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FIGHTING THE RESISTANCE: ALGAE AS KEY ORGANISMS AGAINST ANTIBACTERIAL RESISTANCE

AN OVERVIEW OF RESISTANCE MECHANISMS, ALGAE COMPOUNDS AND COMMERCIAL CHALLENGES

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Dissertação no âmbito do Mestrado em Biologia celular e molecular orientada pela Professora Doutora Gabriela Conceição Duarte Jorge da Silva e pela Professora Doutora Paula Maria de Melim Vasconcelos de Vitorino Morais e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Muito obrigado a todos.

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List of Abbreviatures

ABR	**Antibacterial resistance**
WHO..	**World Health Organization**
CDC..	**Center for Disease and Control**
GHT..	**Genetic Horizontal Transfer**
MRSA..	**Methicillin-resistant *Staphylococcus aureus***
CAFO	**Concentrated Animal Feeding Operations**
NIH	**National Institute of Health**
MGE	**Mobile Genetic Elements**
MLS	**macrolidelincosamide-streptoGramin**
ESBL	**Extended Spectrum Beta-Lactamases**
PBP	**Penicillin binding proteins**
EPS	**Extra Polymeric Substance**
LPS	**Lipopolysaccharide**
PIA	**polysaccharide intracellular adhesin**
CF	**Cystic Fibrosis**
QS	**Quorum-sensing**
AHL	**Acyl Homoserine Lactone**
AIP	**Auto Inducer Peptide**
AI-2	**Auto Inducer-2**
BHL	**N-butanoyl L-homoserine lactone**
PA	**Phenolic Acid**
HBA	**hydroxybenzoic acid**
HCA	**Hydroxycinnamic acid**
EAE	**Enzyme Assisted Extraction**
UAE	**Ultrasound Assisted Extraction**
SFE	**Supercritical fluid extraction**
MAE	**Microwave Assisted Extraction**

Resumo

A resistência bacteriana é um dos problemas de saúde pública do século XXI. No geral, o abuso e consumo de antibacterianos nos diversos setores, como o da agricultura e cuidados de saúde, promoveu a resistência de bactérias a praticamente todos os compostos antibacterianos disponíveis no mercado. Além dos mecanismos de resistência, amplificados a partir da pressão seletiva constante deste consumo exacerbado (por exemplo, bombas de efluxo, alteração mutacional do alvo e inibição enzimática do agente antibiótico), o desafio imposto por infeções relacionadas com formação de biofilmes, caracterizado por forte recalcitrância, requer soluções alternativas na exploração e desenvolvimento de novos agentes terapêuticos. Os compostos bioativos isolados de ambientes aquáticos (marinhos ou de água doce), macro- e microalgas, apresentam grande potencial terapêutico, devido à sua variabilidade de compostos, que se apresentam como novos agentes antibióticos alternativos. Os metabólitos primários e secundários produzidos por esses organismos apresentam uma miríade de propriedades bioativas com reconhecido potencial para serem introduzidos no mercado comercial. No entanto, a variabilidade inerente desses organismos e consequente variabilidade na extração é um sério desafio para a padronização dos métodos de extração, etapa essencial na transferência de tecnologia para o setor industrial. Este trabalho apresenta uma visão geral dos desafios atuais na resistência bacteriana, como a utilização de antibióticos em ambiente antropogénico e mecanismos de resistência, com uma exploração mais profunda da ecologia da estrutura de biofilmes e a sua ligação à infeção e resistência à terapêutica. Além disso, são apresentados compostos bioativos encontrados em algas, com discussão dos seus efeitos antibacterianos e o desafio da transferência desta tecnologia e conhecimento do laboratório para o ambiente industrial e comercial.

Palavras-chave: Resistência bacteriana, mecanismos de resistência, Ecologia Biofilmes, Compostos bioativos de algas, Métodos de extração;

Abstract

Antibacterial resistance (ABR) is one of the public health problems of the XXI century. Overall, abuse in use and consumption of antibacterials in several sectors, such as agriculture and healthcare, has promoted bacterial resistance to practically all therapeutic compounds available in market. Apart from conventional resistance methods, amplifyed from the constant selective pressure (e.g. efflux pumps, mutational alteration of target of enzymatic inhibition of antibiotic agent), the challenge imposed by biofilm-related infections, characterized by strong recalcitrance, requires alternative solutions in exploration and developing of new therapeutic agents. Bioactive compounds isolated from aquatic (marine or freshwater), from macro- and microalgae species, contain great potential in compound variability, that hold future as new alternative antibiotic agents. The primary and secondary metabolites produced by these organisms show a myriad of bioactive properties with recognized potential to be introduced in the commercial market. However, inherent variability of these organisms and compounds produced are a serious challenge for extraction standardization, an essential step in the transference of technology to the industrial sector. This work overviews current challenges in global bacterial resistance, such as anthropological antibiotic uses and bacterial mechanisms of resistance, with deeper exploration of bacterial biofilm ecology and connection to infection and therapeutic resistance. Additionally, it is examined bioactive compounds present in algae, with discussion of their antibacterial effects and the challenge of technological transference from the laboratory to the industrial and commercial setting.

Keywords: Bacterial resistance, mechanisms of resistance, biofilm ecology, Bioactive algae compounds, Extraction methods.

The antibacterial resistance problem

Antibacterial resistance (ABR) has emerged as one of the principal public health problems in the 21st century, affecting all areas of health and consequently the whole of society (Collignon, 2015; Dadgostar, 2019; Prestinaci et al., 2015; Ventola, 2015; WHO & World Health Organization, 2015).

Shortly after antibiotic discovery, Sir Alexander Fleming advised against overuse of antibiotics, as he had already observed bacterial resistance following prolonged exposure (Dadgostar, 2019; Fleming, 1929). Since then, ABR has been found to nearly all antibiotics that have been developed and used in healthcare (Blaskovich, 2018; Chandler, 2019; Collignon, 2015; Dadgostar, 2019; Kinlaw et al., 2017; Klein et al., 2018; Maxwell et al., 2019; Nikaido, 2009; Nikolaidis et al., 2014; Prestinaci et al., 2015; Samanta & Bandyopadhyay, 2020; Schultz, 2018; Ventola, 2015; Viswanathan, 2014; WHO et al., 2019; WHO & World Health Organization, 2015).

Exacerbated usage of these compounds, in diverse sectors such as agriculture, animal production and healthcare, alongside loose management and misinformation regarding their proper usage, has led to a constant selective pressure on bacterial communities, therefore pushing development of resistance. ABR is a leading cause of mortality and morbidity globally (Klein et al., 2018), as it threatens prevention and treatment of wide range of infections, results in severe illnesses and longer hospital stays, increases in healthcare cost specially in second-line drugs, and recurring treatment failures (Dadgostar, 2019). Pathogenic resistant strains, such as methicillin-resistant *Staphylococcus aureus* and multidrug resistant *Pseudomonas aeruginosa*, members of the *Enterobacteriaceae* family, such as *Klebsiella pneumoniae, Escherichia coli* and *Acinetobacter baumannii* are some of the several bacteria that pose a serious threat in the healthcare setting (WHO & World Health Organization, 2015).

This problem as long been recognized by the World Health Organization (WHO), that in 2001 provided a global framework of interventions with the objective of slowing down the emergence and reduce the spread resistant organisms. CDC declared in 2013 that humanity in now in the post-antibiotic era, and in 2014 WHO warned that the antibacterial resistance crisis is becoming dire (Ventola, 2015) and that a strategies and compromises between countries are required, in order to maintain under control the development and spread of these resistances (Cerceo et al., 2016; Collignon, 2015; Prestinaci et al., 2015; WHO et al., 2019; WHO & World Health Organization, 2015).

However, the global antibiotic consumption has increased dramatically over the last decade, with an overall growth on antibiotic consumption of 39% per person and globally an increase in 65% consumption, in the period of 2000 to 2015 (Klein et al., 2018). If the same trend continues, an overall increase of 200% consumption compared to 2015 will be observed by 2030 (Blaskovich, 2018; Klein et al., 2018). Additionally, it was observed an overall increase in consumption of newer and last-resort antibiotics classes, such as glycylcyclines, oxazolidinones, carbapenems and polymixins. Of concern are lower-middle income countries reaching for consumption levels of high income countries (Blaskovich, 2018; Klein et al., 2018). For example, India had the greatest increase of lower-middle income countries, and a large portion of this increase was consumption of cephalosporins to fight increasing resistant infections. Globally, there is an observed increase in consumption of last-resort antibiotics, specifically carbapenems and colistin, which is consistent with the well documented increase of infections resistant to these compounds (Blaskovich, 2018; Klein et al., 2018).

The overuse of antibiotics drives evolution of resistance and epidemiological studies demonstrate a direct relation between antibiotic consumption and emergence of resistant bacterial strains (Blaskovich, 2018; Dadgostar, 2019; Klein et al., 2018). Antibiotics remove sensitive competitors, allowing surviving resistant bacteria to spread, as a result of natural selection (Ventola, 2015).

Subinhibitory and subtherapeutic concentrations can increase development of resistances through genetic alterations, leading to changes in gene expression, horizontal gene transfer (HGT) and mutagenesis (San Millan, 2018; Ventola, 2015). It has been shown strain diversification in *Pseudomonas aeruginosa* in presence of subinhibitory antibiotics concentration (Viswanathan, 2014) as well as proteomic alterations in *Bacteroides fragilis* in the presence of subinhibitory concentrations of piperacillin and tazobactam (Dadgostar, 2019; Ventola, 2015; Viswanathan, 2014).

How far has antibiotic resistance spread?

Of note, these reports mostly account for human antibiotic consumption, and are shy in describing the presumable higher dimension of the resistance scenario. Other environments and sectors, with much higher usage of antibiotics, such as animal and agricultural practices, are vectors by which bacterial resistance can develop and spread (Figure 1). A serious implication for development and dissemination of bacterial resistance are wastewater treatment plants (Rowe et al., 2017).

Wastewater plants are considered hubs of resistance, as effluents from agriculture, animal farms and human consumptions, often containing remnants of antibiotics, offer a "pool" of residual selective pressure, promoting growth or tolerance of resistance mechanisms (Gallert et al., 2005; Karkman et al., 2018). Additionally, some facilities of water treatment are not efficient in eliminating antibiotic resistant strains from wastewater, acting as permanent suppliers of antibiotic resistant bacteria to the environment, providing a continuous dissemination and accumulation of resistant organisms in environmental water (Martins da Costa et al., 2006). *Enterococcus* spp. resistant to vancomycin have been identified as a major issue of concern due to associations with nosocomial infections and food production.(Chapin et al., 2005; Da Silva et al., 2006; Karkman et al., 2018). Additionally, wastewater treatment does not eliminate resistant strains from residual waters, especially in stations consisting only of primary and secondary activated sludge processes (M. F. Da Silva et al., 2006; Karkman et al., 2018). It was observed that *Enterococcus faecium*, an opportunistic pathogen, was positively selected during wastewater treatment, with an increase in its relative proportion in the treated wastewater. Treated wastewater is usually released to rivers or coastal waters, which can trigger a progressive change in the microbial ecosystem (Da Silva et al., 2006; Karkman et al., 2018)

In livestock, antibiotics are widely used as growth supplements, with the objective of improving overall health of animals and produce larger yields and quality products, as well as prevention infection. Antibiotics used for non-therapeutic purposes such as growth promotion has been shown to select for resistance to high concentration of antibiotics in both pathogenic and commensal bacteria (Chapin et al., 2005; Ventola, 2015).

Along with pork products, more than 110 million tons of swine waste containing antibiotic resistant bacteria are produced in swine concentrated animal feeding operations (CAFO) in the US every year. This waste, left in open air lagoons or applied to land, leads to contamination of soils and nearby surface and groundwaters, with several studies reporting presence of antibiotic residues and antibiotic-resistant bacteria in surface and groundwaters close to CAFO's (Chapin et al., 2005). Land application of antibiotic-polluted manure is agricultural practice in Europe, the US and other parts of the world and consequently antibiotics are transferred to agricultural soils. This has for example been documented for sulfamethazine, tetracycline and chlortetracycline, as well as tylosin (Heuer et al., 2011).

Manure introduces bacteria that spread various combinations of multiple resistance genes, located in mobile genetic elements, that can efficiently transfer on broad-host-range plasmids or other conjugative elements to many species in soil and subsequently other habitats, promoting horizontal transfer of antibiotic resistance genes in soil (Domingues et al., 2012; Heuer et al., 2011). Additionally, even the air surrounding these farms are a source of antibiotic resistant bacteria, with inhalation of air within swine feeding operations serving as another exposure route to transfer of antibiotic resistant bacteria (Fig1) (Antunes et al., 2019; Chapin et al., 2005; Heuer et al., 2011).

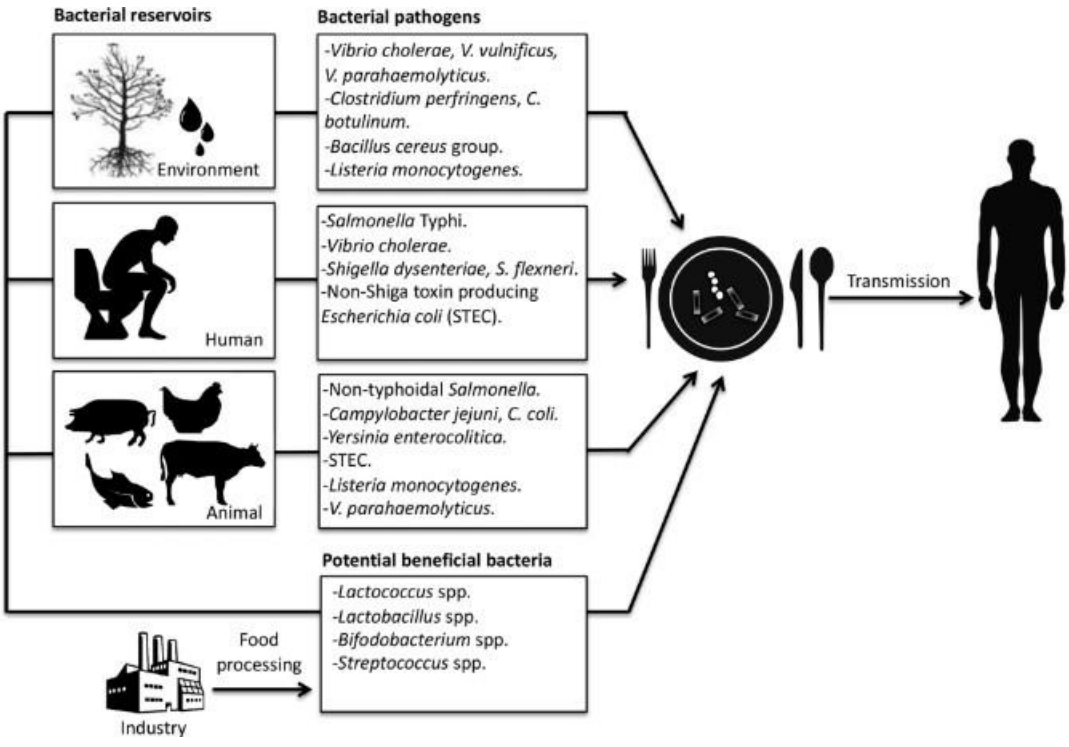

Figure 1-Transmission of bacterial organisms (pathogenic or commensal) to humans (Adapted from Antunes et al., 2019).

Aquaculture, the newest food production sector, may promote development of resistance through the same mechanisms as agriculture. Research has found that most antibiotics used in aquaculture/agriculture are also used in human treatment. Furthermore, classes of antibiotics considered critical by WHO are commonly used in agriculture/aquaculture. Consequently, various zoonotic pathogens isolated from seafood showed resistance to multiple antibiotics on the WHO list (Done et al., 2015).

Antibiotics have allowed for animal health to be improved, increasing economic gain for farmers, as pathogens are greatly reduced with antibiotic usage (Chapin et al., 2005; Done et al., 2015). However, there has been increasing awareness regarding antibiotic usage in farmed species, becoming further under scrutiny because of increasing concern regarding antimicrobial resistance. These imprudent patterns of antibiotic prescribing and use represent a potential risk to human and animal health (P. Davies et al., 2017). In both agriculture and aquaculture, development/persistence of resistance can occur when these bacteria are exposed to sub-therapeutic concentrations of antibiotics. Although the use of antibiotics in human medicine has influenced the emergence of antibiotic-resistant bacteria, the use of antibiotics in animal agriculture has markedly contributed to this critical problem (figure 2) (Chapin et al., 2005).

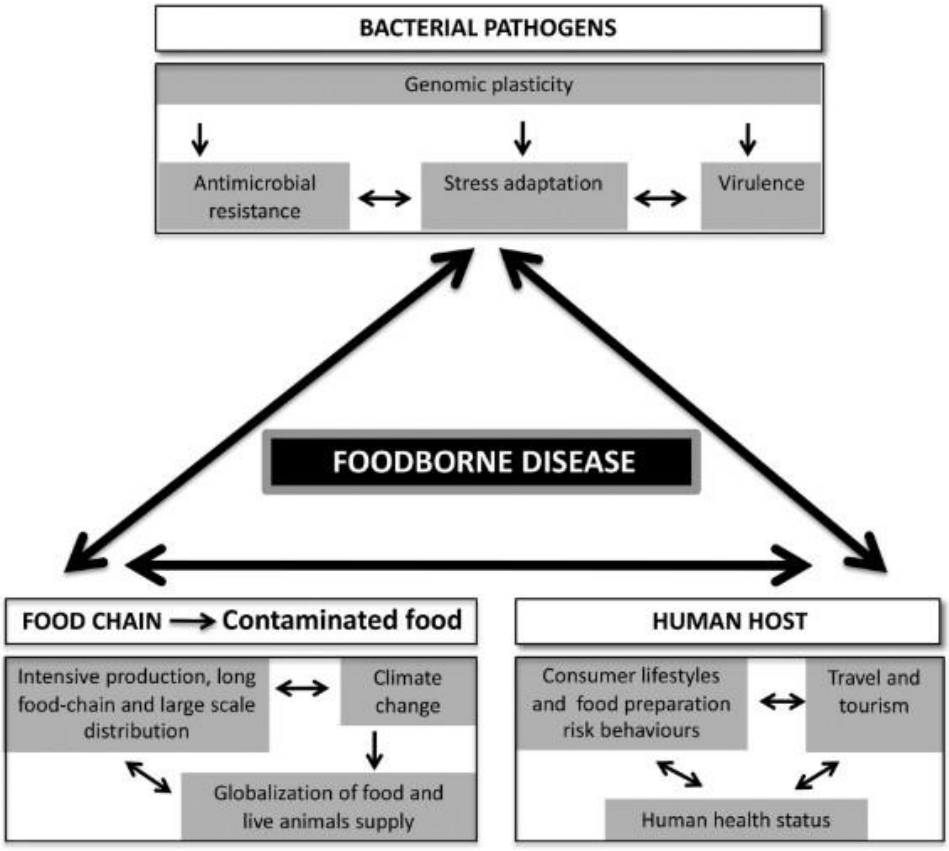

Figure 2-Mechanisms of resistance and implications in foodborne disease (Adapted from Antunes et al., 2019).

Unlike the golden era of antibiotic discovery, the actual threat of resistance mechanisms has not been met by the development of new antibiotics. The current pipeline of antibiotic development is not enough to mitigate the threat of resistant bacterial strains (Theuretzbacher et al., 2019, 2020; World Health Organization, 2019). The 2019 WHO report of antibacterial agents in clinical development states that the clinical pipeline to tackle the challenge of increasing emergence and spread of resistance is still insufficient, with eight new antibacterial agents being approved since 2017, that display limited benefits (World Health Organization, 2019). The pipeline is dominated by derivatives of existing antibiotic classes, with limited innovation, and new drugs without cross-resistances are under-represented, even though the preclinical pipeline is characterized by diversity and innovative options (Theuretzbacher et al., 2019, 2020). However large pharmaceuticals are still abandoning the field of antibacterial research and development, while small and medium sized enterprises are driving antibacterial development (World Health Organization, 2019).

For further understanding the resistance mechanisms by bacteria and how potential alternatives can be implemented in treatment options, an overview of these mechanisms is provided below

Key-messages:

Anthropological overuse of antibiotic compound in diverse sectors (agriculture, livestock, healthcare) has created a permanent selective pressure on bacterial species, amplifying mechanisms of resistance;

Although there have been efforts to control and regulate this consumption, an overall increase in global antibiotic consumption is observed;

The current antibiotic pipeline development is inefficient in pushing back the prevalence of resistant organisms, due to lack of innovation and diversity. The prevalence of antibiotic agents that are derivatives from preexisting classes have limited benefits and don't mitigate the development of resistances.

Resistance mechanisms: Efficiency at its finest

Indeed, some strains have become resistant to all commonly available agents. A hallmark case of resistant bacterial strain is methicillin-resistant *S. aureus* (MRSA). These strains are resistant to beta-lactamic antibiotics and often resistant to aminoglycosides, macrolides, tetracycline, chloramphenicol and lincosamides. As these strains can also resist disinfectants, they are a high source of hospital acquired infections (Nikaido, 2009; Prestinaci et al., 2015). Another serious threat is the emergence of Gram-negative bacteria resistant to essentially all the available agents. The emergence of these “pan-resistant” strains, notably those belonging to *Pseudomonas aeruginosa* and *Acinetobacter baumannni* is disconcerting, as there are almost no agents that can be used against these strains, in which an outer membrane barrier of low permeability and an array of efficient multidrug efflux pumps are combined with multitudes of specific resistance mechanisms (Figure 3) (Nikaido, 2009; Prestinaci et al., 2015; Silva et al., 2020).

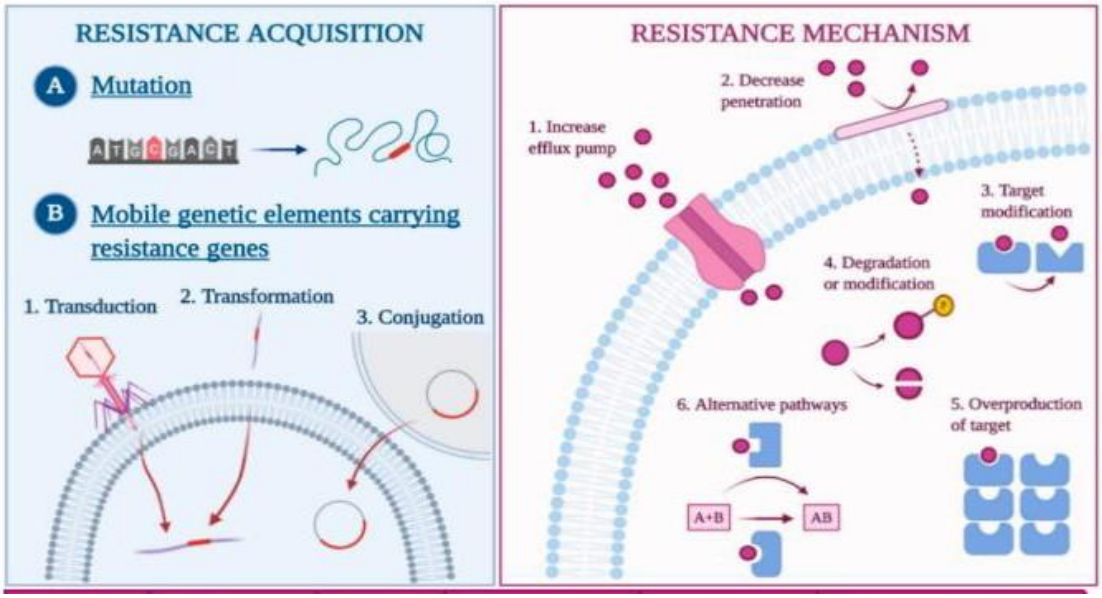

Figure 3-Resistance acquisition and consequent mechanisms expressed by resistant organisms (Adapted from Silva et al., 2020).

Another serious threat to the healthcare setting is formation of a bacterial structure known as the biofilm. Biofilm-producing bacteria are responsible for the majority of

nosocomial chronic infections. The national institute of health (NIH) statistics estimate that biofilm formation is involved in 65% of all bacterial infections and 80% in chronic infections. These structures, besides possessing “conventional” resistance mechanisms, such as enzymatic inactivation of antibiotics or efflux pumps, are also capable of physiological changes that grant tolerance to antibacterial compounds (Preda & Săndulescu, 2019).

Resistance mechanisms can be intrinsic, acquired or adaptative. While intrinsic resistance in organisms is found when certain strains inherently display lower membrane permeability or constitutive efflux pumps, acquired mechanisms result from integration of mobile genetic elements such as plasmids, transposons and naked DNA (Babakhani & Oloomi, 2018; Domingues et al., 2012; San Millan, 2018). Adaptative mechanisms are usually metabolic changes, lowering expression of specific genes and proteins, according to environmental pressures and sub-inhibitory concentrations of antibacterial drugs (Cerceo et al., 2016; Domingues et al., 2012; San Millan, 2018). Adaptative changes can be plasmid or chromosomal mediated, and the location of resistance genes on mobile genetic elements (MGEs) facilitates their spread to other bacteria, taxonomically or not similar (Cerceo et al., 2016; Domingues et al., 2012). These genes can then express several mechanisms by which bacteria survive antibiotic action (fig3). The section below overviews some of the “conventionally” encountered mechanisms of antibiotic resistance by bacteria.

Mutational alteration of target protein

There is an alteration (usually acetylation or methylation of a specific nucleic base) of the drug target, rendering ineffective a antibiotic compound with a proteic target (Nikaido, 2009). One example of resistance attributable to protein modification is that conferred by the *erm* gene, which is usually plasmid coded and produces methylation of adenine at position 2058 of the 50S rRNA, causing resistance to macrolides, lincosamide and streptoGramin, also known as the macrolidelincosamide-streptoGramin (MLS) phenotype (Nikaido, 2009).

Enzymatic inactivation of drug

Common resistance mechanism against antibiotics of natural origin, such as aminoglycosides (kanamycin, tobramycin and amikacin), which are inactivated by enzymatic phosphorylation, and beta-lactamases) that through enzymatic hydrolysis by beta-lactams (penicillin, cephalosporins and carbapenems such as imipenem), prevent their bioactive action (Nikaido, 2009).

Aminoglycosides

Aminoglycosides are inactivated by modifications that reduce the net positive charge of these polycationic antibiotics. There are now many dozens of aminoglycoside-modifying enzymes known; for example, AAC (3)-II, which is a designated aminoglycoside acetyltransferase acting on position 3 of the substrate and belonging to the second phylogenetic grouping among these enzymes (Chiang et al., 2013; Nikaido, 2009; Pekarek & Debono, 1981).

Beta-lactamases

A few years after the introduction of penicillin into clinical practice, *S. aureus* developed resistance caused by a beta-lactamase coded by a plasmid gene. Although the problem was solved by the introduction of methicillin and similar compounds that resists enzymatic hydrolysis, another enzyme, TEM beta-lactamase, was reported in Gram-negative bacteria, in strains containing multiple-drug-resistant resistance (R) plasmids. This enzyme became widespread throughout the world, making penicillin activity, such as ampicillin ineffective (Kong et al., 2010; Nikaido, 2009; Samanta & Bandyopadhyay, 2020).

Beta-lactamases are classified into several phylogenetic families/classes, according to several classifications. Ambler classification classify beta-lactamases as: Class A beta-lactamases include penicillinases like TEM enzyme; Class B beta-lactamases represent metalloenzymes that hydrolyze carbapenems; Class C beta-lactamase enzymes represent chromosomally coded enzymes, such as ampC, present in many Gram-negative bacteria; and Class D beta-lactamases, or oxacillin (OXA; oxacillinases) beta-

lactamases, that confer additional resistance to oxacillin (Nikaido, 2009; Samanta & Bandyopadhyay, 2020).

Especially troublesome among the extended-spectrum beta-lactamases (ESBL) enzymes are those called CTX-M. The genes coding for these enzymes appear to have originated from the chromosome of an infrequently encountered Gram-negative bacterium *Kluyvera* and have transferred to R plasmids. This mobilization appears to have occurred many times, consequently spreading the enzyme among R-plasmid-containing pathogenic bacteria (Nikaido, 2009).

Acquisition of exogenous resistance genes

Sequencing for the genes coding for the targets of penicillin, DD-transpeptidase or penicillin binding proteins (PBP), revealed that penicillin resistance among *Streptococcus pneumoniae* was due to the production of *mosaic* proteins, parts of which came from other organisms. Note that *S. pneumoniae* is capable of natural transformation and may import foreign DNA (Cerceo et al., 2016; Nikaido, 2009). A case of this scenario is the generation of MRSA. MRSA contains a new methicillin-resistant PBP, called PBP-2A, whose expression is induced by methicillin or other beta-lactams. The gene for this new PBP apparently came from an organism other than *S. aureus* and contains other antibiotic resistance genes. *S. aureus* is not naturally transformable and it is unclear how this horizontal transfer of a large DNA segment occurred (Cerceo et al., 2016; Nikaido, 2009).

Bypassing the target

Vancomycin, a fermentation product from *Streptomycetes*, has an unusual mode of action. Instead of inhibiting an enzyme, it binds to a substrate, the lipid linked disaccharidepentapeptide, a precursor of the cell wall peptidoglycan. Because of this mechanism, many would assume it would be impossible to generate resistance against vancomycin. However, vancomycin resistance is now prevalent in *Enterococci* (Cerceo et al., 2016; Collignon, 2015; Hemmati et al., 2020; Nikaido, 2009). When vancomycin resistance was studied, it was found that the substrate to which vancomycin binds was replaced in the resistant strain by an ester structure, which is not bound by vancomycin (Cerceo et al., 2016; Collignon, 2015; Hemmati et al., 2020; Nikaido, 2009).

Preventing drug access to targets

Drug access to target can be reduced locally but also by active efflux by multidrug efflux pumps. In Gram-negative bacteria, access can be reduced generally by decreasing influx across outer membrane (Cerceo et al., 2016; Collignon, 2015; Hemmati et al., 2020; Nikaido, 2009).

Local inhibition of drug access

TetM or TetS proteins, produced by plasmid-coded *tet* genes in Gram-positive bacteria, bind to ribosomes with high affinity and change ribosomal conformation, preventing association of tetracyclines to ribosomes. Plasmid coded Qnr proteins protect DNA topoisomerases from (fluoro)quinones (Cerceo et al., 2016; Collignon, 2015; Hemmati et al., 2020; Nikaido, 2009).

Non-specific inhibition of drug access

Mutation within coding sequences of porins, reducing permeation rates of bulky beta-lactams without affecting those of smaller nutrient molecules (Cerceo et al., 2016; Collignon, 2015; Hemmati et al., 2020; Nikaido, 2009).

Efflux pumps

Drug resistance owing to active efflux was discovered in the common tetracycline resistance protein TetA in Gram-negative bacteria, which catalyzes a proton-motive-force-dependent outward pumping of a tetracycline-Mg complex (Cerceo et al., 2016; Collignon, 2015; Hemmati et al., 2020; Nikaido, 2009). There is a vast family of efflux pumps that actively export specific or non-specific antibiotic compounds from the intracellular bacterial milieu or periplasmic space to the external medium. These efflux pumps are an important factor in the bacterial resistance scenario (Nikaido, 2009).

Although these “conventional” resistance mechanisms are a crucial factor in the resistance to antibacterial therapeutics, focus towards biofilms is increasing. Biofilms

pose a very serious threat to the effectiveness of antibiotic treatment, especially in the healthcare system.

The biofilm structure: Elegant evolution

Pure culture planktonic growth is rarely how bacteria exist in nature, as demonstrated by complex bacterial communities responsible for driving the biochemical cycling that maintains the biosphere. Direct observation of a wide variety of natural habitats has established that most microbes persist attached to surfaces, within a structured biofilm, rather as free-floating organisms (Alford et al., 2019; Christophersen et al., 2020; Crabbé et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018; Donlan & Costerton, 2002; Gajdács, 2019; Hall & Mah, 2017; Hemmati et al., 2020; Pei et al., 2010; Pourcel et al., 2020; Preda & Săndulescu, 2019; Tahrioui et al., 2019; Yan & Bassler, 2019).

It is becoming clear that these natural assemblages of bacteria within the biofilm matrix function as a cooperative consortium, in a complex and coordinated matter. As such, and although microorganisms can have an independent planktonic existence, an interdependent lifestyle in which they function as an integral part of a population or community is in fact more typical (Davey & O'toole, 2000; Del Pozo, 2018; Yan & Bassler, 2019). Metabolically similar populations (Sulphur-sulphate reducing, fermentative, methanogenic, etc.) are known as guilds, and sets of guilds can conduct interdependent physiological processes within a biofilm (Davey & O'toole, 2000; Donlan & Costerton, 2002).

Van Leeuwenhoek proposed the general theory of biofilm predominance, promulgated in 1978 (Costerton et al., 1978). In this theory, he stated that most bacteria grow in matrix enclosed biofilms, adherent to surfaces in all nutrient-sufficient aquatic ecosystems and that these sessile bacterial cells differ profoundly from their planktonic (free floating) counterparts (Davey & O'toole, 2000). At the time, most of the data for this theory came from aquatic environments, in which direct observational methods and direct quantitative recovery techniques revealed that 99.9% of bacteria grow in biofilms, on a wide variety of substrates. This predominance of biofilms was established for all natural ecosystems,

and we now realize that these sessile populations account for most physiological processes in ecosystems (Davey & O'toole, 2000).

Costerton posteriorly stated in his biofilm observations a series of cascading conclusions, that lead to general knowledge regarding simple biofilm ecology (Costerton et al., 1978; Donlan & Costerton, 2002). He initially observed that these bacterial communities found in aquatic systems, were encased in a "glycocalyx" matrix, found to be polysaccharide in nature, and this matrix material had shown to mediate adhesion (Donlan & Costerton, 2002).

It was also observed that biofilms could adhere to surfaces and interfaces and to each other, including in the definition microbial aggregates and adherent populations within pore spaces of porous media, and that adhesion triggered gene expression responsible for production of the necessary components for adhesion and biofilm formation (Figure 4). The process of biofilm formation was regulated by the genetic expression activated moments upon adhesion to a surface (Costerton et al., 1978; Donlan & Costerton, 2002). Biofilms can form in a vast array of biotic and abiotic surfaces and also form natural assemblages at air-water interaction and in suspensions, such as anaerobic digestors, in which they aggregate as flocs or granules (Alford et al., 2019; Christophersen et al., 2020; Crabbé et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018; Donlan & Costerton, 2002; Gajdács, 2019; Grimes et al., 2019; Hall & Mah, 2017; Hemmati et al., 2020; Pei et al., 2010; Pourcel et al., 2020; Preda & Săndulescu, 2019; Tahrioui et al., 2019; Yan & Bassler, 2019).

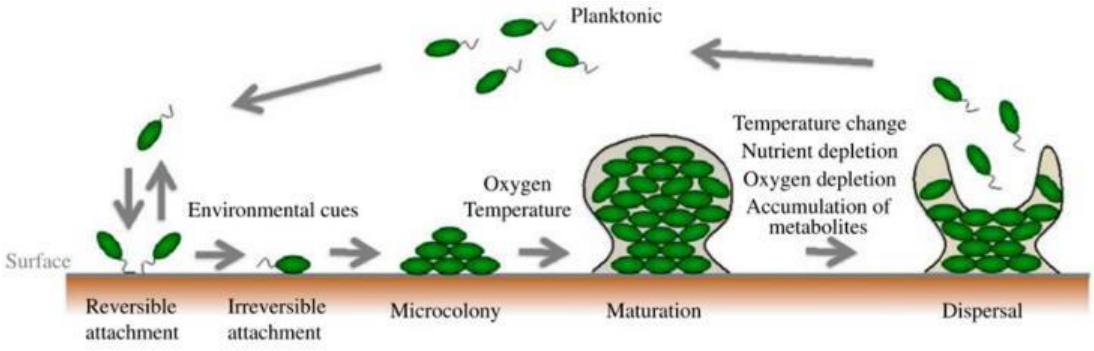

Figure 4-Biofilm formation is characterized by sequential mechanisms triggered by environmental factors and environment bacteria are. These steps begin with adhesion to a substrate, formation of microcolonies through aggregation, maturation and finally dispersal, in order to colonize other surfaces (Adapted. from López & Soto, 2019).

Their ability to persist is in part due to their versatility and phenotypic plasticity. One of the key elements is the ability to position themselves in a niche they can propagate. Most common mechanisms are flagellar motility and other methods of surface translocation (Alford et al., 2019; Christophersen et al., 2020; Crabbé et al., 2019; Del Pozo, 2018; Donlan & Costerton, 2002; Gajdács, 2019; Grimes et al., 2019; Hall & Mah, 2017; Hemmati et al., 2020; Pei et al., 2010; Pourcel et al., 2020; Tahrioui et al., 2019).

Bacterial adhesion: the first step

This phenotypic switch from a free swimming, planktonic cell to a sessile, attached cell is a highly regulated process, dependent on numerous environmental and genetic factors, that vary from species to species (Alford et al., 2019; Crabbé et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018; Hall & Mah, 2017; Yan & Bassler, 2019). The classic biofilm formation model indicates that motile planktonic cells, under certain environmental conditions, attach to a surface. One example of such environmental conditioning and genetic response, is the biofilm formation in *P. aeruginosa* and *E. coli,* that is triggered by exposure to subinhibitory concentrations of aminoglycosides. In *P. aeruginosa*, transcription of *alg*C, a gene involved in biosynthesis of alginate, is reportedly linked to a downregulation of flagellum synthesis. *Alg*C gene is induced soon after bacteria adhere to a surface (Cady et al., 2012; Crabbé et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018; Hall & Mah, 2017; Nikaido, 2009; Pei et al., 2010; Preda & Săndulescu, 2019; Tahrioui et al., 2019; Yan & Bassler, 2019). Davies and Geesey (Davies et al., 1993), have shown that *alg*C, a gene controlling expression of phosphomannutase, related to production of alginate, is an important EPS component that is upregulated within minutes of bacterial adhesion to a surface (Davey & O'toole, 2000; Del Pozo, 2018; Hall & Mah, 2017). Other studies have shown that *alg*D, *alg*U, *rpo*S and genes controlling polyphosphokynase synthesis, are all upregulated in biofilm formation and that as many as 45 genes differ in expression between sessile cells and their planktonic counterparts (Davey & O'toole, 2000; Donlan & Costerton, 2002).

These attached cells proceed then to the production of a hydrated matrix that is composed of exopolysaccharides, extracellular DNA (eDNA), proteins and lipids and overtime, formation of microcolonies and maturation into mature microcolonies form the mature biofilm. Lastly, planktonic cells detach from the mature biofilm to subsequently

colonize new surfaces (Alford et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018; Pei et al., 2010; Yan & Bassler, 2019).

Additionally, other conditions such as surface and interface properties, nutrient availability, composition of microbial community and hydrodynamics can affect biofilm structure. Biofilms have been examined under conditions such as laminar and turbulent flow, and biofilm structures are altered in response to flow conditions (Yan & Bassler, 2019). Biofilms are polymorphic and structurally adapted to changes in nutrient availability. Interstitial voids are also an integral part of the biofilm structure, as water flows through these channels working the lifeline of the system, providing a mean of circulating nutrients as well as exchanging metabolic products with the bulk fluid layer (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

There are many specific species-requirements for the formation of biofilms, according to the organisms being studies. However, some general concepts regarding biofilm initial formation hold true to several species, such as *P. aeruginosa*, *E. coli* and *Vibrio cholerae*. These organisms have become prominent models in biofilm studies (Davey & O'toole, 2000; Del Pozo, 2018; Hall & Mah, 2017).

The environmental cues controlling transition from free-swimming to immotile vary greatly among organisms. While *P. aeruginosa* forms biofilms under most conditions that allow grow, some strains of *E. coli* require additional supplementation of aminoacids in the medium, while other strains form biofilms in conditions of low-nutrient availability (Davey & O'toole, 2000). One example is *P. fluorescens*, to which an analysis of this organism identified multiple genetic pathways for the initiation of biofilm development. Mutants unable to form a biofilm when grown on glucose could bypass this defect by displaying growth on citrate, suggesting a citrate-alternative pathway to biofilm formation (Davey & O'toole, 2000; G. A. O'Toole & Kolter, 1998b). *V. cholerae* also possesses several pathways for initial attachment, according to the surface the organisms is colonizing (Davey & O'toole, 2000). For example, the Tcp pilus is required for colonization of the intestinal lumen, but this pilus plays no role in the attachment of *V. cholerae* to abiotic surfaces (Davey & O'toole, 2000). On the other hand, the pilus encoded by the *msh* locus, that has no role in pathogenesis, is required for initial adhesion to abiotic surfaces. Other environmental signals, such as pH, osmolarity, iron availability, oxygen tension and temperature may have a profound impact on the transition between planktonic and biofilm growth (Figure 5) (Davey & O'toole, 2000).

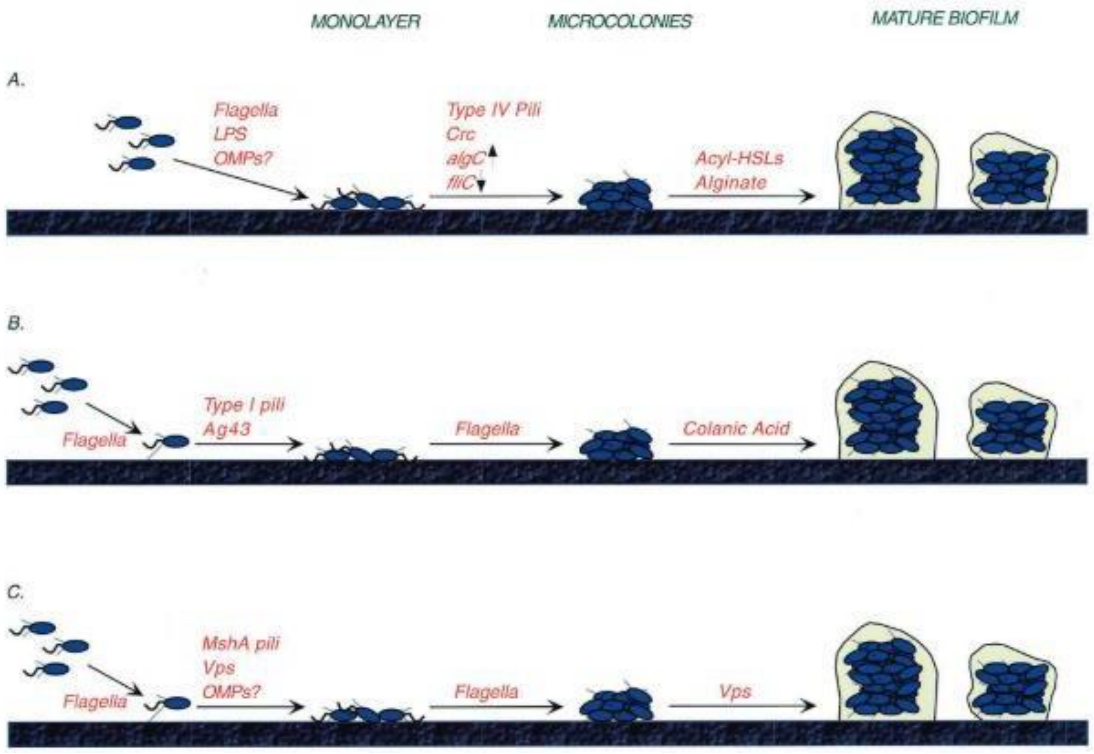

Figure 5-Biofilm attachment and molecular mechanisms of adhesion. (A)-*Pseudomonas aeruginosa*; (B)-*Escherichia coli,* (C)-*Vibrio cholerae* (Adapted from Mah & O'Toole, 2001).

Several experiments with mutant organisms, with the objective of identifying functions related to biofilm development has shed light on the mechanisms of development of the biofilm structure. *P. aeruginosa* mutant strains, designated "*sad*" for surface-attachment defective have been described (O'Toole & Kolter, 1998a). Strains defective in flagellum-mediated mobility appear to be blocked in initial interaction with a surface. Other class of *sad* mutants are defective in the biogenesis of type IV pili, known to be involved in surface- associated movement, known as twitching motility. Strains with defective or non-functional type IV pili, attach to a surface and create a monolayer biofilm similar to wild-types, however these biofilms are unable to form the characteristic microcolonies, a hallmark of biofilm development in *P. aeruginosa* (Davey & O'toole, 2000; G. O'Toole et al., 2000; G. A. O'Toole & Kolter, 1998a). Additionally, the *crc* locus, which codes for a catabolite repressor protein, is also presumably involved in biofilm development (Davey & O'toole, 2000; G. A. O'Toole et al., 2000). The Crc protein is required for the repression of sugar metabolism in the presence of organic acids and also been shown to regulate *pil*A and *pil*B, that encode the main structural proteins of type IV pili and accessory factors for pilus assembly (O'Toole et al., 2000). The mechanism by which Crc regulates metabolism and pilus synthesis is unclear, but

interestingly draws a link between nutrient availability and biofilm formation (O'Toole et al., 2000). Additionally, lipopolysaccharide (LPS), a major component of bacterial outer membrane, also plays a role in initial surface adhesion (Makin & Beveridge, 1996). Loss of LPS B-band reduced cell's ability to interact with hydrophobic surfaces (Davey & O'toole, 2000; Makin & Beveridge, 1996).

E.coli also requires flagella and pili to initiate early attachment processes (Davey & O'toole, 2000; O'Toole et al., 2000). Type I pili is mandatorily essential to the attachment process to succeed, but does not have impact in surface-motility (Davey & O'toole, 2000; Pratt & Kolter, 1998). The phase-variable outer membrane protein of *E. coli,* Ag43, is also required for biofilm development, playing a direct role in bacterial interaction with a surface (Dove, et al., 2000; Davey & O'toole, 2000). Similarly to *P. aeruginosa*, loss of LPS in *E. coli* results in decreased ability to attach to a surface (Pratt, & Kolter, 2000). The biofilm mutant phenotype of *E. coli* is different from *P. aeruginosa*, as attachment is not eliminated in flagellar mutant strains, although it is severely impaired. However, the resulting biofilm from these mutants consists of isolated microcolonies, without the characteristic aggrupation architecture. This means that unlike *P. aeruginosa*, *E. coli* biofilms flagellar mobility is used for parallel- surface motility, highlighting how the roles of flagella in the formation of *E. coli* biofilms and *P. aeruginosa* are quite different (Davey & O'toole, 2000; O'Toole et al., 2000;
O'Toole et al., 1999; O'Toole & Kolter, 1998a).
After biofilm adhesion, this structure maturates, with the characteristic cell aggregation, formation of microcolonies and production of the extracellular polymeric substances (EPS) matrix.

Biofilm maturation

After bacterial adhesion, cellular aggregation and extracellular polymeric substances (EPS) are produced, forming microcolonies. Observation has led to conclude that developed biofilms are not structurally monolayers of microbial cells on a surface, they are heterogeneous in both time and space. The basic building block or structural unit of the biofilm is the microcolony (Davey & O'toole, 2000; de Kievit, 2009; Donlan & Costerton, 2002; O'Toole et al., 1999; Pritchard et al., 2017; Yan & Bassler, 2019).

Using confocal laser scanning microscopy (CLSM), direct observations of living biofilms have shown that the basic community structure is universal (Pei et al., 2010). Established microcolonies mature over time, forming finally macrocolonies (usually displayed as towers), with the characteristic architecture (figure 6) (Davey & O'toole, 2000; Del Pozo, 2018; Hall & Mah, 2017; Pei et al., 2010; Yan & Bassler, 2019). On course of the maturation, bacteria adhere to each other and produce EPSs, forming the matrix (Arciola et al., 2018). EPSs include exopolysaccharides, techoic and lipoteichoic acids (in case of Gram-Positives), proteins and extracellular DNA (eDNA). The interaction between these components, coupled with the physiochemical properties of the matrix itself result in a highly complex structure (Crabbé et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018; Pei et al., 2010; Yan & Bassler, 2019).

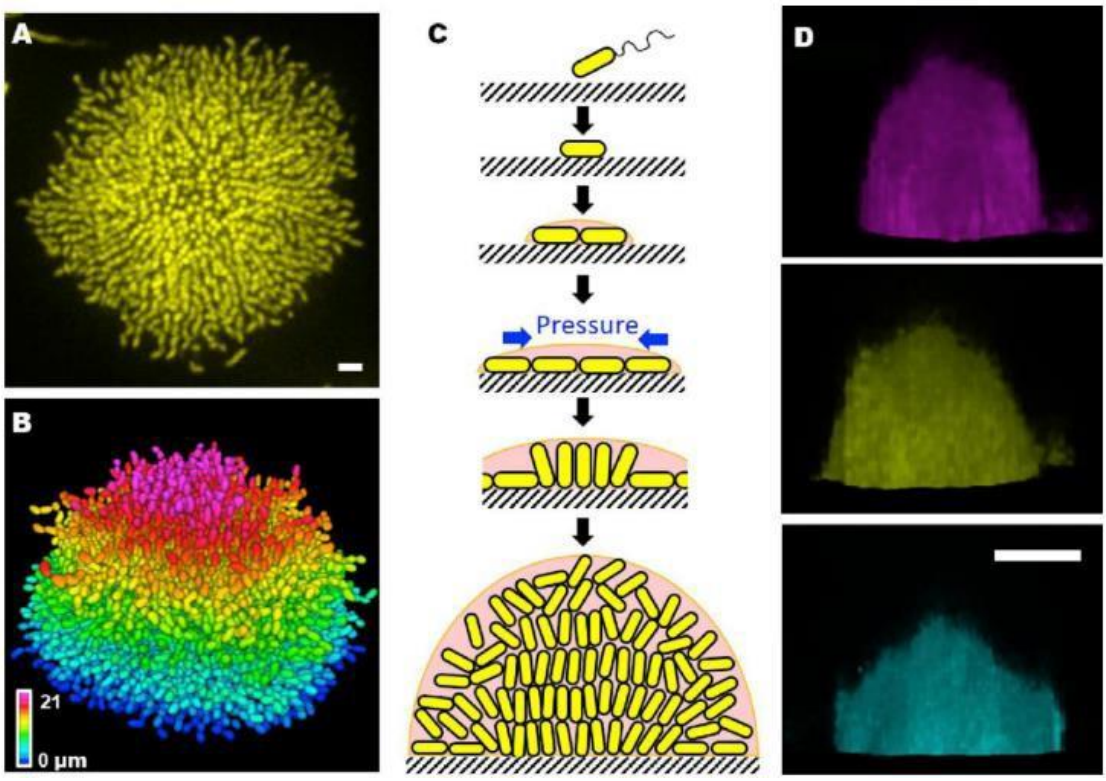

Figure 6-Maturation and bacterial organization in *Vibrio cholerae* biofilms forming the characteristically towers. (A) represents a cross-section of the bottom layer of a 18h *V. cholerae* biofilm, and the corresponding image in (B), demonstrating color coding for Z position. (C) representation of steps involved in *V. cholerae* biofilm formation. Yellow represents bacterial cells and pink the EPS matrix. (D) Side views of 7-h-old biofilms grown with 0.4 mg/mL A22, magenta; without treatment. Yellow; and with 4 mg/mL cefalexin, cyan. A22 and cefalexin cause the cells to become shorter and longer, respectively (Adapted from Yan & Bassler, 2019).

In *S. epidermidis* and *S. aureus,* the main polysaccharide of the biofilm matrix is polysaccharide intracellular adhesin (PIA) (Arciola et al., 2018). PIA production is responsible for providing higher resistance to antibiotics, specifically aminoglycosides.

In *S. epidermis*, environmental stress increase PIA synthesis and biofilm formation. PIA production is additionally increased when iron availability and oxygen tensions are low. In *S. aureus*, biofilms exposed to stressful environments, there is an increase in the rates of horizontal gene transfer and mutation, promoting the appearance of resistance mutations (Arciola et al., 2018).

In *P. aeruginosa* strains, the main polysaccharides produced are Pel, Psl and alginate and combinations of these three are found in the biofilm matrix (Davey & O'toole, 2000; Yan & Bassler, 2019). In specific, Pel-eDNA interactions are believed to drive overall biofilm dynamics (Yan & Bassler, 2019). In cystic fibrosis patients (CF), *P. aeruginosa* strains tend to produce Psl and alginate, and in *V. cholerae*, the major biofilm component is the *Vibrio* polysaccharide (VPS) and three matrix proteins: RmbA, Bap1 and RmbC (Yan & Bassler, 2019).

Extracellular DNA

Extracellular DNA (eDNA) present in the matrix is proposed to have four roles: stabilization and strengthening of the biofilm matrix; gene transfer between cells; modulation of the inmate immune response and nutrient supply. eDNA can be endogenously derived through quorum-sensing mediated release, outer membrane vesicles and altruistic or fratricidal lysis (Arciola et al., 2018; Crabbé et al., 2019; Davey & O'toole, 2000; Hall & Mah, 2017). In the case of the latter, *S. aureus* biofilm cells can be divided into altruists and survivors, in which the former "commits suicide" for the sake of community, and in *E. faecalis* biofilm cells, this mechanism occurs through differentiation of cells into "attackers and targets", in which the "attackers" releasing killing factors and target other cells, while "attacker" cells are immune to the factor (Yan & Bassler, 2019). It has been reported that eDNA, a crucial component of biofilm development and establishment, is generated by lysis of bacterial population in two different mechanisms: one pathway linked to QS, that results in a larger bacterial lysis and consequently larger releases of DNA, and a QS-independent pathway that liberates a basal level of eDNA to the medium (Allesen-Holm et al., 2006; de Kievit, 2009).

Regardless of whether the source of eDNA is endogenous or exogenous, there is a link between eDNA and increased biofilm resistance to antibiotics (Yan & Bassler, 2019). Addition of exogenous DNA to *P. aeruginosa* biofilms from became incorporated in the

biofilm matrix, resulting in an increased resistance to tobramycin and gentamycin (Chiang et al., 2013; Hall & Mah, 2017).

Quorum sensing

Within the biofilm, bacteria communicate with each other through quorum-sensing (QS) molecules. QS has been demonstrated to modulate cellular functions, population density, pathogenesis, nutrient acquisition, transfer of genetic material between cells and motility and synthesis of secondary metabolites in biofilm structures (Crabbé et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018; Hall & Mah, 2017; Tahrioui et al., 2019; Tait et al., 2009; Yan & Bassler, 2019).

There are at least three main types of quorum sensing systems: the acyl-homoserine lactone (AHL) system in Gram-negative bacteria; the autoinducing peptide (AIP) system in Gram positive bacteria; and autoinducer-2 (AI-2) system both in Gram-negative and Gram-positive bacteria (Alford et al., 2019; Crabbé et al., 2019; Davey & O'toole, 2000; Del Pozo, 2018).

N-acylhomoserine lactones (AHLs) are the best understood signal molecules in Gram-negative bacteria and are known to modulate a diversity of genes involved in biofilm formation, motility, exchange of genetic material and virulence (Tait et al., 2009). In *P. aeruginosa*, a well-studied Gram-negative producer of AHL signaling molecules, approximately 300 genes are responsible for pathogenic factors, and studies have shown that 11% of *P. aeruginosa* genome is subjected to AHL regulation (de Kievit, 2009). The best studied QS systems in *P. aeruginosa* are *las* and *rhl* systems (Allesen-Holm et al., 2006; de Kievit, 2009; Tahrioui et al., 2019).
The *las* system consists of *las*I, an AHL synthase responsible for the synthesis of OdDHL, and *las*R, which encodes a *lux*R-type transcriptional regulator. The *las* system has been shown to regulate expression of several virulence factors; such as extracellular enzyme LasB elastase, LasA protease, alkaline protease; secondary metabolites such as pyocyanin, pyoveridin, hydrogen cyanide; toxins (exotoxin A); and *las*I itself (Allesen-Holm et al., 2006; de Kievit, 2009; Tahrioui et al., 2019).

In the *rhl* system, the *rhl*I gene promotes synthesis of N-butanoyl L-homoserine lactone (BHL), that in conjunction with *rhl*R gene activates transcription of the *rhl*AB rhamnolipid

biosynthesis gene and *rhl*I gene itself. The *rhl* system is also responsible in the modulation of several virulence genes controlled by the *las* system (Hentzer et al., 2002). N-3-oxododecanoyl homoserine lactone (3O-C12-HSL) and N-butyryl homoserine lactone (C4-BSL) are the autoinducer signal molecules regulating *P. aeruginosa* population, by binding to receptors that consequently activate cognate transcriptional regulators. Other pathogenic factors, such as production of pyocyanin, pyoveridin, rhamnolipid and motility all contribute to strengthen the pathogeny and virulence of *P. aeruginosa* biofilms (Oscar et al., 2018). The *pel* biosynthetic operon has been definitively identified as being subjected to QS regulation, this gene regulating production of glucose-rich biofilm exopolysaccharides (De Kievit, 2009).

P. aeruginosa mutants lacking *las*, which confers to a defect in production of acyl-HSL, are unable to synthesize the major quorum sensing molecules, producing a radically altered biofilm structure, evidencing the role of these molecules in the regulation of biofilm structure. The mutant biofilm was characterized by the absence of colonies and lacking resistance to 0.2% SDS (sodium dodecyl sulphate), characteristic traits of wild biofilms from *P. aeruginosa* (de Kievit, 2009).

Rhamnolipids are amphipathic glycolipids that act as biosurfactant and its production is regulated through the *rhlAB* operon and *rhlC* (de Kievit, 2009). Studies have linked rhamnolipids to multiple roles in the establishment and maintenance of *P. aeruginosa* biofilms (Boles et al., 2005; de Kievit, 2009). Specifically, rhamnolipids are responsible for maintaining open channels structures within the biofilm (Boles et al., 2005; de Kievit, 2009) and involved in the detachment of cells from the biofilm (de Kievit, 2009). It was reported that a hyper-detachment mutant had overexpression of rhamnolipids (Boles et al., 2005), and inactivation of *rhlAB* genes stopped accelerated detachment (Boles et al., 2005; de Kievit, 2009).

In Gram-positive bacteria, QS occurs though production of auto-inducer peptides (AIPs) (Novick & Geisinger, 2008). AIPs are signal molecules secreted by membrane transporters and synthesized by Gram-positive bacteria. In comparison to the HSL of Gram-negative bacteria, the overall dynamics of QS are similar, but differ greatly in detail (Novick & Geisinger, 2008). While extracellular HSL diffuses into the cell, binds to a specific intracellular receptor protein, usually a transcriptional activator to act on regulated genes (de Kievit, 2009), extracellular AIPs in Gram-positive bacteria do not enter a bacteria, but bind instead to a membrane receptor, triggering a classic signal

transduction pathway that results in the specific expression or repression of regulated genes (Novick & Geisinger, 2008). As environmental concentrations of AIPs increase, these bind to the histidine kinase sensor, consequently promoting phosphorylation and altering gene expression. These genes are then responsible for the production of toxins and degradable exoenzymes (Tait et al., 2009).

As part of their cooperation and communication mechanisms, microorganisms also have the capacity to sense and translate signals from distinct strains in AI-2 interspecific signals, catalyzed by the *Lux*S synthase. *Lux*S is involved in the activation of methylation cycles, controlling the expression of genes associated with processes of biofilm formation, in processes such as surface adhesion, detachment and toxin production (Preda & Săndulescu, 2019; Tait et al., 2009).

Relationship between biofilm formation and disease

Biofilm eradication is remarkably difficult. As biofilm-encapsulated bacteria can be up to 1000-fold more resistant to antibiotic treatment than the correspondent planktonic counterparts, clinical biofilm infections are marked by symptoms that typically recur after repeated antibiotic treatment (Allesen-Holm et al., 2006; Cepas et al., 2019; Donlan & Costerton, 2002; Garrett et al., 2008; Hentzer et al., 2002; Mah & O'Toole, 2001; Pei et al., 2010; Pratt & Kolter, 1999; Odile Tresse et al., 2008).

Standard antibiotic therapy is not able to completely eradicate bacterial cells, leaving sessile forms to propagate within the biofilm and to disseminate when therapy is terminated. In specific, the role of biofilm in contamination of medical implants has been well documented (Arciola et al., 2018). Among patients who develop these infections, mortality rates are as high as 70% and in patients that survive, some may have sequelae to life (Cerceo et al., 2016; Dadgostar, 2019). Millions of catheters are inserted every year and these implants serve as potential surfaces for attaching bacteria and biofilm formation. Overall, is it thought that upwards of 60% of all nosocomial infections are due to biofilms. These biofilm-based infections can increase hospital stays by 2 to 3 days and cost upwards of billions of dollars in added costs (Cerceo et al., 2016; Dadgostar, 2019).

The processes by which biofilm elicit disease mechanisms have been suggested as: (i) detachment of cells and cell aggregates from indwelling medical devices biofilms, resulting in bloodstream or urinary tract infections; (ii) production of endotoxins; (iii) resistance to host immune systems; and (iv) provision of niche for generation of resistant organisms through mechanisms of exchange, such as plasmid transfer through conjugation.

(i)-Detachment of cells and aggregates. Cells may detach individually from biofilms as a result of cell growth and division within the biofilm, or cell aggregates and clusters may detach or be sloughed off from the biofilm. Studies have shown that an increase in sheer stress, as would occur during changes in direction and rate of flow, result in an increased biofilm erosion. detachment can also occur due to alterations in substrate concentration (Boles et al., 2005; Hentzer et al., 2002).

(ii)-Production of endotoxins. Endotoxins are al bacterial component that contributes to the inflammatory process. In addition to direct effects of cell detachment or antimicrobial resistance, Gram-negative bacteria established within a biofilm will produce endotoxins (Tran et al., 2018). However, very few studies have documented the levels or kinetics of endotoxin release from biofilms.

(iii)-Resistance to host immune system. It was reported that the extracellular slime produced by *S. epidermidis* interfered with macrophage activity. They showed that opsonic antibodies made by patients with CF were ineffective in mediating phagocytosis and elimination of bacterial cells growing in biofilms (Shiau & Wu, 1998). In a study with a rabbit model to show that bacterial growth within a biofilm on an implanted peritoneal device was unaffected by the vaccinated animals immune system. Vaccinated animals had a 1000-fold higher titer of the antibody, but it appeared that the antibody could not reach the surface of bacterial cells within the biofilm. These results lead to the conclusion that organisms detaching from a biofilm, on a medical device or causing other infections, can overcome the immune system, causing disease (Mah & O'Toole, 2001).

(iv)-Provision of a niche for the generation of resistant organisms. It has been shown that bacteria can exchange plasmids by conjugation with biofilms and resistance factors can be carried on a plasmid and conjugation can occur between different bacterial genera (Babakhani & Oloomi, 2018; Domingues et al., 2012; Nikaido, 2009; San Millan, 2018).

Tolerance and persistence of biofilm structures

As well as “conventional” mechanisms of resistance that can be expressed in biofilm bacteria, mechanisms associated to the nature of the biofilm structure are also relevant in conferring antimicrobial resistance of the biofilm (Figure 7) (Levin-Reisman et al., 2019; Yan & Bassler, 2019). Biofilm disease is characterized by a strong recalcitrance to antibiotic treatment, often leading to prolonged treatment regimens or extreme measures, like removal or replacement of colonized devices (Arciola et al., 2018; Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

One feature of this recalcitrance is proposed to be due to tolerant and persistent cells within biofilm communities (de Kievit, 2009; Hall & Mah, 2017; Yan & Bassler, 2019). Bacteria dispersed from biofilms are susceptible to antibiotics, which suggests that tolerance to antimicrobial agents within a biofilm is not only the result of mutations or mobile genetic elements (de Kievit, 2009; Hall & Mah, 2017; Yan & Bassler, 2019)

Biofilm bacteria are physiologically different from the planktonic counterparts, and bacteria in microcolonies employ specific regulatory mechanisms to resist antimicrobial action (de Kievit, 2009; Hall & Mah, 2017; Yan & Bassler, 2019). Highly structured biofilms are not homogenous and consequently physiology and metabolism can be different in different parts of the biofilm (Arciola et al., 2018; Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

Tolerance to an antimicrobial agent are the physiological changes within of a microorganism to survive a transient exposure to high concentrations of an antibiotic. Biofilm tolerance mechanism include reduced growth rate, persistent cells, and the mechanisms that handle antibiotic-induced oxidative stress. Tolerant bacteria grow slower and have longer non-growing phases (lag times) when they exit stationary phase, than their planktonic counterparts (Arciola et al., 2018; Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

Common targets of antibiotics exhibit low-activity in non-growing cells, thus evading antimicrobial killing (Arciola et al., 2018; Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019). Tolerant cells display longer minimum duration of killing, when compared to non-tolerant cells and possess a selective advantage during transient or periodic

antibiotic treatment (Arciola et al., 2018; Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

Tolerant *E. coli* cells were found to rise spontaneously after repeated cycles of ampicillin treatment (Fridman et al., 2014; Hall & Mah, 2017; Yan & Bassler, 2019). It is hypothesized that a sequential relationship exists between bacterial tolerance and bacterial resistance and that tolerance mutations occur more frequently than resistance mutations, due to the molecular size of the former, as there are many genes that when mutated confer tolerance, while only mutation on a few genes yields resistance to specific antibiotics (Levin-Reisman et al., 2019; Yan & Bassler, 2019). Once a tolerant mutation has stablished in the community, chances increase of rarer resistance mutations that confer resistance to a specific antibiotic (Levin-Reisman et al., 2019; Yan & Bassler, 2019).

Other form of tolerance is the formation of persister cells. Time-dependent antibiotic killing essays demonstrate that actively growing bacteria are killed first, while persister cells are killed in a second-phase, much slower compared with the growing individuals (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019). It is important to acknowledge that biofilms can employ tolerance and resistance mechanisms cooperatively to withstand antibiotic pressures. Tolerance prolongs the duration of treatment that bacteria can sustain, for example by remaining dormant. This protects bacteria from lethality of many antibiotics, as beta-lactams and quinolones (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

Visualization of individual cells stablished that exponential growing bacterial population contains a fraction of non-growing cells. This population survives antibiotic treatment and regrows when antibiotic treatment is withdrawn (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019). Other source of persister cells are those that became dormant during stationary phases, that are carried to a new culture upon sub culturing (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019). Although the mechanisms driving subpopulations to enter persistent state are an intense subject of discussion and research, some mechanisms have been discussed (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

One mechanism identified in a high persistent *E.coli* strain is a mutation in the *hipAB* that codes for a toxin-antitoxin (TA) module (Yan & Bassler, 2019). In this mutation, regulation

of HipA is impaired, and when levels of HipA reach a threshold in a cell, cell growth is arrested. Consequently, these growth-arrested cells become persistent (Yan & Bassler, 2019). Additionally, some *S. aureus* strains stochastically enter the stationary phase earlier than others to become persister cells. This entry into stationary phase is characterized by a decrease in intracellular ATP levels, reducing the activity of ATP-dependent antibiotics. As such, stationary-phase *S. aureus* are naturally prone to becoming persister cells (Yan & Bassler, 2019). Persister cells make up for from 10^{-2} to 10^{-5} of a bacterial population within a biofilm (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

Recalcitrance is the reduced susceptibility of biofilm cells to antibiotics, due to a mixture of resistance and tolerance mechanisms. Antibiotic persistence prolongs the duration of treatment that bacteria can sustain only for a subpopulation, even if the population is clonal (Hall & Mah, 2017). Isolation and re-culturing of these persistent populations and subsequent re-exposure to the treatment demonstrates the same heterogeneous response, indicating that this persistence phenomena is not a heritable change in the population, but a bacterial response to pressuring agents (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019). However, genetic mutations can increase or decrease the size of tolerant subpopulations. The importance of diffusion limitation within the biofilm appears to be variable depending upon the experimental setup, bacterial strain and biofilm growth conditions, and even the antimicrobial used. The role of reduced antibiotic penetration in promoting biofilm recalcitrance is not clear, as studies have demonstrated that even antibiotics that rapidly penetrate the biofilm do not cause significant cell death, and it has been proposed that antibiotics with slower penetration time can give time for a more efficient adaptative phenotypical response, with potential to increase tolerance (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

Slow-growing biofilm *E. coli* cells were more resistant to cetrimide than they planktonic counterparts (Pei et al., 2010). At growth rates higher than 0.3 per hour, biofilm and planktonic cells were equally susceptible. In *S. epidermidis,* growth rate strongly influenced susceptibility. The faster the rate of cell growth, more rapidly the rate of inactivation by ciprofloxacin. 10-day old chemostat grown *P. aeruginosa* biofilms were significantly more resistant to tobramycin and piperacillin than younger (2-day old) biofilms. A dosage of 500ug piperacillin + 5ug tobramycin per ml completely inactivated both planktonic and young-biofilm cells of *S. epidermidis*. However, 10-day old biofilm

cell count was reduced by approx. 20% by exposure to this dosage (Christophersen et al., 2020; Tahrioui et al., 2019).

Other physiological changes due to biofilm mode of growth

Gram-negative bacteria respond to nutrient limitations and environmental stresses by synthesizing sigma factors. In *E. coli,* sigma factors are under control of the *rpo*S regulon, that regulates the transcription of genes whose products mitigate stress effects on the cell. By studying wild and mutant *rpo*S *E. coli* biofilms, it was found that wild *E. coli* had higher densities and higher number of viable organisms, comparing to the inactive *rpo*S regulon in the mutant strains. Since *rpo*S is activated during slow growth of this organism, it appears that conditions that elicit slowing bacterial growth such as nutrient limitations or build ups of toxic metabolites favor the formation of biofilms (Adams & McLean, 1999). Nutrient limitation and increase of toxic metabolites concentrations might be particularly acute within the depths of stablished biofilms. In agar entrapped *E. coli,* cells were more resistant to aminoglycosides as oxygen tensions were decreased. They suggested the effects were due to a lowered uptake of antibiotic in oxygen starved cells (Jouenne et al., 1994; O. Tresse et al., 1994, 2003).

The EPS matrix has the potential to physically prevent access of certain antimicrobial agents into the biofilm, acting as an ion exchanger, therefore restricting diffusion of compounds. This characteristic appears to be most pronounced with antibiotics that are highly hydrophilic and positively charged, such as aminoglycosides. It has been demonstrated EPS ability to sequester metals, cations and toxins. EPS has also been reported to provide from a variety of environmental stresses, such as UV radiation, pH shifts osmotic shock and desiccation (Crabbé et al., 2019; Stewart, 2002).

The ability of biofilms to maintain tolerant and persister cells is proposed to be responsible for the difficulties eliminating biofilm infections. Reduced antibiotic penetration in biofilms was initially proposed to be responsible for most of this tolerance, however it is now known that the biofilm mesh size is much larger than antibiotic molecules, and that most antibiotics do not interact strongly with biofilm matrix components (Levin-Reisman et al., 2019; Yan & Bassler, 2019). Rather, this increased tolerance and resistance can be due to altered physiological processes within the biofilm

communities. Cells deep within the biofilm can be in stationary phase, as it is known that penetration of nutrients and oxygen are limited, mostly due to consumption in the periphery of the biofilm. There is increasing evidence supporting similarities between deep-dwelling bacteria inside biofilms and stationary-phase planktonic bacteria (Crabbé et al., 2019; Hall & Mah, 2017; Yan & Bassler, 2019).

This makes the study of biofilms challenging since many experimental procedures such as susceptibility testing and transcriptomic profiling assess the biofilm as a whole, instead of distinct biofilm subpopulations. Planktonic and biofilm cells do not share identical transcriptomes and proteomes, giving rise to phenotypical differences between the two lifestyles (Arciola et al., 2018; Crabbé et al., 2019; Hall & Mah, 2017; Stewart, 2002; Yan & Bassler, 2019).

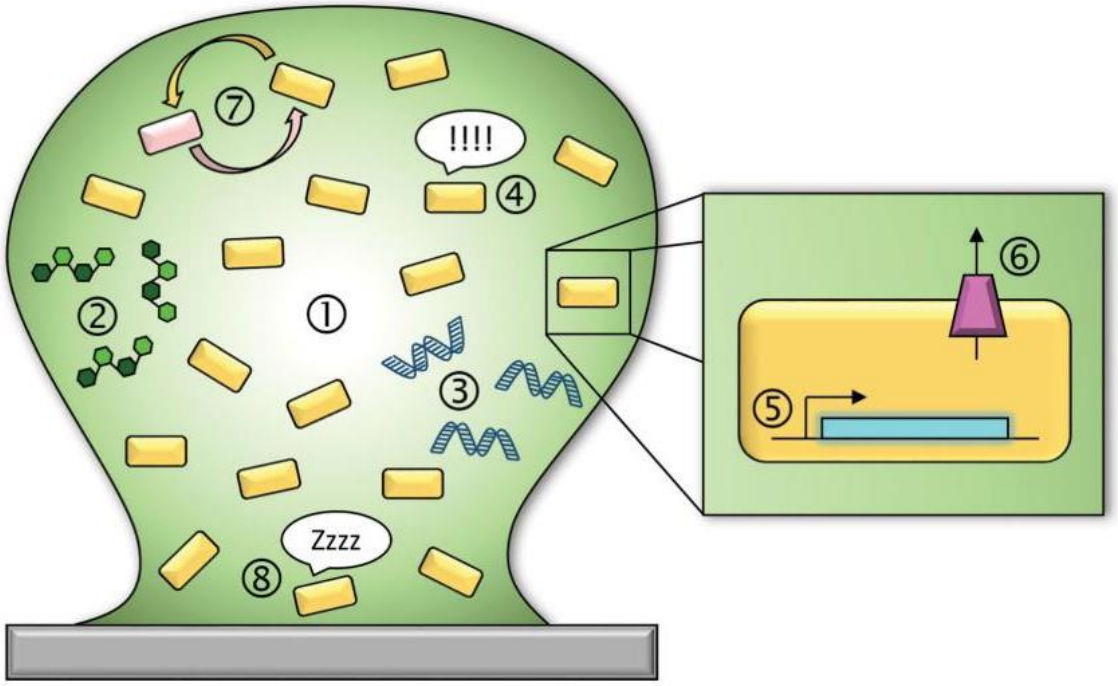

Figure 7-Overview of the major antimicrobial resistance and tolerance mechanisms employed by bacterial biofilms. Biofilm cells (yellow rectangles) are embedded in the EPS matrix (shown in green). The biofilm is attached to a surface (grey rectangle), biotic or abiotic. Resistance mechanisms are numbered as follows: (1) nutrient gradient (color-intensity gradient) with less nutrient availability in the core of the biofilm, (2) matrix exopolysaccharides, (3) extracellular DNA, (4) stress responses (oxidative stress response, stringent response, etc.), (5) discrete genetic determinants that are specifically expressed in biofilms and whose gene products act to reduce biofilm susceptibility via diverse mechanisms (ndvB, brlR, etc.), (6) multidrug efflux pumps, (7) intercellular interactions (horizontal gene transfer, quorum sensing, multispecies communication, etc.) and (8) persister cells (Adapted from Stewart, 2002).

Facing this myriad of mechanisms (Figure 7), “conventional” antibiotics alternatives are further losing effectiveness, while the number and complexity of resistances and structures increases. The clinical pipeline is mostly adaptations of existing classes of antibacterial compounds (Mayer et al., 2010; Theuretzbacher et al., 2019, 2020), that currently offer no long-term solution to the control of resistance development and spread. New sources and alternatives to “conventional” therapeutics are required.

Key messages

Many mechanisms can generate resistance in bacteria. Chromosomal mutations or horizontal gene transfer promote spread of resistance genes within bacterial communities;

Resistance genes are expressed as a vast array of mechanisms that inhibit antibacterial actions by compounds;

A serious threat to healthcare settings are biofilm-producing strains. Biofilms confer resistance to bacteria residing within, through “conventional” resistance mechanisms and through phenotypical differences

Biofilms are characterized by bacterial communities living in colonies, residing within a protective matrix of extra polymeric substances, with different phenotypes than planktonic counterparts.

Biofilm establishment is a stepwise procedure, starting with adhesion, formation of microcolonies and maturation, formation of the mature biofilm. Mature biofilms can detach to colonize other sites.

Biofilms tolerance through persister cells is a key factor in infection recalcitrance of patients. These metabolic distinct cells within a biofilm can resist antibiotic treatment and regrow, causing re-infection.

A blue approach: Seeking solutions from aquatic environments

Since demonstration of antibacterial action in *Chlorella vulgaris* by (Pratt et al., 1944), there has been an increasing interest is unraveling potential bioactive properties of algal and cyanobacterial organisms, with large screenings and discovery of relevant compounds every year (Lauritano et al., 2019). Currently, many compounds extracted from aquatic algae are fully integrated in the nutraceutical, pharmaceutical, cosmetic, food preservation and other industrial sectors.

The term alga (pl. algae) corresponds to a functional group commonly defined as containing all distant related eukaryotic organisms that possess capacity to perform oxygenic photosynthesis, except higher (vascular) plants (Pierre et al., 2019). Oxygenic photosynthesis evolved only once during the history of life, in cyanobacteria, and appeared much later in eukaryotes via primary endosymbiotic incorporation of a cyanobacterium into a unicellular heterotrophic eukaryote host, early in the evolution of the Archaeplastida lineage, now comprising Glaucophytes, red and green algae and vascular plants (Pierre et al., 2019). During history of the Archaeplastida, algae from this lineage have been incorporated through a secondary endosymbiotic event, into a heterotrophic host from distant evolutionary lineages, forming plasmid-containing sub-lineages (i.e. algae) within these groups. Consequently, algae are a remarkably taxonomic diverse, with representatives such as macroalgae and microalgae in all but one group, that is the Unikonts (Pierre et al., 2019).

All chloroplast-containing organisms are descendants of cyanobacteria, highly modified by massive gene transfer to the hosts. While multicellular algae (macroalgae or macroalgaes) only occur in sub-lineages of Archaeplastida (red and green macroalgae) and Heterokontophyta (brown macroalgae), microalgae are single celled algae found in all main eukaryotic lineages except Unikonts, as mentioned above (Pierre et al., 2019). This increasing concern and demand for effective antimicrobial solutions has led to research and identification of novel classes of compounds these aquatic organisms. Micro- and Macroalgae harbor a multitude of compounds with potential to be employed in several industrial fields (Fabris et al., 2020; Khan et al., 2018; Koyande et al., 2019; Kim, et al., 2015; Salvador et al., 2007). These compounds are in their majority physiological metabolites, primary or secondary (Hentati

et al., 2020). Primary metabolites are directly involved in physiological functions under normal growth conditions, such as reproduction, photosynthesis, etc., while secondary metabolites are mainly excretory products occurring under stressful conditions, as exposure to ultraviolet radiation, changes in temperature and salinity, nutrient scarcity and environmental pollutants (Hentati et al., 2020).

The compositional content of algal primary metabolites are usually proteins, polysaccharides, and lipids, whereas secondary metabolites that are produced are phenolic compounds, halogenated compounds, polyketides, terpenes, peptides, alkaloids, shikimates and sugars small peptides, among other bioactive compounds (Mayer et al., 2013; Rocha-Santos & Duarte, 2014; Rosa et al., 2020; Schmitz et al., 1993).

The existence of bioactive compounds in algae is to be expected due to cooccurrence of these organisms in aquatic natural communities, where an inhibitory interaction occurred between producers and competitors in the same habitat (Amaro et al., 2011; Barrera & Mayfield, 2013; Chanda et al., 2019; de Vera et al., 2018; Lauritano et al., 2019; Magdalena & González-Fernández, 2019; Molino et al., 2018; Shannon & Abu-Ghannam, 2016; Tran et al., 2018).

Macroalgae: multicellular aquatic flora

Macroalgae (or macroalgaes) are a diverse group of marine organisms that have developed complex and unique metabolic pathways to ensure survival in a high competitive environment (Harnedy & Fitzgerald, 2011). These organisms can be categorized into three major groups: (i) brown macroalgaes (Phaeophyceae) (ii) red macroalgaes (Rhodophyceae) and (iii) green macroalgaes (Chlorophyceae), and can be a key source of functional metabolites, such as proteins, polysaccharides, peptides, lipids, amino acids, polyphenols and mineral salts (Hentati et al., 2020). Macroalgae constitute the richest source of non-animal biological compounds in nature and have historically been employed as fertilizers, in human food, medicine and animal feed (Harnedy & Fitzgerald, 2011; Hentati et al., 2020).

Their consumption also falls in line with consumers awareness and perception towards organic and environmentally friendly products. Of all three types of macroalgae, brown algae are the most consumed (66,5%), followed by red algae (33%) and green algae (5%) (Afonso et al., 2019). Only six species of macroalgae represent 96% of global production volume, such as *Euchema*, *Laminaria*, *Gracillaria*, *Undaria* , *Porphyra* and

Kappaphycus. Due to the massive macroalgae diversity, compositional content is highly diversified, as it results of a combination of biotic factors, such as species, reproductive status, and age, as well as abiotic factors of the environment, such as light availability, grazing pressure and nutrient availability (Hentati et al., 2020).

Microalgae: Microscopic factories

Microalgae are microscopic unicellular organisms capable of converting solar energy in chemical energy as a result of photosynthesis and contain a multitude of bioactive compounds that can be harnessed for commercial use. Microalgae can produce proteins, lipids, carbohydrates, carotenoids and vitamins. The first use of microalgae dates to the Chinese, that used *Nostoc* to survive during famine (Cardozo et al., 2007; Molino et al., 2018; J. Singh & Saxena, 2015; Stengel et al., 2011).
Microalgae are a rich source of widely distributed bioactive compounds with commercial importance. These metabolites can be synthesized from secondary metabolism or directly from primary metabolism, and these compounds include proteins, vitamins, fatty acids and pigments with bioactivity as antibiotic, antifungal, antiviral, anticancer, antiprotozoal, antialgal and antienzymatic (de Vera et al., 2018; Gouveia et al., 2010). In most microalgae, these compounds are accumulated in the biomass while other cases are known to secrete metabolites into the medium (exometabolites). Microalgae have an extra advantage of significant metabolic plasticity, which is dependent of their physiological state (i.e. stressed vs non stressed conditions), meaning that their secondary metabolism can be easily triggered (Chamberlain et al., 1991; Raposo et al., 2015; de Vera et al., 2018; Gouveia et al., 2010).

The variety of compounds generated from microalgae have a broad spectrum of application, from pharmaceuticals to food industry or wastewater management (Figure 8). Several compounds have shown potent biological activities and the possible use of the compounds as probiotics, nutraceuticals or chemotherapy agents demonstrated promising results (Chamberlain et al., 1991; Raposo et al., 2015; de Vera et al., 2018; Gouveia et al., 2010) The main obstacle for their commercial exploitation remains the production cost, bypassed with optimization of mass culturing. Microalgae are a promising source of high value compounds and their application as antibacterials are far from fully developed (Falaise et al., 2016).

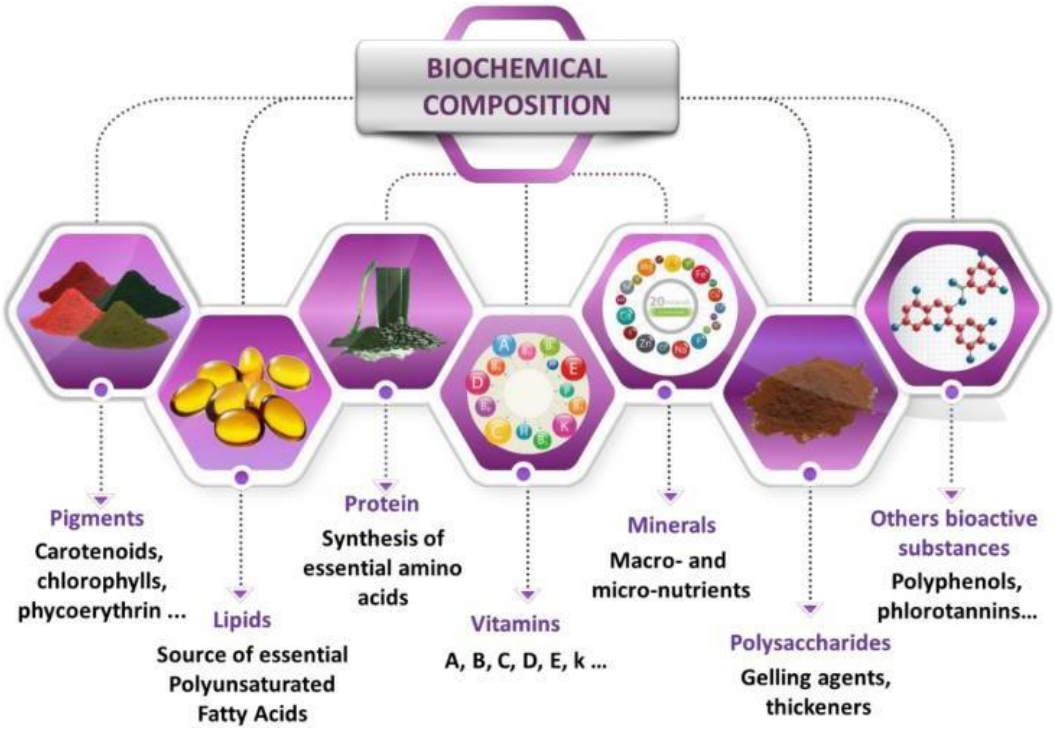

Figure 8-Bioactive components found in algae biomass (Adapted from Hentati et al., 2020).

Cyanobacteria: Where it all started

Cyanobacteria (blue-green algae) have also been identified as one of the most promising groups of organisms with potential bioactive compounds (Figure 9). Therapeutic characteristic of these organisms dates back to 1500 b.c,, where *Nostoc* species were used to treat gout, fistula and several forms of cancer (Burja et al., 2001; Tran et al., 2018; Zerrifi et al., 2018). Regards using cyanobacteria bioactive compounds require substantially more information, as they are producers of several toxins to mammals, with serious toxicity, specially hepatotoxicity, as these hepatotoxins can inhibit protein phosphatases and raised the possibility that human exposure to non-lethal dosages of these toxins might contribute to the development of cancer (Burja et al., 2001).

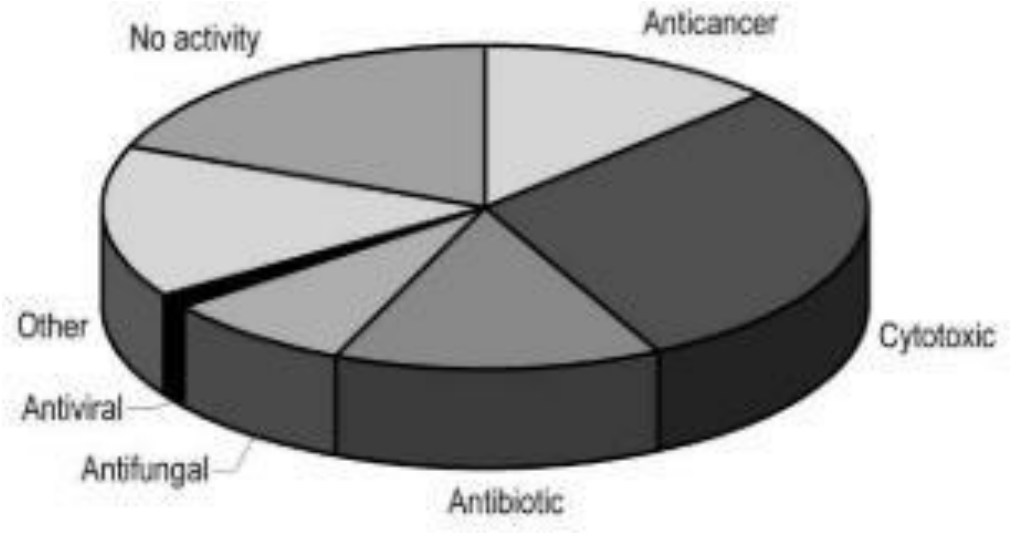

Figure 9-Bioactivity of compounds identified from cyanobacteria (Adapted from Burja et al., 2001).

Algal secondary metabolites in their natural form will not necessarily lead to commercialization, but activity of these natural compounds may be explored in structure-function studies, to develop analogues with greater and more focused activities (Burja et al., 2001; Moore, 2011; Tran et al., 2018; Zerrifi et al., 2018).

Compounds produced by algae

Carbohydrates

Macroalgaes are considered the most abundant source of polysaccharides, that can be sulphated and non-sulphated. These polymers are structurally variable and are polymers of repeating monomeric carbohydrates units joined via glycosidic linkages. As constituents of the algal cell walls, these compounds provide rigidity and strength to the algal cell (Bhowmick et al., 2020).

Carbohydrate content in macroalgaes vary from 5% to 75% DW depending on species, period and harvesting site, and consist mainly of polysaccharides and few amounts of disaccharides and monosaccharides. Algal polysaccharides are mainly found in sulphated and non-sulphated forms (Hentati et al., 2020).

Green macroalgae are rich in ulvans, brown macroalgae are rich in alginates/alginic acids, laminaran/laminarins and fucoidans, while red macroalgae are rich in carrageenans, agar, xylogalactans, sulphated galactans, xylans, porphyran and floridean starch (Hentati et al., 2020).

The reserve polysaccharides result from the photosynthetic pathway and are stored in algae plastids, reusable on demand to maintain basal metabolism. On the other hand, polysaccharides from the matrix are substantially different from one algal class to another. These phycocolloids, with numerous applications in the food industry as texturizers, are hydrocolloids capable of changing the rheological properties of aqueous solution that contains them, hence their utilization as thickeners or gelling agents. These characteristics are strongly influenced by the specific structure of these polysaccharides, such as monosaccharide composition, anomeries, branching degree and glycosidic bonds, as well as the molar masses. As such, these polymers can be linear (alginates, cellulose) or branched (fucoidans, sulphated galactans) and can be replaced by proteins and organic groups such as acetate, lactate pyruvate and succinate, or inorganic groups like phosphate, sulphate and amine and in this case are called aglycones (Hentati et al., 2020).

Brown macroalgae

- Alginates, available in acidic form (alginic acid) or in salt (alginates), are the major components of brown macroalgae cell walls and the intracellular matrix. Alginates are anionic polysaccharides, composed of Beta-D-mannuronic-acid (M) and alpha-L-guluronic-acid (G).
- Laminarans are the principal storage PS of brown macroalgae. Content can represent up to 32-35% dry weight.
- Fucoidans are the main water-soluble polysaccharide of brown algae. Fucoidans are a complex group of polysaccharides, which contribute to intracellular mucilage and are sulphated polysaccharides composed of L-fucose and sulphate ester groups with minor amounts of different molecules. They can differ from monosaccharides (mannose; arabinose; glucose), acidic monosaccharides, acetyl groups and proteins. Fucoidans composition varies according to species and geographical origin, even within the same species.

Red macroalgae

- Carrageenans are the major component of red macroalgae cell walls.

- Carrageenans are available in 3 forms, according to the degree of sulphation. These forms can be kappa, yota and lambda and intermediate phases such as kappa/yota, yota/lambda and kappa/lambda (Cardoso et al., 2019; Pangestuti & Kim, 2014; Pereira et al., 2007).
- Agar is a mixture of at least 2 polysaccharides (agarose and agaropectin). It is extracted from the red macroalgae and have structural and functional properties like carrageenans. In agar, agarose is the predominant fraction.
- Sulphated galactans are the main EPS (extracellular polysaccharides) of red algae, found as well in brown and green algae.

Green macroalgae

- Ulvans. Water soluble sulphated polysaccharides extracted from the intracellular space and fibrillar wall of green macroalgae (mainly *Ulva* sp). Accounts for 18-29% of algal dry weight.

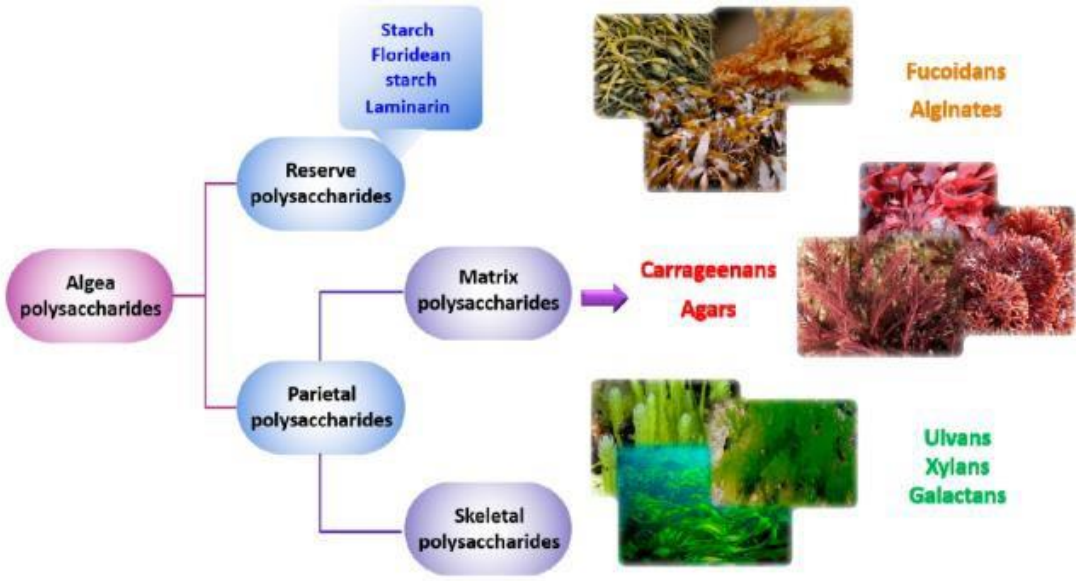

Figure 10-Polysaccharides present in algae and cellular functions (Adapted from Hentati et al., 2020).

Macroalgal polysaccharides and their structural diversity represent a potential source of bioactive properties with interest for alternative therapeutic options and industrial applications (Figure 10). These are present in a variety of products, such as pharmaceutical, cosmetic and food preservation technologies. However, and despite the usage of alginate gels and wound healing agents among others, these compounds still depict a passive role due to extraction and purification costs (Hentati et al., 2020).

The underlying mechanism behind the antimicrobial properties of these polysaccharides has been proposed to be through the presence of glycoproteins receptors in the polysaccharide, with capacity to bind to molecules present in the bacteria cell wall, cell membrane and nucleic acids (Bhowmick et al., 2020). These interactions cumulatively result in the disruption of the bacterial cell wall and consequently cell lysis (Bhowmick et al., 2020). However, the bioactivity of the polysaccharide compounds can vary according to extraction procedures (Bhowmick et al., 2020), supported by a study regarding hot and cold water polysaccharide extraction inhibited the growth Gram-negative and Gram- positive bacteria (Abou Zeid et al., 2014; Bhowmick et al., 2020).

In microalgae, polysaccharides vary greatly from one species to another, reflected in the high number of enzymes involved in polysaccharide synthesis (Chanda et al., 2019; Rossi & De Philippis, 2016). With the exception of *Gyrodinium impudicum* and *Chlorella vulgaris*, that produce homopolymer polysaccharides of galactose and B-(1-3)- glucan, respectively (Chanda et al., 2019), microalgal polysaccharides are heteropolymers of mainly galactose, xylose and glucose at different proportions and bound by glycosylic linkages (Chanda et al., 2019; De Jesus Raposo et al., 2015). This characteristic proportion difference of these neutral sugar is presumed to act as variability of bioactivity in these compounds (Chanda et al., 2019), although bioactive properties are also linked to the degree of polysaccharide's sulphation, uronic acid content and correspondent molecular weight (Chanda et al., 2019). Additionally, other sugars such as rhamnose, fucose, fructose and methyl sugars are also constituents of microalgal polysaccharide fractions (Chanda et al., 2019).

Amino acids and peptides

Algae are a potential source of protein, although their protein values varies among phyla (Bhowmick et al., 2020). The proportion of essential amino acids in the algae *Palmaria palmata*, such as valine, methionine and leucine, is much higher, with values comparable to ovalbumin, than when compared to *Ulva rigida*, whose proportion of essential amino acids were comparable to those of legumes (Bhowmick et al., 2020). Such volumes of essential amino acids confer algae a potential food supplement. Additionally, several antimicrobial peptides (AMPs) can be derived from either short chain peptides or highly complex large proteins, that typically demonstrate a wide range of activity against pathogenic bacteria (Guzmán et al., 2019; Harnedy & Fitzgerald, 2011; Shannon & Abu-Ghannam, 2016; Smith et al., 2010).

This is most likely due to their amphiphilic nature, which allows these compounds to interact with both polar and apolar molecules in the bacterial cell membrane, causing pores in the surface of the bacterial cell, promoting lysis and disruption of the bacterial cell (Bhowmick et al., 2020). Protein concentrated fraction of a green algae *Tetraselmis suecica* demonstrated high antibacterial activity against MRSA, *Bacillus cereus* and *E. coli*. Identification of compounds in these fractions identified presence of (AMPs) that were later isolated and synthesized for posterior antibacterial bioactivity essays. Subsequent substitution of single residues in these AMPs enhanced antibacterial activity, with no cytotoxicity on the tested human cell line (Guzmán et al., 2019; Bhowmick et al., 2020).

A mechanism for successful isolation of these bioactive peptides from their mother proteins is through enzymatic hydrolysis. These isolated peptides display higher bioactivity levels than the original molecule (Bhowmick et al., 2020). Additionally, marine sources have proven to be sources of these peptide hydrolysates (Fan et al., 2014). Protein isolates from the brown macroalgae *Saccharine longicruris* inhibited the growth of *Staphylococcus aureus* (Beaulieu et al., 2015). Further identification of these compounds by LC-MS/MS indicated that these peptides were precursors of algal enzymes related with macroalgae immune system (Beaulieu et al., 2015). With further advancements, these algal peptides can display potent antibacterial activities, with additional modifiable characteristics that can further enhance their potential as therapeutic agents (Bhowmick et al., 2020).

Lectins

Lectins are a natural ubiquitous group of carbohydrates-binding proteins. Lectins interact with specific glycan structures connected to membrane-bound and soluble glycoconjugates, participating in a wide array of biological processes, such as host-pathogen interaction, cell-cell communication, among others (Harnedy & Fitzgerald, 2011). Most of algal lectins display higher specificity to oligosaccharides and/or glycoproteins, rather than monosaccharides. Based on the binding properties of lectins, these are categorized as three major groups: complex type-N glycan specific lectins; high mannose (HM) type N-glycan specific lectins; and lectins specific to the none above (Pérez et al., 2016). Lectins from marine organisms can also be categorized as C-type lectins, F-type lectins, galectins, intelectins and mannose-binding lectins (Pérez et al., 2016).

Lectins isolated from the red alga *Solieria filiformis* inhibited growth of pathogenic Gram-negative bacteria, such as *P. aeruginosa*, *K. pneumoniae* and *Salmonella enterica* serovar *typhi*, among others (Lima et al., 2005). Lectins extracted from the red algae *Galaxaura marginata* and *Euchema serra* inhibited the growth of the pathogen *Vibrio vulfinicus* (Zhao et al., 2003). This specific antibacterial activity of lectins is proposed to be through the binding of these lectins to mannan found on the cell-surface of Gram-negative bacteria (Bhowmick et al., 2020).

Lipids, fatty acids and sterols

Fatty acids (FAs) are essential membrane components, required for the maintenance of membrane integrity and cellular organization (Figure 11). Fatty acids from algal cells are released upon conditions in which membrane integrity is lost, correlating to defensive behavior observed against predators and pathogenic bacteria, revealing potential as new antibiotic agents (Bhowmick et al., 2020). FAs are found primarily on lipids that constitute cell membranes and energy storage structures, but during cellular disintegration, large quantities of free fatty acids (FFA) are released from cellular lipids to host lipolytic enzymes (Messyasz et al., 2018; Smith et al., 2010; Tran et al., 2018).

Fatty acids are carboxylic acids with aliphatic chains and prevalent even carbon numbers (C4-C28). These can be straight or branches, saturated or unsaturated. According to double bonds, these fatty acids are classified as monounsaturated (MUFAs; one double bond) and polyunsaturated (PUFAS; two or more double bonds). PUFAs can be classified as n-3 or n-6, according to the position of the first double bond from the methyl end (Pérez et al., 2016).

Lipid content in algae range from 0.12% to 6,73% (dry weight), composed primarily by phospholipids, glycolipids, non-polar glycerolipids (neutral lipids) and sterols (Pérez et al., 2016).
Phospholipids are in extra-chloroplast membranes, accounting for 10%-20% of total fatty acids in algae. These are characterized mainly by n-6 fatty acids, and the majority of fatty acids present are oleic, palmitic, stearic, arachidonic and eicosapentanoic acids. In green algae, the most dominant fatty acid is phosphatidylglycerol, while in red algae is phosphatidylcholine. In brown algae, the prevalent fatty acids are phosphatidylcholine

and phosphatidylethanolamine (Cavonius et al., 2014; Chanda et al., 2019; P. Kumar et al., 2020; Pérez et al., 2016).

Glycolipids are in photosynthetic membranes, constituting more than half the lipids present in all algal groups. They are characterized by high n-3 polyunsaturated fatty acids, and the 3 major types of glycolipids found are monogalactosyldiacylglycerides, digalactosyldiacylglycerides and sulfoquinovosyldiacylglycerides. Regarding glycerolipids, the most prevalent is trycylglicerol, with content ranging from 1% to 97%, with functions of storage and energy reservoir. Green algae are rich in C18 PUFAs, mainly α-linolenic (C18:3 n-3), stearidonic (C18:4 n-3) and linoleic (C18:2 n-6) acids; red algae are rich in C20 PUFAs, mainly arachidonic (C20:4 n-6) and eicosapentaenoic (C20:5 n-3) acids. Brown algae exhibit both these fatty acids (Shannon & Abu-Ghannam, 2016).

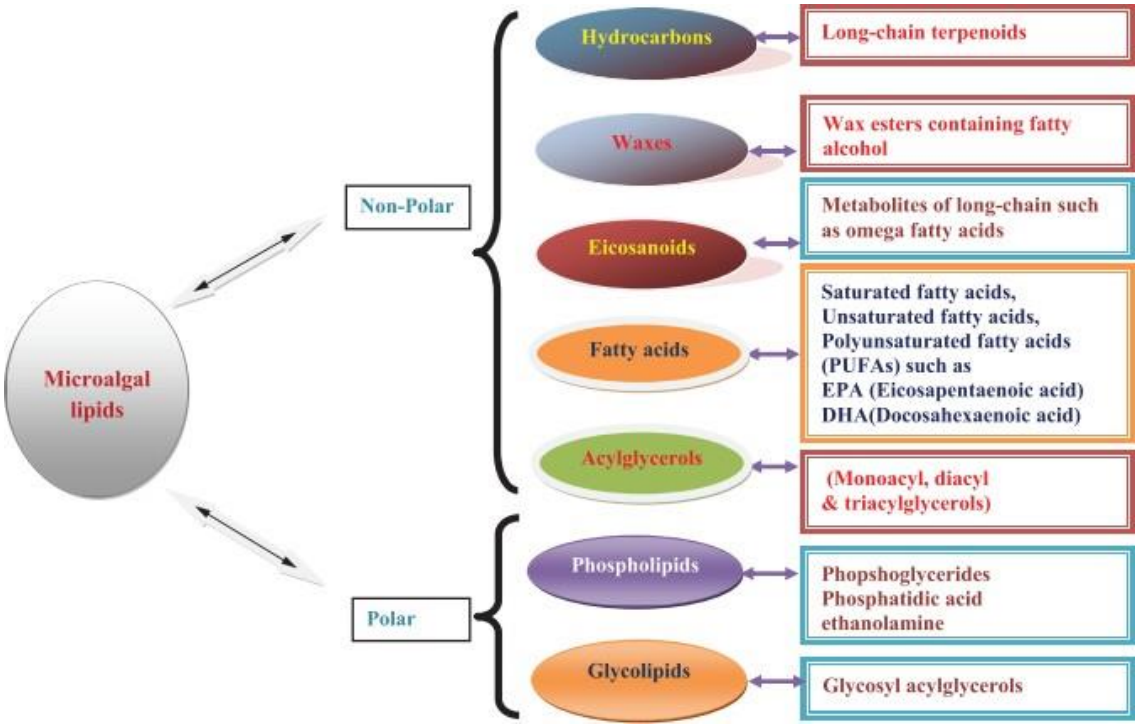

Figure 11-Lipid fractions found in microalgae species (Adapted from Ranjith Kumar et al., 2015).

In general, unsaturated FFAs tend to be more bioactive that saturated FFAs, with the same carbon chain length. Within series of monounsaturated FFAs, the most potent usually have 14 or 16 carbon atoms and there is often a direct correlation between the number of double bonds in an unsaturated FFA chain and its antibacterial efficacy. For saturated FFAs, the most active have 10 to 12 carbons in the chain, and antibacterial efficacy tends to decrease as the chain gets longer or shorter. Workers have reported

that FFAs with 14,16 or 18 carbon atoms can be more potent than FFAs with 10 or 12 carbon atoms, depending of the bacterial species (Desbois & Smith, 2010).

Additionally, oxygenated products of these acids (oxylipins), derived mainly from C16,C18,C20 and C22, are proposed to participate in immune responses when algae face biotic and abiotic stresses, such as predation or pathogenic bacterial colonization (Pérez et al., 2016). Fatty acid profile of macroalgae contain wide quantities of PUFA's, such as docosahexaenoic acid (DHA, C22:6 n-3), eicosapentanoic acid (EPA C20:5, n-3), alpha-linoleic (ALA C18:3 n-3), linoleic acid (LA C18:2 n-6), octadecatetraenoic acid (SDA C18:4, n-3) and arachidonic acid (AA, C20:4 n-6) (Desbois & Smith, 2010).
The antibacterial activity of each FFA is influenced by its structure and shape. In turn, these are a function of the carbon chain length and orientation and the presence, number and orientation of double bonds (Tran et al., 2018).

Sterols are organic molecules with four fused rings (A-D) as their molecular core structure, with a hydroxyl group in carbon-3, two methyl groups at C18 and C19 and a side chain at C17 (Bhowmick et al., 2020; Pérez et al., 2016). The presence of a hydroxyl group in the third position of ring A imparts polarity, while the remaining non-polar nature is due to the aliphatic chains (Chanda et al., 2019; Iglesias et al., 2019; Ren et al., 2017). Sterols are essential lipid molecules present in eukaryotic cells, maintaining membrane fluidity and acting as signaling molecules to signal transductor signals, as important precursors to several fat soluble vitamins and hormones (Bhowmick et al., 2020; Pérez et al., 2016).

Sterol content in algae is mainly represented by fucosterol, clionasterol, isofucosterol, and cholesterol (Bhowmick et al., 2020; Pérez et al., 2016). Fucosterol and its derivatives are found in brown algae, while desmosterol, cholesterol and its derivatives are abundant in red algae. Sterol content in green algae is characterized by the presence of 24-ethylcholesterol and ergosterol (Bhowmick et al., 2020). These sterols have important nutritional and biological properties, such as anticancer, antioxidant, antiobesity, antitumor, antiviral and are effective against cardiovascular disease (Pérez et al., 2016). Additionally, Sterols exhibit antibacterial activity thought interaction with negatively charged phosphate groups in the bacterial membrane, causing lipid exchange and resulting in loss of integrity and instability in osmoregulation, causing cell lysis (Bhowmick et al., 2020).

Pigments

Three basic type of pigments are found in algae and these are chlorophylls, carotenoids and phycobiliproteins (phycobilins) (Figure 12) (Abou Zeid et al., 2014; Beaulieu et al., 2015; Bhowmick et al., 2020; Fan et al., 2014; Silva et al., 2020). Pigment function is wide, varying from being essential photosynthetic components to conferring photoprotective nature to algae, along with characteristic coloration (Abou Zeid et al., 2014; Beaulieu et al., 2015; Bhowmick et al., 2020; Fan et al., 2014; Silva et al., 2020). Due to this coloring, classification of algae is made according to these characteristics, as Chlorophyta (green algae) with its characteristic green coloration due to chlorophyll a and b; Phaeophyta (brown algae), with its greenish-brown coloration attributed to fucoxanthin, chlorophyll a and c; and finally Rhodophyta (red algae), with the characteristic red coloration attributed to chlorophyll d and phycobillins, such as phycoerythrin and phycocyanin (Bhowmick et al., 2020; Pérez et al., 2016; Silva et al., 2020).

Phycobilins are water-soluble fluorescent pigments, consisting of a proteic backbone, to which prostetic billin chromophores bind covalently (Harnedy & Fitzgerald, 2011), forming arranged macromolecules known as phycobillisomes. Phycobiliproteins include phycocyanins (blue pigment), phycoerytrins (red pigment) and allophycocyanins (light-blue pigment) (Harnedy & Fitzgerald, 2011).

Carotenoids are linear polyenes and are classified in carotenes, structurally containing a chain end with a cyclic group, containing carbon and hydrogen atoms only (α, y, β-carotene, lycopene) (Silva et al., 2020; Pérez et al., 2016); and xanthophylls or oxycarotenoids, that have at least one oxygen atom as a hydroxyl group, as an oxygroup or combination of both. Xantophyls include fucoxanthin, violaxanthin, zeaxanthin, lutein, neoxanthin (Pérez et al., 2016; Silva et al., 2020).

- Chlorophyta common pigment composition is β-carotene, lutein, violaxanthin, neoxanthin and zeaxanthin;
- Rhodophyta contains B-carotene, lutein and zeaxanthin;
- Phaeophyta contain β-carotene, violaxanthin and fucoxanthin, this latter being restricted to Phaeophyta (Bhowmick et al., 2020; Pal et al., 2014; Pérez et al., 2016; Rosa et al., 2020; Silva et al., 2020). Fucoxanthin is the most abundant pigment in brown algae, conferring the characteristic brown coloration (Silva et al., 2020; Bhowmick et al., 2020);

Vitamins and minerals

Vitamin content in macroalgae is mainly composed of hydro- and liposoluble vitamins, with potential for improving food and feed vitamin status. These consist of water-soluble vitamins such as vitamin B (B1, B2, B3, B6 and B12), C, niancin, folic acid, pantothenic acid and riboflavin, as well as fat-soluble vitamins, such as vitamin A, D and E and carotenoids as provitamin forms of vitamin A (Beta-carotene) (Ali et al., 2016; Singh et al., 2005). Regarding mineral content, it ranges between 7% to 40% DW, again according to criteria such as species, time and harvest location and season. There is a significant amount of macroelements such as Ca, K, P, Na, Mg, Mn, Fe and microelements, such as Pb, Cu, Zn, Sc, Sd, As, Sr and Cr produced by algae species (Lauritano et al., 2019; Singh et al., 2005).

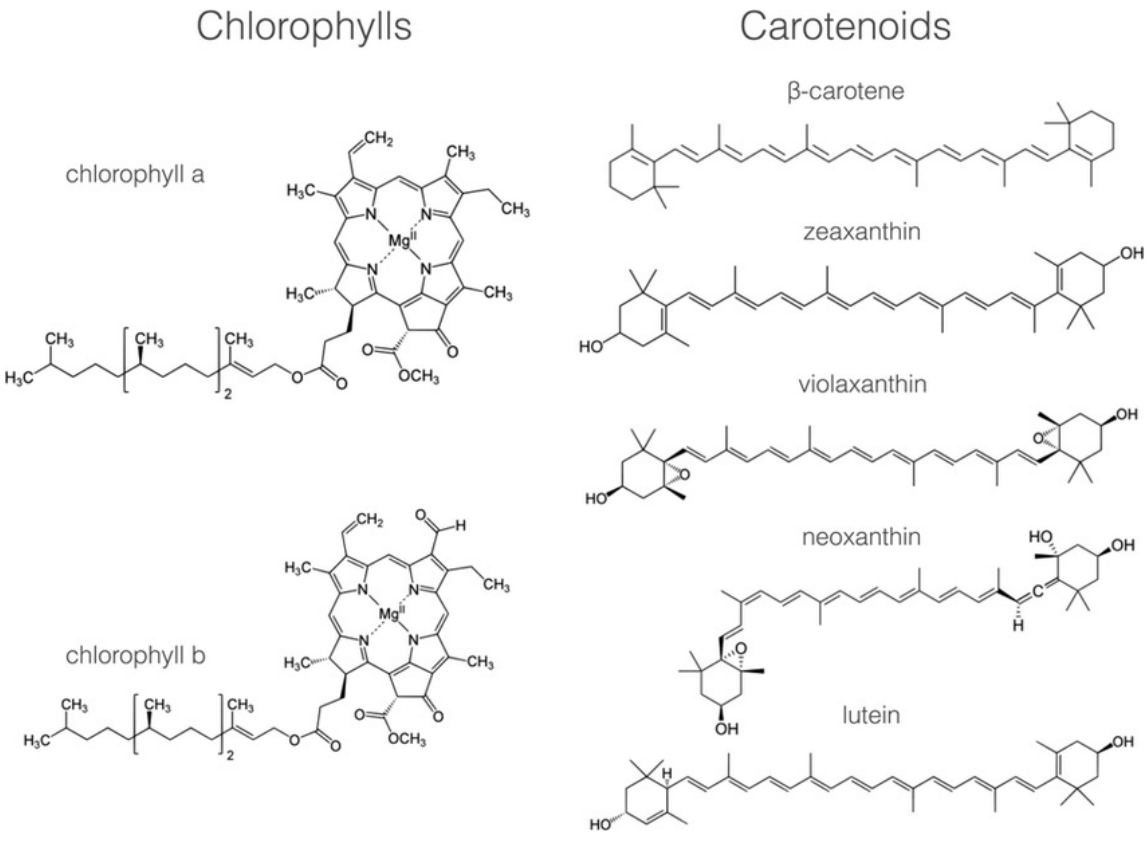

Figure 12-Main types of pigments found in algae species (Adapted from Johnson, 2016).

Secondary metabolites

Secondary metabolites production occurs in response to predators, niche colonization and several abiotic factors, such as pH alterations, UV exposure, among others. Functional groups with antibacterial activity in these compounds include phenols, peptides, terpenes, polyacetylenesindole alkaloids, aromatic organic acids, shikimic acids, polyketides, hydroquinones, alcohols, aldehydes, ketones and halogenated furanones, alkanes and alkenes (Shannon & Abu-Ghannam, 2016; Bhowmick et al., 2020; Chanda et al., 2019; Cho & Rhee, 2019; Harnedy & Fitzgerald, 2011; Pal et al., 2014; Pérez et al., 2016; Silva et al., 2020).

Phenols

Phenolic molecules are characterized by the presence of an aromatic ring and one or more hydroxyl groups. These structures can range from single molecules, such as hydroxycinnamic acid or flavonoids, to more complex polymers, with a wide range of molecular sizes (126-650 kDa) (Cotas et al., 2020). According to the characteristic substructure, a phenol is represented by one phenolic hydroxyl group. Cathecol and resorcinol (benzenediols) are characterized by the presence of two phenolic hydroxyl groups; and pyrogallol and phloroglucinol are characterized by the presence of three phenolic hydroxyl groups (benzenetriols) (Cotas et al., 2020).

Phenolic acids

Phenolic acids (PAs) are bioactive compounds, involved in functions such as nutrient absorption, protein synthesis and enzymatic activity, among others (Bhowmick et al., 2020; Cotas et al., 2020; Fernando et al., 2016; Rosa et al., 2020). Regularly, these compounds are found bound to other molecules, such as simple or complex carbohydrates, organic acids and other biomolecules such as terpenoids and flavonoids. PAs are formed by a single phenol ring and at least a functional carboxylic group and are classified according to the number of carbon chains attached to the phenolic ring (Figure 13) (Cotas et al., 2020). Phenolic acids are therefore classified as C6-C1 for hydroxybenzoic acid (HBA), which is typically found with one carbon chain attached to the phenolic ring; C6-C2 for acetophenones and phenylacetic acids, found with two

carbon chains attached to the phenolic ring; and C6-C3 for hydroxycinnamic acids (HCA), found with three carbon chains attached to the phenolic ring (Cotas et al., 2020). HBA acids include gallic acid, p-hydroxybenzoic, vanillic, syringic acids and protocathecins, among others. There are variations in the basic structure of HBA, as the methoxylation and hydroxylation of the aromatic ring. These can be detected as free acids, however they occur mainly as conjugates, as esterification of conjugates can yield interesting compounds, as hydrolysable tannins. HCA acids are trans-phenyl-3-propenoacids that differ in their ring constitution. The HCA derivatives include caffeic, ferulic, sinapic, p-coumaric, with wide distribution of these compounds as conjugates, as esters of quinic acid (chlorogenic acids CGA). Additionally, though variation of the position and number of the acyl residue, the acids can be classified in additional subgroups: (1) mono-esters of caffeic, ferulic, and p-coumaric; (2) di-, tri-, and tetra-esters of caffeic acid; (3) mixed di-esters of caffeic-ferulic acid or caffeic-sinapic acids; (4) mixed esters of caffeic acid with dibasic aliphatic acids, such as oxalic or succinic. Additionally, cinnamic acids can condense with molecules other than quinic acid, including rosmaric and malic, with aromatic amino acids and choline, among others (fig13) (Cotas et al., 2020; Fernando et al., 2016).

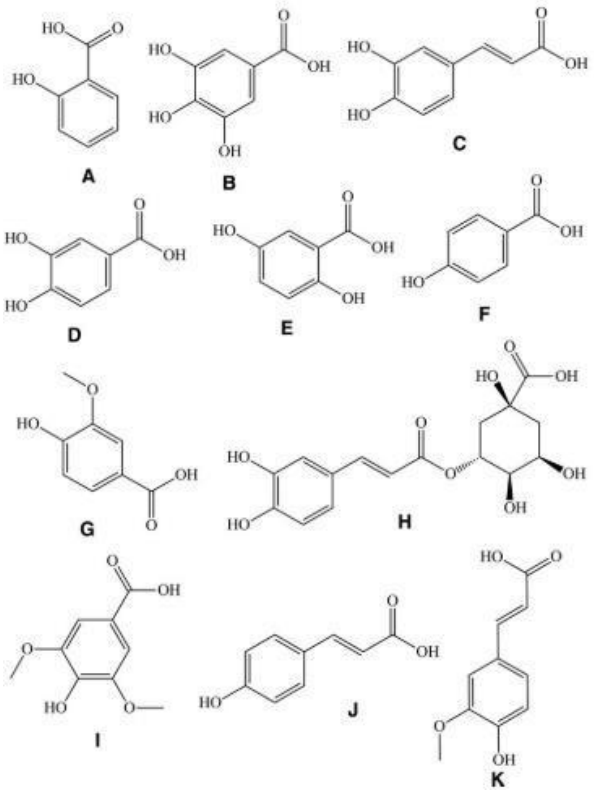

Figure 13-Structures of some phenolic acids found in macroalgaes. (A) Salicylic acid, (B) gallic acid, (C) caffeic acid, (D) protocatechuic acid, (E) gentisic acid, (F) p-hydroxybenzoic acid, (G) vanillic acid, (H) chlorogenic acid, (I) syringic acid, (J) p-coumaric acid, and (K) ferulic acid (Adapted from Fernando et al., 2016).

Of all phenolic compounds, attention has shifted towards tannins, due to their interesting bioactive properties (Arnold & Targett, 2002; Bhowmick et al., 2020; Cotas et al., 2020; Luna-Guevara et al., 2018; Mukherjee et al, 2019).

Phlorotannins

Phlorotannins are a polyphenolic molecule restricted to brown macroalgaes (Afonso et al., 2019; Cotas et al., 2020). Structural oligomers of phloroglucinol, these compounds are formed thought the acetate-malonate pathway (polyketide) and found in cellular structures known as physodes, located either in the periphery of the cell or in perinuclear regions (Bhowmick et al., 2020; Cotas et al., 2020). A description of the process behind the formation of phloroglucinol molecules is well explained in (Cotas et al., 2020).

The polymeric molecules of phloroglucinol are structurally heterogeneous, due to the variability of linkages between phloroglucinol and the hydroxyl groups present. Phlorotannins can be subdivided in: (1) phloretols (aryl-ether bonds); (2) fucols (aryl-aryl bonds); (3) fucophloretols (ether or phenyl linkage); (4) eckols (dibenzo-1,4-dioxin linkages); (5) fuhalols (*ortho-/para*- arranged ether bridges containing an additional hydroxyl group on one unit); and (6) carmalols (dibenzodioxin moiety) (Bhowmick et al., 2020; Cotas et al., 2020). Additionally, the binding of phloroglucinol monomers can take place at a different position within each of the classes described above, leading to the formation of structural isomers in addition to the conformational ones (Figure 14) (Fernando et al., 2016; Imbs & Zvyagintseva, 2018; Santos et al., 2019).

This leads to other criteria of classification of phlorotannin molecules, such as linear and branched phlorotannins (Cotas et al., 2020).

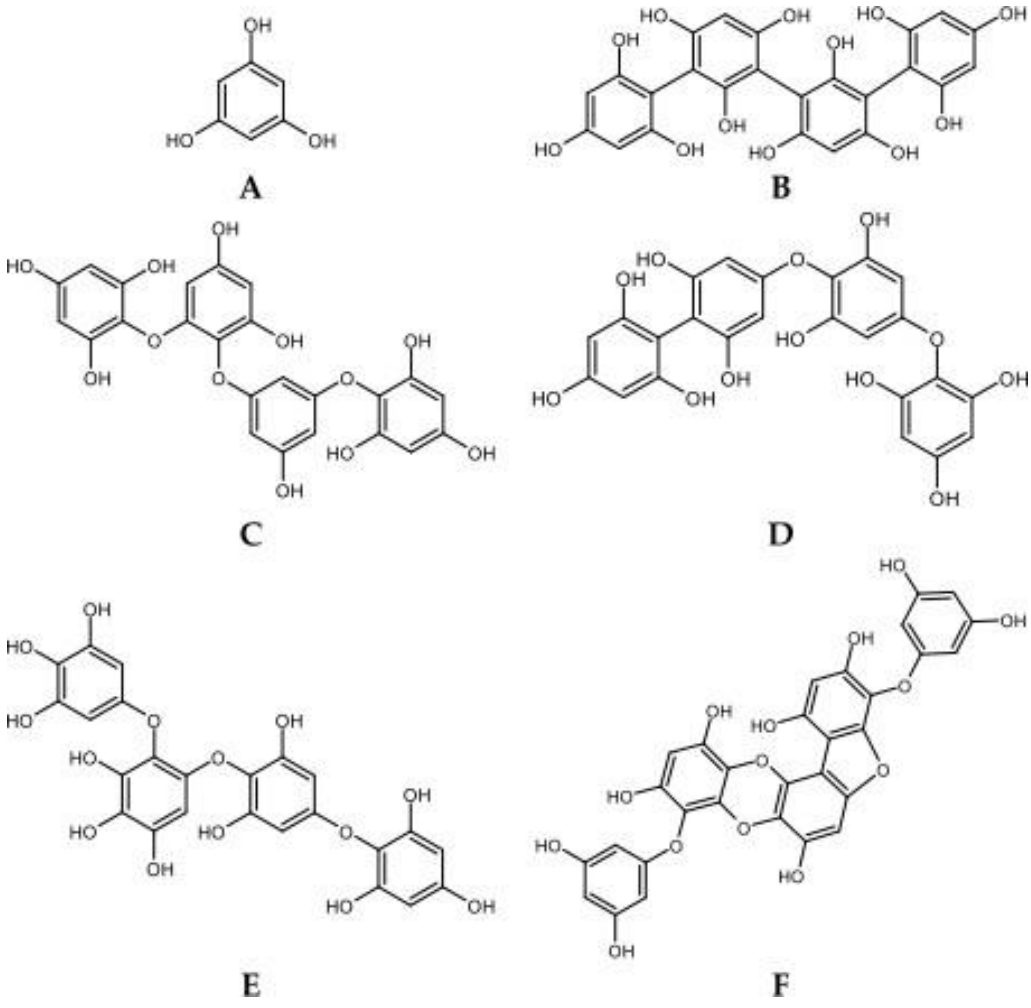

Figure 14-Chemical structures of phlorotannins: (A) Phloroglucinol; (B) Tetrafucol A; (C) Tetraphlorethol B; (D) Fucodiphlorethol A; (E) Tetrafuhalol A; and (F) Phlorofucofuroeckol (Adapted from Cotas et al., 2020).

Bromophenols

Bromophenols are organic compounds, containing one or more bromine atoms covalently bound to the phenol backbone (Figure 15), and are secondary metabolites present in all algal species. These compounds are responsible for ecological functions, such as ecological defense and deterrence (Bhowmick et al., 2020; Cotas et al., 2020). The first bromophenol compound was isolated from the red algae *Neorhodomela larix*, and soon thereafter bromophenols were identified in all taxonomic groups of marine macroalgae (Bhowmick et al., 2020; Cotas et al., 2020). It has been reported antibacterial

effect of bromophenols against several strains of bacteria, such as *Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli* and *Pseudomonas aeruginosa* (Bhowmick et al., 2020). Additionally, substitution of extracted bromophenols with methyl, glycosyl and benzene revealed significant docking score along with increased stability and enhanced pharmacokinetic effects (Bhowmick et al., 2020). Bromophenols show immense potential to be used as either as a candidate for development of new antibacterial compounds (Bhowmick et al., 2020; Cotas et al., 2020).

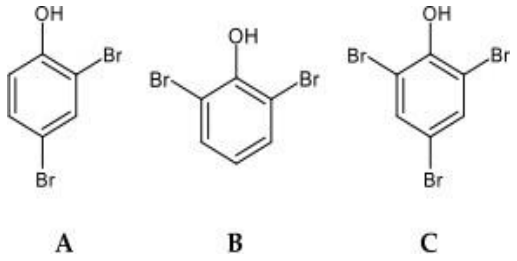

Figure 15-Chemical structures of bromophenols: (A) 2,4-bromophenol; (B) 2,6-bromophenol; (C) 2,4,6-bromophenol (Adapted from Cotas et al., 2020).

Flavonoids

Flavonoids are phenolic compounds structurally characterized by a heterocyclic oxygen bound to two aromatic rings, that vary according to the degree of hydrogenation (Figure 16) (Cotas et al., 2020; Fernando et al., 2016; Liwa et al., 2017; Yonekura-Sakakibara & Saito, 2014). Flavonoids are widely distributed in terrestrial plants, with over 2000 compounds identified. These were divided in major categories such as flavones, flavanol, flavanones, flavonols, anthocyanins and isoflavones (Cotas et al., 2020). Some studies have identified flavonoids such as rutin, quercitin and hesperidin among other, identified in several green, red and brown species (Cotas et al., 2020). However, some contradictions between studies indicates that isolation and characterization of flavonoid species needs further exploration. One example of such incongruity is a study from (Yonekura-Sakakibara & Saito, 2014) that states that macro-and microalgae species do not present flavonoid content due to the lack of two primary enzymes required for the synthesis of flavonoids, but on the other hand, genes encoding enzymes for the shikimate pathway are described in algae (Bowman et al., 2017; Cotas et al., 2020). Additionally, presence of flavones, isoflavones and flavonols were identified in various

microalgae evolutionary lineages (Goiris et al., 2014). However, there is still a need for further insights into characterization, isolation and bioactivity of this wide range of compounds (Cotas et al., 2020).

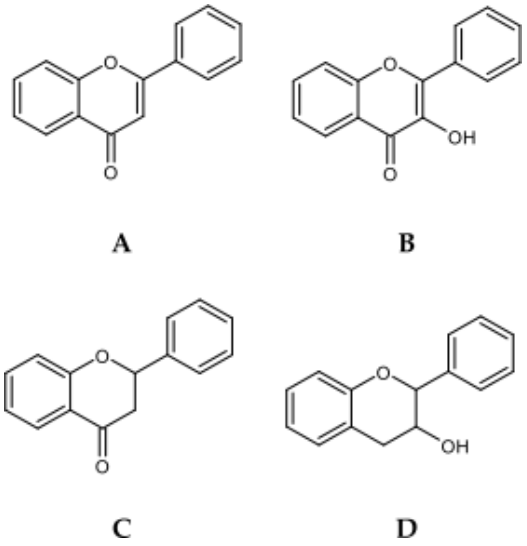

Figure 16- main classes of flavonoids found in algae. (A) Flavones; (B) Flavonols; (C) Flavanones; (D) Flavan-3-ol (Adapted from Cotas et al., 2020).

Phenolic terpenoids

Phenolic terpenoids (Figure 17) have been identified in brown and red macroalgae species. Brown macroalgae phenolic terpenoids are characterized as meroditerpenoids, which in turn are divided in plastoquinones, chromalols and chromenes, almost solely in the *Sargasseum* sp. These meroditerpenoids are structurally characterized by a propenyl chain that is bound to a hydroquinone moiety ring (Cotas et al., 2020; Lu et al., 2019; Pérez et al., 2016). However, even though these compounds have been identified, little is known about their formation and there is no evidence that these compounds follow the same biosynthetic pathway as other terpenes and terpenoids. In the case of meroditerpenoids, these are partially formed by the mevalonic pathway, but further insight into these biosynthesis pathways is required for proper extraction, identification and characterization (Cotas et al., 2020).

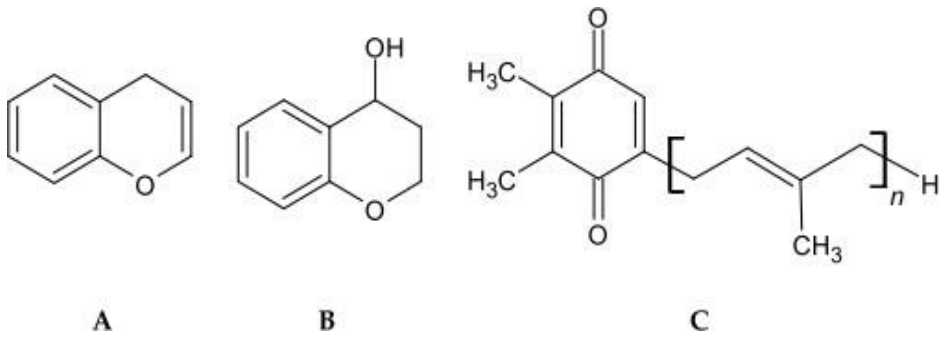

Figure 17-Main classes of phenolic terpenoids found in algae: (A) Chromene; (B) Chromanol; (C) Plastoquinone (Adapted from Cotas et al., 2020).

Mycosporine-like aminoacids (MAAs)

Mycosporine-like aminoacids (MAAs) are a class of compounds present in a wide variety of aquatic organisms, with primary cellular function of protection from UV-induced cellular damage. These compounds were first identified in fungi, in a role in UV-induced sporulation. Later, a wide range of MAAs have been found in several aquatic organisms. These compounds are primarily present in the intracellular space, are water-soluble and have low-molecular weight (<400Da) (Cotas et al., 2020). The chemical structure of these compounds is characterized by a ciclohexenone or cyclehexenine ring with amino acid substituents (Figure 18). Consequently, these substituents result in a broadband absorption of different wavelengths within the MAA molecule. Literature support that MAAs are synthesized via the Shikimate pathway (Carreto & Carignan, 2011; Cotas et al., 2020; Figueroa et al., 2008; Llewellyn & Airs, 2010; Wada et al., 2015).

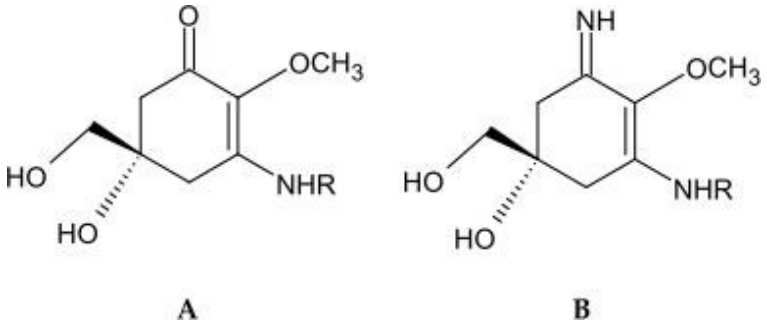

Figure 18-Mycosporine-like amino acids (MAAs) found in algae. (A) Aminocyclohexenone; (B) Aminocyclohexeniminone (Adapted from Cotas et al., 2020).

Non-phenolic secondary metabolites

Terpenes

Terpenes are organic compounds with five repeated carbo isomers units with substitutent groups attached to the backbone. They are synthesized via the melavonic acid pathway in terrestrial plants while in the case of algae is via the melavonic acid-independent pathway. Terpenes are chemically derived from the five-carbon precursor isopentenyl pyrophosphate, and are classified as hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30) and polyterpenes (>C30) (Bhowmick et al., 2020; Pérez et al., 2016).

Terpenes extracted from algae using different solvents and indicated the presence of bromophycolides, commonly known as diterpene-benzoate macrolides. Additionally, other compounds from the bromophycolides family showed antibacterial activity against several antibiotic resistant strains. The mechanisms of action by which these compounds possess antibacterial properties can be related to the structural stringency. These compounds show diverse properties with antimicrobial potential with promising future as new therapeutic strategies (Bhowmick et al., 2020).

Alkaloids

Alkaloids are organic compounds that are structurally diverse, containing one or more nitrogen atoms attached to a ring (Bhowmick et al., 2020; Pérez et al., 2016). Alkaloids have been known since the 18th century, and the first algal alkaloid extracted was hordenine, from *Phyllophora nervosa* (Bhowmick et al., 2020). Alkaloids in marine algae can be classified as phenylethylamine alkaloids, indole and halogenated alkaloids and other alkaloids, such as 2,7-naphtyridine derivatives (Pérez et al., 2016; Bhowmick et al., 2020). Halogenated alkaloids are restricted to algal species and marine organisms (Pérez et al., 2016; Bhowmick et al., 2020).

Halogenated compounds

Halogenated compounds are a group of bioactive macroalgal secondary metabolites. Of these, interesting compounds such as halogenated terpenes, furanones and bromophenols have gained interest due to their potential bioactive properties (Rosa et al., 2020).

As demonstrated above, algae contain a myriad of physiological components resulting from primary or secondary metabolism. Several molecules from algae organisms have been extracted and essayed for antibacterial potential, with many demonstrating positive results, both in planktonic, functioning as biocidal compounds, as well as demonstrating antiproliferative activities against biofilm structures.

Key messages

Algae organisms are a valuable source or natural compounds, with potential to be implemented as solutions to the global bacterial resistance challenge.;

Many of these compounds are metabolites from primary and secondary metabolism. Primary metabolites are polysaccharides, amino acids and peptides, lipids, micro- and macroelements. Secondary metabolites are synthesized in stressful conditions to help mitigate environmental. Characterized as phenolic acids, tannins, terpenes, alkaloids and halogenated compounds, among others;

These compounds have been recognized as potential therapeutics against "conventional" and biofilm resistances, due to anti-proliferative activities;

Discussion: Can algae solutions solve the problem?

Algae compounds with antibacterial action

The search for natural, non-antibiotic, therapeutic alternatives has led to a boom in the search for bioactive antimicrobial compounds, especially from the marine environment. The increasing number of studies regarding extraction and purification of algal organisms has shown a steady increase, in specific sequential extraction of compounds with organic solvents, with numerous reports focusing on bioactive molecules that exert growth inhibition and bactericidal effects (Cepas et al., 2019; Kim, et al., 2015; Sánchez-Lozano et al., 2019; Vasconcelos & Pomin, 2018). However, most preliminary studies show little exploration of the mechanisms of action of specific compounds in extracts, regardless of the overall activity of the whole extract.

Studies reporting extracts bioactivity reveal tremendous variability of effects, due to the innate variability within the extraction process. One issue is the evaporation of volatile components, (e.g. terpenoids) upon heat extraction (Demirel et al., 2009). Terpenoids have shown important microbial inhibiting properties, that can be lost in extraction, diminishing the reported activity of a specific extract (Demirel et al., 2009). Additionally, there is little replication studies of reports that have previously demonstrated efficiency, which contributes to the uncertainty in compound extraction and purification. This compound variability is demonstrated by the overall differences in extract activity across the literature, with reports demonstrating previous organisms or extract techniques positively or negatively efficient (Cepas et al., 2019; Cortés et al., 2014; Dantas et al., 2019; Ferreira et al., 2019; Jafari et al., 2018; Jha et al., 2013; Lauritano et al., 2016; Lu et al., 2019; Mariottini, & Coppo, 2015; Salta et al., 2013; Salvador et al., 2007; Shanmughapriya et al., 2008; Yap et al., 2019).

Different algae have different compositions, a result of several combinations of environmental factors, demonstrated in several macro and microalgae species by the differences according to pH, temperature, time of harvest (Stengel et al., 2011), algae life cycle that are found inter ,and sometimes, intraspecies (Khan et al., 2018; Ramanan et al., 2016).

A sequential solvent extraction from the genus *Scenedesmus subspicatus*, for assessment of antimicrobial compounds against Gram-positive and Gram-negative bacteria, has revealed that the antimicrobial effect of this genus has a lower activity against Gram-negative bacteria, in comparison to Gram-positive. However, in accordance to literature, disparities were observed in extraction results and antibacterial effects of solvents, as the above-mentioned study demonstrated low inhibition values for the algae tests, especially with no activity observable from ethanolic and methanolic extracts (Dantas et al., 2019). Interestingly, a study with algae samples obtained from paddy fields found that methanolic and ethanolic extracts of the genus *Scenedesmus* demonstrated the highest values of antimicrobial activity (Mitra & Mishra, 2019). This light up the question if antibiotic activities of isolated cultured algae from high polluted waters have higher effects than samples from "clean environments". Some studies propose higher antimicrobial activities in algae collected from wastewater and human-contaminated water, than when compared to algae grown in pristine environments (Amaro et al., 2011). Ecologically, this may be due to the defense mechanisms of algae in a highly competitive environment with bacteria.

Sequential solvent extraction of several brown algae assessed for antibacterial activity against Gram-negative and Gram-positive bacteria, revealed a higher activity of the dichloromethane extract than the methanolic and ethanolic extractions (Dantas et al., 2019; Demirel et al., 2009).

In another study, 675 extracts from cyanobacteria and microalgae species were screened for antibacterial and antiproliferative bioactivity against seven bacterial species and two candida strains. The group performed sequential extraction (hexane, ethyl acetate and methanol) and determined the antiproliferative effects of extracts in terms of biofilm inhibition ratio (%). Overall, the results demonstrate a higher number of effective hexane extracts than methanol or ethyl-acetate, but overall, there was no significant difference between methods of extraction, to which the conclusion was the wide range of compounds that were covered by the specific solvent extraction (Cepas et al., 2019). Understanding of the mechanisms behind this bioactivity, isolation and purification of specific compounds is essential.

The antibacterial properties of 2 green macroalgaes, *Caulerpa racemosa* and *Caulerpa lentillifera* were assessed for their antibacterial potential against methicillin-resistant *S. aureus* and neuropathogenic *E. coli* K1. The antibacterial assays (agar diffusion growth)

revealed that the chloroform extraction of *C. racemosa* shows the higher antibacterial activity (growth inhibition) against MRSA in a dose-dependent way, but not a significant effect on *E. coli* K1. Molecular analysis of the components responsible for these activities were analyzed, specifically in the chloroform extract and major compounds with bioactivity were identified as non-polar, such as monounsaturated and polyunsaturated fatty acids, terpenes and alkaloids (Yap et al., 2019).

Some relevant work has been developed in the field of molecules found in extracts. Compound purification, like fatty acids or secondary metabolites have demonstrated antimicrobial and antiproliferative activity (Amaro et al., 2011; Desbois et al., 2008; Desbois & Smith, 2010; Mayer et al., 2013). It has been found that quorum sensing inhibitors increase the susceptibility of microbial biofilms to antibiotics in vivo and in vitro Fatty acids (FA) have emerged as a potential alternative to “conventional” antibiotics. Several FAs mimic diffusion signal factors (DSFs) and control motility, fimbriae and biofilm development, as well as virulence characteristics of diverse microbes (Amaro et al., 2011; Desbois et al., 2008; Desbois & Smith, 2010; Mayer et al., 2013; Smith et al., 2010). Whilst they antibacterial mode of action is still poorly understood, the prime target of FFA action is the cell membrane, where it disrupts the electron transport chain and oxidative phosphorylation (Desbois & Smith, 2010). FFA mode of action may also result from the inhibition of enzyme activity, nutrient uptake impairment, generation of peroxidation and auto-oxidation degradation products or direct lysis of bacterial cells (Desbois & Smith, 2010). FFAs may further affect expression of bacterial virulence factors, that are essential to the establishment of infection, probably by disturbing quorum sensing mechanisms. Saturated and unsaturated FFAs can prevent initial bacterial adhesion and subsequent biofilm formation (Desbois et al., 2008; Desbois & Smith, 2010; Le & Desbois, 2017; Smith et al., 2010). For example, swarming behavior of the urinary tract pathogen *Proteus mirabilis* is inhibited by medium and long chain saturated FFAs (liaw et all 2004). The -OH group of the carboxyl group seems to be important for the antibacterial activity of FFAs, as methylated FFAs often have reduced or no activity (Desbois et al., 2008; Desbois & Smith, 2010; Le & Desbois, 2017; Smith et al., 2010).

A functional extract from the red macroalgae *Gracillaria fishery* has demonstrated antiproliferative properties against *Vibrio harvery* and *Vibrio parahaemolynticus*, two biofilm forming strains. To evaluate the inhibition effectiveness, an ethanolic extract of the macroalgae and furanone, a biofilm inhibitor used in this experiment as a positive

control, were tested for biofilm inhibition in two clinically relevant *Vibrio* species. *Vibrio* is a marine pathogen responsible for large mortalities in aquaculture production systems. The characteristic pathogenesis of this species is the formation of biofilm structures resulting in survival and proliferation within the system, consequently causing high economic losses in the aquaculture industry (Karnjana et al., 2019).

A study with where 14 macroalgae species were tested against ten human pathogen bacteria, revealed seven algal species with bioactive properties against multiresistant strains. Of these 7 potential algal species, *Acrosiphonia ocidentalis* and *Stochoespermum marginatum* displayed bactericidal activities. The results revealed a positive skewedness of bioactivity towards Gram-negative bacteria, in opposition to previous literature statements (Shanmughapriya et al., 2008). Brown algae had the highest percentage of active species, and in specific the extract of *Sargassum marginatum* showed activity towards multiresistant *K. pneumonia*. The results obtained in the study suggested that lipid soluble extracts from marine algae are a source of pharmacologically active substances (Shanmughapriya et al., 2008). In fact, FFAs such as eicosapentanoic acid (EPA), palmitic acid and HTA possess potent antimicrobial effects against Gram-positive and Gram-negative bacteria (Desbois et al., 2008).

Properties of FFAs and potential ability as bacteriostatic and bactericidal agents have been reviewed (Desbois & Smith, 2010; Le & Desbois, 2017; Ren et al., 2017). Although they act through mechanisms different to most antibiotics and have recognized potential as therapeutic agents, problems such as non-specific binding to proteins can hinder possible commercialization of such agents (Desbois & Smith, 2010). Nevertheless, FFAs show promise when applied in combination therapies for efficient antimicrobial eradication, reducing opportunity for pathogens to develop resistance. Due to its non-specific mode of action reduce appearance of resistance and can posteriorly be treated as possible “lead ” compounds though chemical engineering, potentially improving efficacy and delivery to targets (Desbois & Smith, 2010).

Functional extracts from macroalgaes have also demonstrated potential as efflux pump inhibitors (EPIs) (Lu et al., 2019). Functional extracts of two macroalgaes *Gracilaria* sp and *Porphyria dentata* were tested for activity as EPIs, against a drug-resistant strain of *E.coli.* The results demonstrated the potentializing drug effects of the functional extracts in the resistant strain, specifically the synergistic effect of the extracts with clarithromycin were observed from the onset of kill-time assays, with no observable regrowth (Lu et al.,

2019). Although a specific mechanism of inhibition is not described, bioactivity can be correlated to the presence of compounds such as terpenes, terpenoids, phenolic acids, among others with possibly several compounds that work on bypassing resistance mechanisms, such as efflux pumps (Lu et al., 2019).

Using a N-acyl-homoserine-lactone (AHL) construct with the reporter gene GFP, (Hentzer et al., 2002) tested the effect of a synthetic halogenated furanone derivative, a secondary metabolite from the macroalgae *Delisea pulchra,* and reported the effects of this compound on quorum sensing mechanics of *P. aeruginosa*. This construct responds to AHL activation and expression of QS through fusion of a *las*B promoter to a gene coding for GFP. The furanone was applied to *P. aeruginosa* biofilms established in biofilm flow chambers. The GFP based analysis indicates a loss of expression of important virulence factors, indicating a general effect on target genes of the *las* quorum sensing circuit. Additionally, it revealed that the compound penetrates microcolonies and blocks cell signaling and quorum sensing in most biofilm cells. However, the compound did not affect initial attachment to the abiotic substratum, but it affected biofilm architecture and increases the detachment process, leading to loss of bacterial biomass (Hentzer et al., 2002). The compounds produced by this macroalgae had previously been demonstrated to interfere specifically with AHL-regulated bacterial processes without affecting bacterial growth (Hentzer et al., 2002; López & Soto, 2019; Tran et al., 2018). The hypothesis is that furanone compounds antagonize AHLs by competition to the binding site, as this halogenated furanones at concentrations produced by algae can displace molecules from the *lux*R receptor protein (Hentzer et al., 2002). Additionally, the synthetic furanone compound reduced production of two virulence factors, elastase and chitinase. Nevertheless, furanone compounds hold promise as AHL antagonists and for the development of new non-antibiotic, antipathogenic agents that interfere with bacterial cell-to-cell communication, rendering these strains less virulent and more sensitive to treatment (Hentzer et al., 2002).

Fucoxanthin, a carotenoid present in several algae species, was studied as a possible antimicrobial agent. Of the bacterial strains investigated, it was observed a stronger effect on Gram positive bacteria than Gram negative bacteria, with MIC's for Gram-positive *S. aureus* at 125 ug/ml and Gram-negative *P. aeruginosa* and *E. coli* with MICs of 250-500 ug/ml and 125 ug/ml respectively. This selective effectiveness for Gram-positive rather than Gram-negative bacteria is observed in other fucoxanthin literature. However, in the case of infection, fucoxanthin has demonstrated anti-inflammatory

effects, proposed through the inhibition of LPS production, a membrane endotoxin responsible for fever, septic shock and microbial invasion (Bahar et al., 2012; Karpiński & Adamczak, 2019). The possible mechanisms of antibacterial activity of antioxidants is proposed to be by 3 mechanisms: outer membrane permeability, cytoplasm leakage and inhibiting formation of nucleic acids (Karpiński & Adamczak, 2019).

Algae compounds for biosynthesis of active nanoparticles

Nanoparticles, due to their nano-scale size, possess several attractive physiochemical properties, such as low toxicity, high stability and high surface area to volume ratio. Several studies have used biologically synthesized nanoparticles from marine compounds. These “green-made” nanoparticles, such as biopolymers, provide benefits in terms of NP production costs, aggregation, isolation and are environmentally friendly (Bao & Lan, 2019; Khan et al., 2019; Khanna et al., 2019; Mola et al., 2016).

Gold nanoparticles (AuNP) were biologically synthesized using fucoidan (F-AuNP), an active compound from brown macroalgae, for evaluation of inhibitory effect on *P. aeruginosa* bacterial growth, biofilm formation, virulence factor production and bacterial motility. The results of the study reveal that a MIC value of 512ug/mL demonstrated to be bactericidal against *P. aeruginosa* and that sub-MIC levels of F-AuNP effectively prevented biofilm formation and establishment, virulence factors production, such as pyocyanin, rhamnolipid and pyoverdine and motile elements, as well as eradicating pre-existing biofilms (Khan et al., 2019).

Exopolysaccharides from green algae *Botrycoccus braunii* (EPBb) and *Chlorella pyrenoidosa* (EPCp) were tested as reducing and stabilizing agents of silver nanoparticles (AgNP). These particles were posteriorly tested for their antibacterial activities against Gram-positive *S. aureus* and Methicilin resistant *S. aureus*, and against Gram-negative bacteria *E. coli*, as well as for cytotoxicity (fibroblast) assessment. The results demonstrated that, when treated with the particles, the tested strains required an extended time period to adapt, before entering exponential phase, and when reaching the log phase, demonstrated slower growing and lower cell density, when compared to

control (Navarro Gallón et al., 2019). This represents an important clinical advantage, as an extension of the lag phase can slow the adaptation of bacteria to new conditions, giving time for the immune system to fight the infection. The study reports observed action potential at the level of the cell membrane, demonstrated by membrane disruption, lower cell envelope integrity and morphological changes, to which they refer to binding of sulphur-containing and phosphorus-containing intracellular molecules, due to silver ions activity. Additionally, cytotoxicity tests demonstrated only a slight reduction in dermal fibroblast proliferation, which is a promising sign for use of nanoparticles in biological applications (Navarro Gallón et al., 2019).

Disruption of biofilm structures in a *P. aeruginosa* mucoid phenotype was demonstrated by an alginate oligomer (OligoG). Alginate is a polysaccharide extracted from brown macroalgaes and found in the EPS matrix of pseudomonads biofilm (Christophersen et al., 2020; Danese, Pratt, & Kolter, 2000; Khan et al., 2012; Powell et al., 2018). When administrated, OligoG demonstrated inhibition of biofilm formation with a significant reduction in biomass and height, additionally diffusing into established biofilms (24h), significantly reducing EPS and eDNA, with a consequently increase in nanoparticle diffusion and antibiotic efficiency (Powell et al., 2018). The mechanism by which OligoG acts is through disruption of DNA-Ca2+-DNA bridges, by interacting with Ca2+ (Powell et al., 2018).

On “conventional” and current methods for extraction

Of critical importance is a proper standardized method for the correct extraction of the purified bioactive component. There have been methods for the selection and extraction of overall compounds or extracts from algae historically applied that are currently still used for the assessment of bioactivity, such as hydric extraction and solvent-phase extraction (Bhowmick et al., 2020; Khan et al., 2018; Rocha-Santos & Duarte, 2014). However, these methods are time-consuming and have a wide range of variability, due to the inherent biochemical characteristics of the algae being studied. Compound purification obtained from water or solvent extraction is as well time consuming and labor intensive, furthermore with the low quantities of a specific content extracted (Farasat et al., 2014; Michalak & Chojnacka, 2014; Pane, Kim, et al., 2015). Extraction and purification of relatively high compound concentrations requires

high volume of algae for extraction, with additional efforts not achievable without a specialized lab (Ciko et al., 2018; Cotas et al., 2020).

Regardless of the confirmed potential as alternative therapeutic solutions, the transference of these results to the industrial scenario is challenging. Traditional mechanisms of extraction are not viable as a commercial mechanism, as the volume of solvent needed for a commercial yield is not sustainable, as well as toxic due to the nature of these components (Sosa-Hernández et al., 2018; Thiyagarasaiyar et al., 2020).

However, modern extraction and isolation techniques for compounds or natural sources are continuously being developed due to the potential of algae compound wealth. These mechanisms include Enzyme-assisted extraction (EAE), Ultrasound-assisted extraction (UAE), supercritical-fluid extraction (SFE), microwave-assisted extraction (MAE) and Solid-phase Extraction (SPE) are novel techniques have been applied to a more efficient extraction of amino-acids, pigments, fatty acids and other metabolites from cyanobacteria, macro-and microalgae (Azmi et al., 2020; Costa et al., 2020; Dobrinčić et al., 2020; Baky et al., 2020; Gallego et al., 2018; Kadam et al., 2013; Khoo et al., 2019; Koyande et al., 2019; Kumar et al., 2020; Kwang et al., 2010; Messyasz et al., 2018; Michalak & Chojnacka, 2014; Mitra & Mishra, 2019; Pérez et al., 2016; Santos et al., 2019; Tran et al., 2018).

Enzyme-assisted extraction has developed interest due to the hydrolytic action on disruption of cell walls structure and breakage of interior storage compounds, allowing release of intracellular compounds such as polysaccharides, peptides and amino acids into the milieu and it has revealed potential to improve extraction yield while maintaining bioactive properties of extracts (Rodrigues et al., 2015). EAE is an economically and sustainable solvent free method with low costs, high extraction rates and high yields. Yield comparison was tested between EAE and UAE, with higher extraction yields for EAE in comparison to UAE, for all the algae tested (Rodrigues et al., 2015). Ultrasound assisted extraction (UAE) is a extraction method based on sound wave migration, generating cavitation within the organisms that when collapsed lead to disruption of cell walls and release of intracellular compounds of interest (Ciko et al., 2018; Kwang et al., 2010; Patras et al., 2018; Rodrigues et al., 2015).

Supercritical fluid extraction is considered ideal for thermolabile compounds. When used with CO2 it has low-viscosity, high diffusion and is also non-toxic (Gallego et al., 2019;

Patras et al., 2018). Additionally, it is gaseous at normal temperature and atmosphere, reducing the solvent evaporation step after extraction. Its non-oxidizing properties prevent extracts from degradation. A good example is the utilization of super critical CO2 for the extraction of several components in *Scenedesmus obliquus* (Ciko et al., 2018; Gallego et al., 2019; Kadam et al., 2013; Kwang et al., 2010; Otero et al., 2018; Patras et al., 2018; Silva et al., 2020).

Microwave assisted extraction (MAE) is an extraction method that through microwave irradiation, accelerates rate of extraction substantially (Cao et al., 2020; Patras et al., 2018). An experimental study comparing MAE and traditional extraction of from two algal species *Chlorella sorokiniana* and *Nannochloropsis salina* demonstrated an increase at the extraction rate by 15 times (Ciko et al., 2018; Patras et al., 2018; Sosa-Hernández et al., 2018; Tran et al., 2018).Overall, these new mechanisms demonstrate the potential to optimize extraction mechanisms of interesting compounds as well as potentiating the transference of this knowledge to the industrial setting.

Key messages

There is demonstrated bioactivity of algal compounds, with antibacterial actions against a wide diversity of species;

Bioactive compounds from algae are structurally variable, due to the variability within algae species and classes;

Solvent extraction reports reveal incongruities within reported bioactivity, with some solvent extracts reporting higher efficiency than the same solvent extraction, sometimes for the same algae class;

Biosynthesis of nanoparticles with algae extracts have shown high efficiency when applied to bacterial strains, planktonic and biofilm-forming;

"Conventional" extraction methods, such as hydric extraction and sequential solvent extraction can reveal potential bioactivities of algae compounds, but these methods are not transferable to the industrial setting, as they are time consuming, not standardized due to the variability of compounds extracted and the yield obtained is insufficient;

"Next-generation" extraction methods, such as supercritical CO_2 extraction enzyme assisted extraction are methods that reduce time-consumption from "conventional" extraction methods, at the same time increasing yield of compounds extracted.

These "next-generation" extraction methods show potential for implementation of these compounds in the industrial setting, besides being "cleaner" than traditional sequential extraction;

Conclusion

Antibacterial resistance is a major challenge to the global community, with increasing severity of infections funneling further the therapeutic options available to fight these infections. Due to unproper management and regulation, disinformation regarding therapeutic strategies and consumption, have led to several contamination of natural environments, such as soil and water basins. Excessive utilization in food production jeopardizes our food supplies, further aggravating the problem of persisting resistances. These are especially severe in the hospital environments, where nosocomial infections become more prevalent, risking patients with immunocompromised systems. To worsen the scenario, there are no signs of big pharmaceutical companies returning to mass production of antibiotic solutions. Even through the pharmaceutical preclinical pipeline shows increased compounds that are non-"conventional" and with potential to bypass "conventional" resistances, in the clinical pipeline there is still prevalence for modifications of previous antibiotics classes, that although effective in short term, these compounds are not efficient in controlling developing and spread of resistances.

Beyond "conventional" resistances, attention towards the problem of biofilm development related to pathogenesis has gained considerable interest, due to the problematic nature of this structure. Although awareness and research of biofilm ecology has approximately 100 years, only in the last 50 years its relevance and complexity in the eyes of microbiologists became prominent. Even though biofilm development is currently a hot topic with higher increasing relevance, there is much to learn and understand regarding physiological mechanisms of these bacterial communities. The prevalence of biofilm-related infection in the healthcare environment is a serious concern.

Algae metabolites, either primary or secondary, have demonstrated serious potential as non-"conventional" therapeutic options against biofilm forming bacteria. The vast compound heterogeneity within these organisms offers an unprecedented pool of possible therapeutic options, that can be key in the antibacterial resistance challenge. However, these differences are a double-edged sword, as this intrinsic variability challenges standardization and extraction. "Conventional" extraction yields variable results, and some protocols are biased towards the actual antibacterial power within a specific organism. Nevertheless, the current efforts for development of cleaner and faster technology for compound extraction can offer some stability to the field of bioactive

discovery and extraction, possibly allowing the transference of these antibacterial potential compounds to the industrial setting.

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