgul et al. 2016; Sharma et al. 2016; Goldoni Lazarini et al. 2018; Jun et al. 2018; Wu et al. 2018; Yuyama et al. 2018; Narayana et al. 2019; Narmani et al. 2019; Galdiero et al. 2020; Moazzezy et al. 2020; Roscetto et al. 2020; Song et al. 2020). Further studies will, therefore, be necessary to gain further mechanistic insights into the molecular mechanism of action by which natural compounds exert their antibiofilm activity. Particular attention should be paid to mechanisms of action independent of the antimicrobial activity of the compound by targeting specific molecular pathways that regulate biofilm formation. In this regard, some molecules here discussed, and shown in Figure 1, have been reported to: (i) prevent bacterial adhesion (Overhage et al. 2008; Wang et al. 2009; Lee et al. 2011; Chen et al. 2016; Pompilio et al. 2016; Karkouch et al. 2017; Rashmi et al. 2018; Shurko et al. 2018; Xue et al. 2020); (ii) inhibit QS-related pathways (Overhage et al. 2008; Lee et al. 2011; Burt et al. 2014; Younis et al. 2016; Chang et al. 2017; Özyiğitoğlu et al. 2017; Rashmi et al. 2018; Shurko et al. 2018; Song et al. 2018; Tiwari et al. 2018; Wang et al. 2018; Zhang et al. 2018; Petersen et al. 2019; Song et al. 2019; Sridevi et al. 2019; Kumar et al. 2020; Molina et al. 2020; Lu et al. 2021a); (iii) affect EPS production (Melo et al. 2016; Pompilio et al. 2016; Abdullah Gadhi et al. 2018; Rashmi et al. 2018; Von Borowski et al. 2019; Zhong et al. 2019; Gupta et al. 2020; Lu et al. 2021a); (iv) downregulate genes, others than QS genes, that control biofilm lifestyle (Lee et al. 2011; de la Fuente-Núñez et al. 2014; Zhong et al. 2019; Shang et al. 2020); (v) interfere with bacterial motility (Younis et al. 2016; De Marco et al. 2017; Rashmi et al. 2018; Zhang et al. 2018; Quintieri et al. 2019; Molina et al. 2020; Shin and Eom 2020); and (vi) destabilize biofilm structure (e.g. binding to extracellular DNA) (Wang et al. 2009; Kamiya et al. 2012; Zhu et al. 2013; Chen et al. 2016; Libardo et al. 2017; Zhu et al. 2017; Kumar et al. 2018; Angulo-Zamudio et al. 2019; Divya et al. 2020). These mechanisms often act synergically and seem to be more advantageous as they do not exert selection pressure on bacteria and because they act specifically. However, the use of these antivirulence approaches should be considered with caution since they may favour or select more virulent strains. In this frame, several studies have shown the development of resistance to QSI, although with lower frequency compared to antibiotics (Borges et al. 2016).

Fourth, as for any drug development strategy, *in vivo* studies focussed on the pharmacokinetics and pharmacodynamics are also needed before natural products can be successfully used in clinical practice. Indeed, despite the large number of natural products with antibiofilm potential, not a single FDA-approved drug has been developed. This is generally due to failures in phase II and phase III clinical trials (Lu et al. 2019), probably because of the limited availability of the compound in humans after administration. Another

important aspect to consider is the evaluation of the toxicologic profile. Toxicity is not often reported in research articles, but the few studies evaluating the toxic potential using *in vivo* (Goldoni Lazarini et al. 2018; Von Borowski et al. 2019; Molina et al. 2020) or *ex-vivo* (Borges et al. 2014; Deepa et al. 2015; Oja et al. 2015; Narmani et al. 2019; Mangzira Kemung et al. 2020; Roscetto et al. 2020) models are very encouraging for *in vivo* use of bioactive compounds.

A very promising area of research is the use of natural compounds in coating the surface of medical devices (e.g. catheters) to affect bacterial adhesion, the first step of biofilm formation (Narayana et al. 2019; Lu et al. 2021a). In fact, infections associated with medical devices are the main cause for recurrent surgeries and even patient's death. Further efforts should be made to perform *in vivo* studies and to develop systems in the micro/nano scale (Xue et al. 2020) to allow controlled delivery of the antibiofilm compound at the infection site.

It is also becoming increasingly important to study more in-depth the potential for combinatorial therapy, such as the syncretic coupling between clinically used antibiotics and natural products. Given the ever-increasing prevalence of MDR bacteria, the combinatorial approach could be a powerful tool to select an antimicrobial chemotherapy more effective at low doses, which may reduce both toxicity and emergence of resistant strains. Using this approach several unexpected additive or synergistic interactions with increased antibiofilm activity have already been identified (Mataraci and Dosler 2012; Deepa et al. 2015; Chen et al. 2016; Dosler et al. 2016; Chang et al. 2017; Shurko et al. 2018).

Finally, natural compounds could be used as antibiotic potentiators/adjuvants that can restore the antibiotic susceptibility in MDR pathogens.

In conclusion, the relevant impact of biofilm-associated infections on the national health care system, in terms of high morbidity and huge costs, provides the incentive to direct future research towards the identification of novel therapeutic strategies. Although the applicability of compounds from natural sources in clinical settings remains to be determined, the promising literature data herein reported suggest that the isolation of bioactive molecules should be the next target of a highly integrated interdisciplinary research to develop new antibiofilm drugs and strategies, thus providing a pathway to anti-infective therapeutics.

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ORCID

Arianna Pompilio http://orcid.org/0000-0002-6309-6981
Marco Scocchi http://orcid.org/0000-0003-1247-3641
Maria Luisa Mangoni http://orcid.org/0000-0002-5991-5868
Samira Shirooie http://orcid.org/0000-0002-5000-3387
Annalisa Serio http://orcid.org/0000-0002-0473-9318
Ygor Ferreira Garcia da Costa http://orcid.org/0000-0002-2648-2285
Maria Silvana Alves http://orcid.org/0000-0002-9153-2402
Gökçe Şeker Karatoprak http://orcid.org/0000-0001-5829-6914
Ipek Süntar http://orcid.org/0000-0003-4201-1325
Haroon Khan http://orcid.org/0000-0002-1736-4404
Giovanni Di Bonaventura http://orcid.org/0000-0001-5850-2810

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