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Review

Marine Invertebrates: Underexplored Sources of Bacteria Producing Biologically Active Molecules

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Abstract: The marine bioprospecting could be considered as a new phenomenon, and already potentially more promising than terrestrial one in terms of possible discovery of new compounds. The secondary metabolites produced by associated-bacteria are actually studied for their remarkable role in several fields, including agricultural, industrial, medical, and bioremediation strategies against different contaminants. The use of such renewable sources could be helpful in the streamlining of the patenting process for natural compounds of good quality, produced with lower energy costs and less impact on the environment. Anyway, more improvements in the research and application of bioactive compounds as alternative to the synthetic counterparts have to be carried out for the costs reduction and the large-scale production upgrading. The use of marine invertebrates could help to overcome these difficulties, as hotspots of microbial diversity and favorable matrix for the development of conditions stimulating the production of substances with special activities. This review will deal with the current accepted definitions and recent advances concerning: (*i*) the marine symbiotic relationships in which microorganisms and invertebrates are involved; (*ii*) the principal taxa of marine invertebrates that establish interactions with microorganisms, the biodiversity of these latter, and their role in the symbiosis; (*iii*) we address the state of current literature and knowledge about the bacterial associated communities specialized in biosurfactants (BSs) and extracellular polymeric substances (EPSs) production; and, (*iv*) their potential biotechnological applications reported still now.

Keywords: marine organisms; biosurfactants; extracellular polymeric substances; filter feeders; bioactive molecules; bioremediation

1. Introduction

Associations between higher organisms and microorganisms often occur as mutualistic or symbiotic relationships, by providing benefits for both in terms of protection and nutritional requirements. Microorganisms could live while attached to the surface of other organisms or within their tissues, thanks to the adhesion to biofilm surfaces of the organisms, or by creating biofilm matrices. Associated bacteria support the defensive strategies of the host organism by producing secondary metabolites, in response to the environmental conditions of surrounding water. For this reason, associated bacterial communities have been firstly and deeply investigated for the discovery of new drugs, valid for the application in pharmaceutical and medical fields as antibacterial, antiviral, antifungal, and antiprotozoal functions [1–4]. In addition to biomedicals, among secondary metabolites of microbial origin, extracellular polymeric substances—including biosurfactants and exopolysaccharides—represent a class of eco-friendly compounds that were investigated since several decades for their advantageous applications in numerous and important fields. They are

interesting for the remarkable role that they could assume in agriculture technology, in food, cosmetic, and pharmaceutical industries, in bioremediation strategies against different contaminants (e.g., hydrocarbons, polycyclic aromatic compounds, heavy metals), in medical applications as fungicidal, bactericidal, insecticidal, and anti-viral agents. Despite the recognized and undisputed potentiality of these compounds in the future economy and technology, the large-scale production still meets many limits, due to costs of recovery and the effective raw extracts yield. The exploration of new sources of microbial producers' isolation, with the subsequent possible discovery of new producers and new molecules, actually represents the primary tool for a more effective application and employment. Microorganisms, as a little high-specialized factory, and more strongly microbial communities that are associated with marine invertebrates, are currently considered the major potential pool to draw new metabolites and bioactive compounds. The production of compounds with biological functions could occur in response to environmental stressors, as a result of physiological processes, which require both complex cell-to-cell and bacteria-host interactions. Despite this, biological marine matrices have been scarcely considered for the isolation of microorganisms that are specialized in the production of extracellular polymeric molecules. The present contribute aims at reviewing the role of marine invertebrates as habitat for the establishment of bioactive molecules-producing microbial communities, the biodiversity inside these latter and their biotechnological applications.

2. Relationships between Microorganisms and Marine Invertebrates

Among all of the environmental compartments that are present on Earth, the marine environment represents a complex set of extremely fascinating habitats and ecosystems, but at the same time mostly unexplored. The high degree of variability of the environmental parameters that can be found site by site, the multitude of possible combinations of these with the geomorphological conditions manifest themselves in the several marine ecosystems, from polar to hydrothermal environments, from deep-sea basins to underwater caves, from the seagrass meadows to the coral reefs. All of their complexity is reflected in a great diversity of animal and plant species.

Among the different ecological strategies of survival and adaptation to the environment, the establishment of relationships between different populations often occurs, and it represents the most attractive strategy used by living organisms to overcome hard and stressful conditions. The interactions could be firstly classified in positive or negative, in dependence of the possible benefits or damages that were suffered by one of the organism involved in the relationship. The positive interactions favor survival and adaptation to external conditions, while negative interactions represent a self-regulation system, which limit the population density. Moreover, a positive relationship could be mutualistic or not, depending on whether only one or both organisms benefit from it. Microorganisms are very often protagonists of these relationships, and they enter into association with representatives belonging to most of the phyla actually detected. The term generally used to design associations between different species is *symbiosis*, and means a close and long-term interaction between two different organisms, which are called *symbionts* [5]. The interactions could be intra- or inter-specific and they are commonly classified in mutualistic, commensalistic, or parasitic relationships. Anyway, no strict limits occur between these categorizes, and really often a relation present characteristic across these defined boundaries, in dependence of the environmental conditions, the evolutionary processes and the health state of organisms involved in the relationships, as suggested by Webster et al. [6]. As an example, Horn et al. [7] described the interaction between Chlamydiae and microbes that starts as an infectious relationship, but then evolves in a positive interaction, which is necessary for the host survival. The aquatic ecosystems harbor a wide array of marine symbioses, which are considered as an important selective force behind evolution. From the evolutionary point of view, the acquisition of symbionts from hosts could happen mainly through two mechanisms: an horizontally acquisition from the surrounding environment, a vertically transmission from parental inheritance through reproductive cells and larvae, as observed in some marine organisms, such as sponges and ascidians [7–10], or finally, a mixture of these processes [11]. A horizontal transmission requires equilibrium between the selection

of specific symbionts on the base of their functions and the maintenance of an efficient immune system to front adverse microorganisms. This leads to the establishment of bacterial communities with a high biodiversity rate, contrastingly with the vertical transmission, which favors microbial communities with a reduced taxonomic and function complexity. The transmission mechanism is really a complex and delicate phenomenon at the base of which lies the stability of the interactions, and the key of evolution comprehension.

2.1. Mutualistic Symbiosis

Symbiosis is a positive interaction in which two organisms get a profit, and develop a strict association that is characterized by greater stability and metabolic dependence, so that a lonely survival and accretion are not achievable for each one of relationship member. On the base of the association structure, it is possible to distinguish *ectosymbiosis*, if the symbiont is attached on the surface of the host but not necessarily established in its internal cavities, *endosymbiosis*, if the host internal cavities represent a favorable matrix for the settlement and growth of symbiont. A fascinating example of endosymbiosis is that established in hydrothermal vent environments between the siboglinid tube worms and symbiotic bacteria: the worm is not provided of the intestinal tract, so it is wholly reliant on symbionts for nutrition [12]. The relations of endosymbiosis are really intimate, so that an endosymbiont is strictly dependent from the host's lifestyle.

The advantages originating from a mutualistic symbiotic relationship can be of different types, and they include protection, feeding, and acquisition of recognition systems. On the one hand, microorganisms could find a microhabitat refuge from the external stressful conditions into the host, on the other hand, the macro-organism benefits of a protective function guaranteed by bacterial symbionts. The protective role is supported by the bacterial production of secondary metabolites, with antimicrobial and antifouling properties, which defend the host from the attack of pathogenic or invasive agents. Several examples of microbial symbiosis could be included here. Bull and Stach [13] reported a sponge-associated marine actinobacterial strain, *Salinispora tropica*, as producer of salinosporamide A, a compound that is approved for the treatment of multiple myeloma. A strong antitumor compound is produced by actinobacterial species *Micromonospora* isolated from the marine sponge *Clathrina coriacea* [14], while the sponge *Halicondria panicea* has been the source for associated Actinobacteria strains involved in the production of potent cytotoxic alkaloids, polyketides, and macrolide [15]. The relationships between chemoautotrophic bacteria and their deep-sea hydrothermal vent hosts are also based on symbiosis: the microbial symbionts convert inorganic compounds (i.e., hydrogen sulfide and carbon dioxide) into organic forms, which could be used by their hosts [16].

Bioluminescence, very commonly widespread in marine environments, is the most suggestive form of symbiosis that allow for one of the symbiont to acquire a peculiar behavior expedient, the light emission, useful in the reproductive processes, in the predation and in the defensive mechanisms. An example of this type of interaction is the *Vibrio* sp. symbiont, which lives in the light organ of bobtail squid and provides luminescence as functional strategy for the host involved in predation and camouflage strategy [17].

2.2. Commensalistic Symbiosis

Commensalism is a (symbiotic interaction) unidirectional relationship in which one species benefits from the association while the other is unaffected by it, nor positively or negatively. It generally occurs when one member of interaction could use waste products of the other as substrate, or when one of the organisms makes the environmental conditions better for the survival of the other. A lot of bacterial commensals live on animals and plants surface, which are favored by the presence of biological secretions or by the body temperature. The commensal microbiota is the most currently studied form of commensalism established between bacteria and higher organisms. In place of refuge and food, complex bacterial communities offer to the hosts a contribution for nutrient absorption, protection against pathogens through the production of antimicrobial compounds or the establishment

of a direct competition relationship with them. The bacterial microbiota of some marine invertebrates has been investigated, and also phylogenetically characterized through molecular methods [18–21].

The commensalistic symbiosis occurs also in the form of interaction bacterium-bacterium. Thus, in this kind of relationship, one bacterium metabolizes a substrate useless to the second one and the residual metabolites are used as energy source from this latter, which represents the *commensal*, the only beneficiated of the relationship. An example of commensally interaction is that between *Saccharomyces carlsbergensis* and *Acetobacter oxydans*, which oxidizes mannitol by producing fructose for its host [22].

Commensalism could acquire different forms or ecological functions on the base of the involved organisms and of the habitat. As an example, an interesting form arises in microenvironments between facultative and obligate anaerobic microorganisms, in which the former consume all of the oxygen, by favoring the survival of the others. Such situations can easily happen in the marine environment, for example, in not confined anoxic basins, which could receive oxygen from neighboring habitats. Is a case of commensalism also the bacterial removal of toxic substances or contaminants from the environment allowing for the survival of microbial species that are sensitive to them, or the growth factor production (vitamines and essential fatty acids) favoring the development of auxotrophic populations.

A more complex arrangement is the *multistage* commensalism, in which the benefits of interaction are extended to more partners in a cascade mechanism, as happens in nutrient cycles, when each bacterium (oxidizing, reducer, fixator) needs of the substrate produced by the bacteria of the previous stage, in a precise succession. The establishment of species in response to the positive effects generated from previous living groups characterizes many colonization processes. The colonization of the body surfaces of other organisms by bacterial communities is also considered as a particular form of commensalism.

Despite the undeniable ecological value of commensalism as relationship that is able to regulate the densities of very complex microbial communities, the difficulty in defining this type of relation lies in the arduousness of assess that a species is not affected from the interaction [23].

2.3. Parasitic Symbiosis

In a parasitic interaction, one of the member gains benefits while the other is damaged from the relationship. This interaction is different from antagonism, which consists in the production of chemical compounds with toxic effects towards other populations. Parasitic relationships have been widely described for several animal phyla (i.e., protozoans, nematodes, annelids) and plants, but also bacterial groups have been described [24]. Microbial parasitism has been studied for a long time, with an interest that is due mostly to the possible adverse impacts of pathogens on human health, agricultural, and animal stocks [6]. It is possible to define *endoparasitim* and *ectoparasitim* strategies, and in general parasitism results in an extremely successful lifestyle from an ecological point of view. Indeed, even if the relationship is based on damages inflicted by the pathogen to the host, a parasite has not interest to kill its host, as it would mean to destroy itself. For this reason, generally parasites possess a high degree of specialization, and evolve in response to the defense strategies adopted by their hosts.

Anyway, in this review, parasitism will be less improved, and more attention will be focused on positive interactions.

2.4. Synergism and Sintrofia

The synergism, which is also called protocooperation, is a particular form of interaction in which both members gain benefits, but it is not obligate or specific. This means that they could live separately or another organism could substitute one of them. Overall, these types of relationships occurred between microbial populations, which lived such closely to form consortia aggregates, hardly separable. The most common type of synergism is correlated to the reciprocal nutrition, as in the case of *Lactobacillus arabinosus* (folic acid producer) and *Streptococcus faecalis* (phenylalanine producer), which grow only together by furnishing each other essential elements. Typical is also the

interaction that occurred in anaerobic environments between green sulfur and sulfate-reducing bacteria, such as *Chlorobium* spp. and *Desulfovibrio* spp. Green sulfur bacteria produce carbohydrates through chemoautotrophic reactions, which are favored by light and H_2S released by the sulfate-reducing bacteria, who survive thanks to the organic compounds that are provided by the photosynthetic microorganisms. Despite this review being aimed at treating the interactions between microorganisms and marine invertebrates, here we retained opportune to cite also synergism, because synergistic forms of relationships could be established within the microbial community that is associated to higher organisms, thus having an important ecological role.

3. Marine Invertebrates Involved in Microbial Association Relationships

Many sessile invertebrates build their defensive strategies against predators or competitors on the production of natural compounds, often correlated to associated microbial communities. All the domains of life members are involved in the establishment of close or weak association with several microbial species, and most phyla of marine invertebrates have been studied for microbial symbiosis. Grossart et al. [25] used a strong effective definition of symbiosis in aquatic ecosystems, by defining them as *the rule than the exception*. In the following sections, we will treat three phyla of marine invertebrates that have been studied for association with bacterial communities, on which we pointed our attention because they represent the taxa mostly reported as source of bacterial isolates that are able to produce bioactive metabolites.

3.1. Porifera

Among marine invertebrates, sponges (phylum Porifera) are the main representatives of marine benthos, and certainly the most studied taxa for their ability to host very complex microbial communities. The phylum is divided in three major classes: the Hexactinellida (glass sponges), Calcarea (calcareous sponges), and Demospongiae (demosponges), which include the highest number of species [26,27]. Sponges are sessile, filter-feeding organisms, with a simple body structure but an efficient water filtering system, which is necessary to gain nutrients from the surrounding environment [28]. The filtration processes are granted by an external pores system (*ostia*), and continued in the internal tissues of sponges through a channel structure with different levels of complexity. The sponge body includes several cell layers, from the surface: *pinacoderm*, formed by pinacocytes, epithelial cells; specialized flagellated cells, called *choanocytes*, responsible of capturing food particles; and, *mesohyl*, a connective tissue layer in which food particles are digested via phagocytosis by *archaeocytes*. The mesohyl is generally the part of the sponge body in which dense microbial communities are established, despite bacteria has been found to be associated to both external sponge tissues and internal mesohyl [29].

Sponges are among the first organisms studied for investigations on bacterial symbiosis, and the association between sponges and bacteria has been described in all symbiosis forms, so that relationships of mutualism, nutrition [28], and also parasitism [30,31] have been reported. In relation to the abundance of bacterial symbionts, some authors proposed the classification into High Microbial Abundance (HMA) sponges, which host different bacterial communities, and Low Microbial Abundance (LMA) sponges, whose associated bacterial communities are less abundant and predominantly composed of Proteobacteria and Bacteroidetes [32]. As highlighted and reviewed by Taylor et al. [29], with particular relevance for marine sponges than for freshwater ones, the sponge-associated bacterial communities cover a wide spectrum of diversity, which justify the different types of interactions that are detectable between bacteria and host sponges. Taylor and coauthors carried out a deep phylogenetic analysis of all available sponge-derived 16S rRNA gene sequences, in order to obtain an overview of sponge-associated bacterial diversity and to investigate the existence of sponge-specific sequence clusters. The work resulted in 14 different bacterial phyla and the major archaeal lineages (Crenarchaeota and Euryarchaeota). The bacterial phyla include Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus,

Firmicutes, Gemmatimonadetes, Nitrospira, Planctomycetes, Poribacteria (a sponge-specific candidate phylum, Poribacteria) [33], Proteobacteria (Alpha-, Beta-, Delta-, Gammaproteobacteria), Spirochaetes, Verrucomicrobia, Chlorobi, and for the first time, the presence of sequences affiliated with the phylum Lentisphaerae and the candidate phylum TM6 have been reported. Moreover, the analysis supported the presence of monophyletic, sponge-specific 16S rRNA sequence clusters. More recently, new scientific contributes allowed for describing the presence of about 40 phyla or candidate phyla, with the predominance of Proteobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, and Cyanobacteria [34–37], and confirm the existence of sponge-specific clusters, among which the candidate phylum Poribacteria [33] and Candidatus *Synechococcus spongiarum* [38].

A relevant feature of the interaction that was established by sponges is that these benthic invertebrates are able to host organisms belonging to all three existing domains. Rodríguez-Marconi et al. [39] performed a deep analysis to investigate the three-domain diversity and community composition from eight different Antarctic sponges and concluded that the sponges differ in diversity and similarity patterns between bacterial/archaeal and eukaryote microbial symbionts. Moreover, they assumed that associated bacterial community structures between Antarctic and tropical-temperate environments differed, with the presence of a highly complex microbial eukaryotic community. The great amount of studies about sponges-bacteria associations consents to assume that the sponge bacterial communities are distinct from the surrounding water, host-specific, and with different functional roles [10,40–42]. An aspect that still appears unsolved is the comprehension of the origin of associations between sponges and microorganisms, because several authors have asked themselves if the association has deep roots in the history of evolution or if it is a more recent phenomenon. The occurrence of the same bacterial species in different sponge species and locations led to suppose that the symbiotic relationships between microorganisms and sponges occurred during ancestral periods, prior to the evolution of the current sponge classes. According to Taylor et al. [29], three evolutionary scenarios could be currently assessed to analyze the ancientness of sponges-microbes associations. The first one assumes ancient symbioses maintained by vertical transmission, a second scenario supports the parental and environmental symbiont transmission, finally the third scenario presumes an environmental acquisition. The authors suggest that available data reflects mainly the scenario 2, according to which specific microorganisms are transmitted vertically between generations by the horizontal exchange phenomenon, but also through the scenario 3 mechanism. Nevertheless, the issue—despite very fascinating—remains troubling, and it needs further and deeper analysis to be completely clarified.

3.2. Annellids

Although the taxonomic classification of Annelids is still much discussed, the distinction in two classes—Polychaetes and Clitellates—is currently recognized [43]. Polychaetes represent the most ancient phylogenetic class of Anellida, and include mostly species of marine worms, which are sometimes very different morphologically from terrestrial annelid species. With separate sexes, fertilization occurs into the water and individuals become adults after a free-swimming larval stage. Some species possess a body formed by a tubular part, produced by the same animals and in which they live, and an apical part consisting of a plume of tentacles and sensory ends defined *palpi*. Particularly, Sabellidae family members are well known for their complex tentacular crown that develops out the tube. The tubes are constructed with sand or mud cemented with digestive secretions, or with calcium carbonate and organic molecules, such as proteins and polysaccharides. Sedentary Polychaetes are often filter-feeders, as they use tentacles to filter large volume of water and feed on food particles held from the water.

Recent reports about the filtration process by Polychaetes were aimed at deeply describing the bacterial capturing potential, and proposed these benthic organisms as bioindicators or as monitoring tool for some microbiological targets. This is the case of Stabili et al. [44] who reported good accumulation capacities for *Sabella spallanzanii* specimens. Similarly, Licciano et al. [45,46] showed

efficiency of 98% (after 20 min) and 70% (after 30 min) for *Branchiomma luctuosum* and *Sabella spallanzanii*, respectively, in retention of *Vibrio alginolyticus* from seawater in experimental conditions.

In addition to these reports regarding the improvement of filtration activity of Polychaetes, literature about their associated bacterial fraction remains very scant if compared to that available for other marine organisms [47,48]. To the best of our knowledge, one of the first attempts in this sense was the assessment of the microbial diversity within the associated epibiotic bacterial community in *Alvinella pompejana* from a hydrothermal vent [49]. With a molecular approach, they reported dominant phylotypes as affiliated to the Epsilonproteobacteria. The predominance of this class in *Alvinella pompejana* associated bacterial community was then confirmed by several works [50,51]. Unfortunately, the determination of potential phylotypes within Polychaetes symbiont community results quite hard, and even if a biosulfite reductase genes has been detected in epysimbiotic community [52], it was not correlated to a specific phylotype. A correct interpretation of the role of this associated community is even more difficult, and it is oriented between the involvement in the nutrition of the host and the detoxification from pollutants, but it is probably correlated to the species and to the environmental conditions of the habitats [49]. In 2012, a change in the bacterial community that was associated to the Polychaete *Ophelina* and related to the increase of *Alteromonadales* bacteria was correlated to heavy metal pollution in sediments by Neave et al. [47], similarly to what was described by Jeanthon and Prieur [53] for bacteria that was isolated from *Alvinella pompejana* and *Alvinella caudata* specimens.

The roles of bacteria symbionts of Polychaetes are still mostly unexplored, despite that some evidences have clarified some aspects on these relationships. Indeed, Polychaetes seem to establish symbiosis with bacteria that supply their energy requirements, and gain for them protection against heavy metals [54,55]. This was observed, especially for bacteria inhabiting the epidermis of hydrothermal vent Polychaetes, which resulted in being resistant to different concentrations of heavy metals, i.e., zinc, arsenate, silver, and copper [53]. Generally, the protective role by providing resistance properties to Polychaetes has been proved for bacterial communities that were associated with the host epidermis [54,56–58]. Chemolithoautotrophic bacteria that are hosted in specialized organs are involved in supplying the energy needs of the tube worm *Riftia pachyptila* [59]. In any case, further improvements are required for a better understanding of dynamics in the interaction between Polychaetes and associated bacteria.

Alain et al. [54] and Li et al. [60] presented other attempts of describing the bacterial communities associated with Polychaetes. Li et al. [60] studied the microbiota of the polychaete *Neanthes glandicincta*, showing that the dominant phylogenetic groups were affiliated to Methanomicrobiales I: Methanosaetaceae, Deltaproteobacteria, and Gammaproteobacteria, which resulted widely distributed throughout the entire gut. Alain et al. [54] reported the characterization of mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis*, which resulted in a dominance of Epsilonproteobacteria, followed by phylotypes that were affiliated to the CF group of Bacteroidetes and Verrucomicrobia. Some other reports referring on associated culturable bacteria will be treated in the following sections in relation to secondary metabolites production.

3.3. Cnidaria

The phylum Cnidaria includes many species of animals, mostly inhabiting marine environments, endowed of cnidocytes, i.e., cells used for defensive or capturing scopes. The tissue composition is still primitive, with a jelly-like substance called mesoglea between two one-cell tick epithelium layers. The main morphological forms are represented by swimming medusae and sessile polyps, but some species could also produce colonial aggregations. The animals have a sole cavity that is used for both digestive and respiratory processes, while the nervous system is constituted by a decentralized nerve net and a simple receptor system. The feeding strategies that were used by the phylum members are several: some of them are predators, others absorb dissolved organic substances or filter food particles from the surrounding water. The main classes are represented by Anthozoa (which consists of sea

anemones, corals, and sea pens), Scyphozoa (mainly jellyfish), Cubozoa (i.e., box jellies) and Hydrozoa (which includes freshwater and marine species of sessile and colonial organisms).

With particular regard to Octocorallia and Hexacorallia subclasses, corals are defined holobiont entities, which are able to create complex relationships with a variety of prokaryotic and eukaryotic microorganisms [61]. A '*core microbiome*' [62] was recently designated to indicate the microorganisms strictly associated with a host species, while transient microorganisms are those for which their association is affected by the local conditions [14,63–65]. Interestingly, Hernandez-Agreda et al. [63] proposed and confirmed the existence of three distinct microbial fractions associated to *Pachyseris speciosa*, including an ubiquitous core microbiome associated to the coral with a high degree of spatial homogeneity, a second associated bacterial fraction more influenced from the local environmental conditions and a third highly variable component dependent from the processes occurring at the spatial and temporal scales. Octocorals are the most studied Cnidarians in relation to prokaryotic association. The Octocorallia (Haeckel, 1866) is a subclass within the Anthozoans (Ehrenberg, 1834), including one of the most ubiquitous benthic specimens in marine environments [65], which are able to provide habitats for a great number of minor organisms. Octocorals are represented mainly by soft corals (sea fans and sea whips, Lamouroux, 1812), sea pens (Verril 1865), and blue corals (Bock, 1938).

Recently, they gain a lot of attention as host of stable bacterial communities in addition to other microorganisms (i.e., dinoflagellates, fungi, Archaea, and viruses) [61], which seem to play strategic roles in the nutrition supply, protection, and adaptative response to environmental changes [19,66]. Moreover, specific important functions involved in the nutrient cycles, as well as nitrogen fixation or sulfur cycling, and in antibiotic production have been attributed to bacterial symbionts [67–69].

The interest towards the symbiotic interaction between octocorals and bacteria is relatively recent, because most studies during last decades have been mainly oriented to the study of reef-building scleractinian corals, for which a community of Dinoflagellata, bacteria, fungi, Archaea, and viruses was described [70–72]. Moreover, great part of researches on Octocoral bacterial communities have been carried out with the only aid of culture-based methods, with a consequent important gap of information in the non-cultivable bacterial species. All of these aspects taken together led today to a still fragmentary and incomplete knowledge of the interaction between octocorals and bacteria, if compared to the other symbionts.

The importance gained from bacterial symbionts grew up when several octocoral populations were affected by disease outbreaks, especially in temperate environments. As it was extensively described by van de Water et al. [65] in the Mediterranean Sea, most studies were focused on some sub-orders, i.e., sub-order Scleraxonia (Studer, 1887) and sub-order Holaxonia (Studer, 1887). The bacterial communities associated to *Holaxonia* species resulted mainly dominated by Proteobacteria, with strong predominance of genus *Endozoicomonas* in octocorals [64,65,73–78], and *Cellvibrionales* BD1-7 (previously Alteromonadales), *Mycoplasma*, *Aquimarina*, *Granulosicoccus*, and *Vibrio* species on Gorgoniida [64,65,73]. The candidate phylum NPL-UPA2 seems to be strongly associated with *Paramuricea clavata* (sub-orders Holaxonia) [64]. Some studies performed on associated bacterial communities in octocorals from deep shallow waters reported as main taxa Gammaproteobacteria and Alphaproteobacteria [73–75,79–82]. The same taxa were reported as being predominant in the bacterial communities that are associated with specimens of *Antillogorgia elisabethae*, with high-levels of Cyanobacteria, Flavobacteria, and unclassified Bacteria occurring sporadically [83], and phylotypes related to *Pseudomonas splenii* and *Endozoicomonas* spp. [84]. The core microbiome of *Antillogorgia elisabethae* was also described by Robertson et al. [83], as constituted mainly by Rhodobacteriales, Rhizobiales, Flavobacteriales, and Oceanospiralles taxonomic groups.

While the structure of bacterial communities associated to cnidaria has been widely investigated from the phylogenetic point of view, the stability of these assemblages is still a discussed issue. Some cnidarians have been reported as host of specific bacterial assemblages, as in the case of *Corallium rubrum*, whose holobionts are mainly composed of Spirochaetales, Oceanospirilalles family

ME2, and Parcubacteria, and only a minor contribution of *Endozoicomonas* [78,85]. Despite some authors sustain a host species-specificity [86–88], according to many authors the structural composition of the associated bacterial community is influenced by several factors, i.e., location, species, stages of coral development, and spatial location within the holobiont [79,82,89–94]. In the case of octocorals, in the light of the recent advances, it was proved that they maintain a bacterial community distinct from the surrounding water [73–75,77,79,82,95], not always stable, and dependent from the gorgonian species investigated. For example, studies of deep-sea octocorals revealed no clear pattern of conserved bacterial consortia [79,82], while the bacterial communities of the Mediterranean octocoral *Paramuriceaclavata* were observed to be relatively stable both geographically and temporally [75].

A strong scientific contribute in this issue was provided by Robertson et al. [83]. These authors, in their study on *A. elisabethae*, argued in terms of temporal and spatial variability of the associated bacterial community, by performing a comparison of data with a previous study that was performed on the same octocoral species collected in Colombia [84], and between different octocorals and seawater samples that were collected in the Bahamas. In addition to prove the occurrence of a distinct bacterial community from the surrounding water in all of the specimens, they showed a high variability between and within the Bahamas sites, in contrast with results of Correa et al. [84], who reported a highly stable bacterial community associated to *A. elisabethae*. Moreover, their results displayed a similar taxonomic composition of bacterial communities that were associated with corals collected from San Salvador Island in 2011 and 2013. According to the authors, the variability in the associated microbial communities is expressed equally both on a local and wide scale, and is correlated to the single specimen sample. A strict correlation between associated coral symbionts, environmental stressors, and the occurrence of disease has been also proved. The associated bacterial communities are really sensible to the stressful factors, which could determine changes in the resident microbial community composition and function, by causing moreover the occurrence of transient pathogens with consequent development of disease [70]. The occurrence of disease in the organism is in turn an additional affecting factor for the bacterial community composition, as highlighted by Gil-Agudelo et al. [96], in the study on *Gorgonia ventalina*, thus additionally confirming the strong interdependence between bacterial community and its host.

Some authors pointed their attention on the association stability of some most abundant bacterial genera detected among symbionts [97]. The *Endozoicomonas* genus, mentioned above as predominant in coral microbiome, is actually reported as associated to a large group of marine invertebrates and vertebrates [98–102], and it seems to be able to establish symbiosis relationships with organisms that are widely distributed in all of the marine environments of the world [103]. Neave et al. [97] investigated on the temporal stability of the symbionts with specimens of *Stylophora pistillata* and demonstrated that the genus maintains its dominance for at least 4–5 years. The authors interestingly correlated also the reproductive strategies of organisms to the specie-specificity of associated symbionts. Indeed, they reported that specimens of *Pocillopora verrucosa* harbors *Endozoicomonas* members, because a spawning coral that could acquire its symbionts from the surrounding water, while the coral *Stylophora pistillata* resulted as being associated with distinct genotypes, because it is a brooding organism with a symbiont-packed planula larvae that could determine a vertical transfer of symbionts.

Several studies confirm the presence of a specific microbial consortia associated to temperate cnidaria, as well as *Eunicella verrucosa*, which is distinct from the surrounding water communities. Moreover, warm-coral associated bacteria have a specificity correlated to the location, as supported by the similar structure of communities inhabiting different coral species that are closely geographically related [73,75,77,86,91,104], despite a strong disturbance is in dependence on environmental conditions. The same conclusions have been achieved by van der Water et al. [85], who studied the structure of the microbiome from five closely (*Eunicella* and *Leptogorgia* spp.) and distantly octocorals (*Corallium* sp.), concluding that the bacterial assemblages are quite stable temporally, but are locally influenced. The roles that are played by symbionts in terms of photosynthetic productivity, antibiotic production [105], and nutrient cycling (as well as nitrogen fixation and sulfur

transformations) [67,106] are at the base of reef ecosystem survival and a primary need for the correct functioning of these ecosystems [72,107].

4. Production of Bioactive Molecules by Bacteria Associated with Marine Invertebrates

The interest towards marine invertebrates have increased during last decades thanks to their enormous potentiality as source of bioactive metabolites, which have a number of applications in several industrial fields. The marine environment is widely recognized as a natural reservoir of organisms that are able to produce molecules with biological properties, in virtue of its ecological pressure acting on organisms, driving them to develop different adaptive strategies. The *blue world* is still so unexplored, and this aspect together with its enormous diversity in terms of richness, abundance, and phylogenetic variety continues to feed the interest and the investments of a lot of researchers. Moreover, the possibility that different organisms could interact each other by establishing several kinds of relationships make it a sort of generator of several combinations, resulting in as much number of different useful metabolites. For these reasons, the topic grew up over the time by becoming an independent current, so that today it is possible deal of *Blue-Biotechnology*, with the goal to create a database of biologically active molecules of exclusively marine origin. If marine organisms seem to be the direct source of so important molecules, the interactions among them and their symbionts have often confirmed the real core of the biomolecules production. After a long time in which the production of these compounds has been attributed exclusively to the higher organisms, now we can assess that mostly the bacteria symbionts are responsible for metabolite production, which is necessary to perform their role in the symbiotic relationship. This assumption has been clarified more recently, and scientifically evidenced after the observation that molecules that were produced by bacterial symbionts were slightly different from those that were formerly attributed to their hosts [108,109]. According to some authors, the bioactive compounds derive from the positive relation between hosts and symbionts, and the chemical precursor has to be individuated in a molecule supplied by one relationship member [110]. The chemodiversity of compounds that were produced by associated bacteria is very wide, and it includes in some cases biopolymers, as well as exopolysaccharides and some types of biosurfactants. Despite this, a lot of molecules of bacterial origin with biological functions are not biopolymers, but also smaller or less complex substances with important functional moieties. Some reports are more focused on the description of the functions at the expense of their chemical structure, and they aim at isolating and describing such active moieties. For this reason, we could often know the source of a new bioactive compound, but it can be not classified as polymers or other molecular class. Initially, most of the studies were aimed at detecting molecules that were employable in pharmaceutical or medical fields. Such polymers often result from cell-to-cell interactions that occur within the associated microbial communities. Indeed, these communities have to perform a delicate action of density control assemblages, which requires the production of antagonistic compounds. Despite that a lot of work have been focalized on natural product of marine origin with potential application in medical fields, during last years the attention have turned on bioactive molecules with interest in bioremediation strategies. The idea behind this issue is that a large portion of marine invertebrates is also filter-feeders, and filter large quantities of water to supply nourishment. The high filtration rate could led to the accumulation into the animal tissue of contaminants dissolved in the surrounding water so acting in two possible ways: (i) favoring the establishment of bacterial communities specialized in the removal of the specific pollutant, (ii) stimulating the bacterial symbionts to produce molecules that are able to defend the host from the contaminant accumulation. In the following sections, we will outline the recent studies and current notions regarding the use of marine invertebrates as source of bacteria with the ability to produce biosurfactants and extracellular polymeric substances, by addressing a first evaluation of best model organism useful to this purpose. A short discussion about the production of biomolecules with medical applications by symbiont bacteria will be also addressed, in consideration of the fact that these latter represented the starting point in the bioprospecting research. Lastly, we will try to

highlight the right future perspectives and directions to exploit the potentiality of these organisms in this sense. Table 1 shows a list of marine invertebrates that are used as source of bacterial producers of biosurfactants (BSs), extracellular polymeric substances (EPSs), and antimicrobial compounds.

Table 1. List of marine invertebrates used as a source of bacteria producing bioactive compounds.

Organism	Species	Bioactive Compound	Reference
Sponges	*Callyspongia diffusa*	Biosurfactant	[111]
	Callyspongia diffusa	Biosurfactant	[112]
	Halicondria panicea	Biosurfactant	[113]
	Dendrilla nigra	Biosurfactant	[114]
	Dendrilla nigra	Biosurfactant	[115]
	Dendrilla nigra	Antibacterial	[116]
	Haliclonissa verrucosa	EPS	[117]
	Hemigellius pilosus	EPS	[117]
	Tedania charcoti	EPS	[117]
	Callyspongia	Antibacterial	[4]
	Haliclona sp.	Antibacterial	[4]
	Haliclonissa verrucosa, *Anoxycalyx joubini* and *Lissodendoryx nobilis*	Antibacterial	[118]
Cnidarians	*Sarchophyton glaucum*	Biosurfactant	[119]
	Acropora digitifera	Antibacterial	[120]
	Acropora digitifera	Antibacterial	[121]
	Acropora digitifera	Antibiofilm	[122]
	Acropora digitifera	Antibiofilm	[123]
Polichaetes	*Megalomma claparedei*	Biosurfactant	[124,125]
	Branchiomma luctuosum	Biosurfactant	[124,126]
	Sabella spallanzanii	Biosurfactant	[124,125]
Sea pens	*Pteroeides spinosum*	Biosurfactant	[127]

4.1. Biosurfactants

Biosurfactants (BSs) are amphipatic compounds doted of a hydrophilic and hydrophobic bond, thank to which they can exercise their tensioactive functions, by the mobilization and solubilization of insoluble compounds. The interest towards biosurfactants is still growing in several fields of application in force of their *eco-friendly* characteristics, in addition to a strong specificity of action, which makes them optimal competitors of chemical surfactants [128,129]. Although they were mainly researched and investigated for their removal capacity on contaminants, such as hydrocarbons [113,124,125,130–134] and heavy metals [126,135–138], some authors pointed their attention also on other possible properties, in particular, medical and pharmaceutical activities, i.e., antimicrobial, antifungal, antitumoral, and anti-mycoplasmic ones [139,140].

4.1.1. Main Screening Methods Applied for the Search of Biosurfactants from Marine Invertebrates

At the beginning of research on BSs and their possible applications, several terrestrial and marine environments have been engaged as source for their extraction. Research was aimed at discovering and isolating not only as many producers as possible, but also novel BSs in order to massively extend the range of application. The ecological context and the intrinsic characteristics of BSs drive the researchers to use mainly contaminated water and sediment samples as source for the isolation of bacterial BS producers. Only in relatively recent times, the attention has been moved to new possible sources of isolation, and in this sense, marine invertebrates, mainly filter-feeding organisms, started to be investigated as potential matrix. Currently, the marine organisms mostly used to isolate BS-producing microorganisms are sponges, polychaetes, cnidaria, and, more rarely, sea pens. To the best of our knowledge, the first attempt in this sense was proposed by Gandhimathi et al. [111], who reported the sponge *Fasciospongia cavernosa* from the southwest coast of India as a source of the sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10, able to produce BSs.

Great part of literature available on associated microorganisms that are able to produce BSs is structured with an isolation step, followed by a production screening procedure on a battery of strains or on targeted strains, and finally, with investigations and speculations about the BS chemical characterization and potential functions. The screening procedure tests chosen for experiments are quite or less common to all works, including oil displacement test, drop collapse test, emulsifying activity and stable emulsion production, surface tension reduction, hemolytic test, and cetyltrimethylammonium bromide (C-TAB) agar plate assay [111–114,124,125,127,141]. In some cases, the most common procedure scheme is enriched with the addition of more specific tests, such as lipase production test [122], bacterial adhesion to hydrocarbons (BATH) assay [113], microplate assay, and penetration assay [127] in dependence of the aim of the research. BSs are basically tensioactive substances, acting at the interface between matrices with different polarity. For this reason, each screening test is aimed at detecting one or more specific action of BSs, and could represent a direct or indirect measure of surface tension, measurements on surface/interfacial tension, evaluation of cell surface hydrophobicity, detection and quantification of emulsifying activity, or finally, specific screening test (i.e., C-TAB assay for detection of anionic BSs, or Blood agar assay for detection of hemolytic effect). The principal demand in the practical approach on BS producer discovery is the possibility to screen a large number of candidates in a short time and with low costs, and this is the reason very often for which the more simple tests are inserted in own screening procedure. Unfortunately, a unique rapid and economic test able to identify BS producers with unequivocal results and interpretation have to be still discovered, so for a consistent detection more than one test have to be applied [124,141], even in consideration of the great heterogeneity of BSs [112]. Moreover, some reports deeply investigated the BS properties by testing their stability and function at different external conditions (i.e., temperature, pH, salinity) [113,114]. The marine organisms could be used as natural samples, or in some cases, they were used to perform firstly enrichment cultures, and then the subsequent bacterial isolation. Generally, the treatment of the samples in aseptic conditions contemplate the washing of organisms and the excision of a fragment, followed by the homogenization of tissues with a tissue homogenizer. Homogenates are then serially diluted and aliquots are plated on nutritive media (the most used media are usually Marine Agar (MA), Luria Bertani medium (LB), or mineral medium supplemented with different carbon sources). In the case of sponges, the area of organisms generally used is the internal mesohyl [111,114], while in the case of Polychaetes the branchial plume is used [124,125]. Some authors decided to use the mucus from the biological matrix, as in the case of Padmavathi et al. [122], who isolated BSs producers from the mucus of the coral *Acropora digitifera*. The idea is try to use the portion of organisms that are mostly involved in the hosting of associated bacteria, or in the accumulation of nutrients and contaminants. The utilization of enrichment cultures leads to a more specific selection of BS producers, and this approach was proposed as a faster way for detecting them [113,124,125]. This assumption was in line with the common principle according to which microorganisms that are able to grow in the presence of hydrocarbons are optimal candidates for BS production [142]. This was effectively proved by the results of some authors, with higher bacterial abundance that was detected in enrichment cultures, thus confirming the presence of a bacterial community that is specialized in the hydrocarbon degradation processes, probably for the heavy accumulation of contaminants in the organisms tissue. Literature shows that all of the biological matrices investigated resulted quite valid as source of BS producers. When the authors decided to apply the screening on a battery of strains instead of on a unique one, the percentage of BSs producers on the total of associated bacteria isolated ranged from a minimum of 9% [114] (bacteria associated to the sponge *Dendrilla nigra*) to a maximum of 43% [124] (bacteria associated to Polychaetes), 52% [127] (bacteria associated to the sea pen *Pteroeides spinosum*), and 68% [113] (bacteria associated to the sponge *Halicondria panicea* or isolated from its enrichment cultures). Anyway, this approach allowed for isolating a good number of BS producers also from cnidarians with ranges of about 20% of total isolates [119,122]. In consideration of the higher number of producers that were isolated from enrichment cultures, the preliminary treatment of organism homogenates with

an enrichment passage could surely facilitate the achievement of the goal. The growth of associated bacteria seems to be correlated to the production of BSs, as it occurred generally during the exponential phase [112,119], despite in some cases no correlation between cellular growth and BS production was detected [127]. The responses to the screening tests that were reported for BSs of marine origin are really diverse, and sometimes it could appear contrasting one to each other. The aspect is due to the great availability of different BS molecules, with different chemical structures, reflecting a different functional behavior. The screening tests that are most commonly used to better describe the properties of tensioactive substances are surely emulsification assay and surface tension reduction. This because each BS could act as an excellent surface tension reducer or as optimal emulsifying agent without a necessary correlation between these two separate actions [113,143]. If some authors reported a strong ability to produce stable emulsion, with E_{24} (index) of 50–60%, without a remarkable surface tension reduction [122,124,125], other reported a potent surface tension reduction capability [112]. These reports, which could induce in confusion, could be really considered as a tool to obtain a preliminary idea on the type of BSs produced. Indeed, low-molecular weight BSs (including glycolipids, lipopeptides, flavolipids, corynimycolic acids, and phospholipids) are known as efficient interfacial agents, while high-molecular weight BSs (including polymeric and particulate surfactants) are most effective in emulsion production [144]. Interestingly, some associated bacteria produced BSs which showed different efficiencies in interfacial activities during incubation in rich or minimal media, with higher surface tension reduction exhibited in the latter one [124,125]. The authors suggested different theories to explain the discrepancy: (*i*), a possible stimulation of BS production with interfacial activity during incubation in mineral medium supplemented with hydrocarbons, which probably act as a stressor parameter, (*ii*), the production of BSs with a different chemical structure in function of the carbon source, and so with different activities. The study of BS kinetic production by associated bacteria, and the expression of emulsifying activity in ratio with stable emulsion production [113,125] allowed for confirming that the emulsion stability could be also considered an indicator of strength and BS amount production [145].

4.1.2. Phylogenetic Affiliation of BS-Producing Bacteria from Marine Invertebrates

Bacterial BS producers from marine invertebrates belong to different phylogenetic groups (Table 2). Among BS-producing Proteobacteria, the Gammaproteobacterial genera *Vibrio* [127], *Providencia* and *Psychrobacter* [122], *Halomonas* sp. [112], *Pseudoalteromonas* sp. [125], *Idiomarina* sp., *Marinobacter* sp., *Alcanivorax* sp. [124], and *Acinetobacter* sp. [113] have been frequently isolated from marine invertebrates. Alphaproteobacteria and Betaproteobacteria have been also detected with *Pseudovibrio* [113] and *Alcaligenes* sp. [112] affiliates, respectively. Among Gram-positive bacteria, Actinobacteria members, which are well known as a suitable source of secondary metabolites [116,146], have been isolated from sponge *Dendrilla nigra* by Kiran et al. in 2014 [114], with *Brachybacterium paraconglomeratum* MSA21 able to produce a BS with antimicrobial activity (see below) for an amount of 21 g/L under optimal growth conditions, and in the 2010 with the strain *Brevibacterium aureum* MSA13 [115]. Four additional *Brevibacterium* spp. strains (i.e., PBE178, PBE181, PBE190, and PBE 209) with good emulsifying and interfacial activities, and *Nocardiopsis* sp. MSA10 with antimicrobial properties were isolated from the sea pen *Pteroeides spinosum* [127], and the sponge *Callyspongia diffusa* [111], respectively. Among Firmicutes, *Bacillus* members often occurred in the works about the associated bacteria that are able to produce BSs, both from sponge [112,113] and coral specimens [119,122]. A wider phylogenetic characterization of associated BS-producing bacteria was provided by Rizzo et al. [124,125]. More information has been added with these contributes because the authors performed the characterization on the whole bacterial selection obtained from the screening, consisting of 30 bacterial isolates from Polychaete enrichment cultures and 18 bacterial strains from Polychaete tissues. Interestingly, phylogenetic groups never reported before in relation to BS production were detected by the authors and revealed very strong potentialities. Among the BS-producing strains that were obtained from enrichment cultures, Bacteroidetes and Proteobacteria

(Alpha and Gamma) were well represented, with several members within the genera *Joostella*, *Cobetia*, *Cellulophaga*, *Thalassospira*, *Cohaesibacter*, *Alcanivorax*, *Pseudomonas*, *Idiomarina*, and *Marinobacter* [124]. Similar results were afterwards reported by the same authors [125] from natural samples, despite different genera (e.g., *Pseudoalteromonas*, *Maribacter*, *Cellulophaga*, and *Tenacibaculum*) were retrieved among the same taxa. In addition, Actinobacteria (*Citricoccus* spp.) and Firmicutes (*Staphylococcus* spp.) members were isolated from *Branchiomma luctuosum* and *Sabella spallanzanii* sabellids. BS-producing bacteria that were isolated from marine invertebrates are listed in Table 2 to gain an immediate idea of how much the exploration of new sources can be effectively translated into the discovery of a greater amount of potential producers, and in a respective greater biodiversity. Moreover, these data have been graphically elaborated to perform a statistical analysis, non-metric multidimensional scaling (nMDS), showing the spatial distribution of bacterial phyla in relation to the organism of isolation (Figures 1 and 2). Whereas the primary objective in the BS search is the isolation of new possible producers, to obtain new molecular forms that can have different fields of application, the winning strategy is just testing new matrices, refining the isolation techniques as much as possible. From the reference framework that is currently available, Polychaetes have proved to be the best source of isolation for BS-producing bacteria. This could be linked to the intrinsic properties of sabellids, or to a higher or more efficient filtration rate operated by these organisms with the consequent development of more suitable conditions for colonization by specialized microbial communities. The experimental design acquired in this sense a key role for the success of the research and the achievement of the objectives. Operating since the initial phase focusing on the applications for which BSs research is aimed in place of a generalized approach repays all of the efforts in a more prudent and more expendable result for scientific purposes. The greater the number of potential producers to test, the wider the spectrum of combinations of possible biosurfactants with their respective functions could be explored. Further insights should be moved on to understand the structure of the entire microbial community associated with these organisms, and what role that each member plays in the relationship.

Table 2. List of biosurfactants (BS)-producing bacteria associated with higher organisms.

Phylum or Class	Strain (Accession Number)	Isolation Source	Reference
Actinobacteria	*Brachybacterium paraconglomeratum* MSA21 (GQ153943)	*Dendrilla nigra*	[114]
Actinobacteria	*Brevibacterium* sp. PBE178 (KR185336)	*Pteroides spinousm*	[127]
Actinobacteria	*Brevibacterium* sp. PBE181 (KR185337)	*Pteroides spinousm*	[127]
Actinobacteria	*Brevibacterium* sp. PBE190 (KR185338)	*Pteroides spinousm*	[127]
Actinobacteria	*Brevibacterium* sp. PBE209 (KR1853312)	*Pteroides spinousm*	[127]
Actinobacteria	*Nocardiopsis alba* MSA10 (EU563352)	*Callyspongia diffusa*	[111]
Actinobacteria	*Citricoccus* sp. Bl52 (KF032914)	*Branchiomma luctuosum*	[125]
Actinobacteria	*Citricoccus* sp. Bl54 (KF032915)	*Branchiomma luctuosum*	[125]
Actinobacteria	*Citricoccus* sp. Bl55 (KF032924)	*Branchiomma luctuosum*	[125]
Actinobacteria	*Brevibacterium aureum* MSA13 (GQ153943)	*Dendrilla nigra*	[115]
Alfaproteobacteria	*Pseudovibrio* sp. SpE85 (KY129823)	*Halicondria panicea*	[113]
Alfaproteobacteria	*Pseudovibrio* sp. SpE86 (KY129822)	*Halicondria panicea*	[113]
Alfaproteobacteria	*Pseudovibrio* sp. SpE90 (KY129821)	*Halicondria panicea*	[113]
Alfaproteobacteria	*Pseudovibrio* sp. SpE93 (KY129820)	*Halicondria panicea*	[113]
Alfaproteobacteria	*Thalassospira* sp. A46 (JX298566)	*Sabella spallanzanii* enr.	[124]
Alfaproteobacteria	*Thalassospira* sp. A57 (JX298539)	*Branchiomma luctuosum* enr.	[124]
Alfaproteobacteria	*Pseudovibrio* sp. A27 (JX298538)	*Megalomma claparedei* enr.	[124]
Alfaproteobacteria	*Cohaesibacter* sp. A25 (JX298537)	*Megalomma claparedei* enr.	[124]
Alfaproteobacteria	*Cohaesibacter* sp. A55 (JX298551)	*Branchiomma luctuosum* enr.	[124]
Alfaproteobacteria	*Cohaesibacter* sp. A60 (JX298552)	*Branchiomma luctuosum* enr.	[124]
Alfaproteobacteria	*Cohaesibacter* sp. A49 (JX298548)	*Branchiomma luctuosum* enr.	[124]
Betaproteobacteria	*Alcaligenes* sp. MB-19 (KJ540940)	*Callyspongia diffusa*	[112]
Gammaproteobacteria	*Vibrio* sp. PBN295 (KR185340)	*Pteroides spinousm*	[127]
Gammaproteobacteria	*Providencia rettgeri* U7 (JN315773)	*Acropora digitifera*	[122]
Gammaproteobacteria	*Psychrobacter* sp. U9 (JN315776)	*Acropora digitifera*	[122]
Gammaproteobacteria	*Psychrobacter* sp. U14 (JN315774)	*Acropora digitifera*	[122]
Gammaproteobacteria	*Halomonas* sp. MB-30 (KJ414418)	*Callyspongia diffusa*	[112]

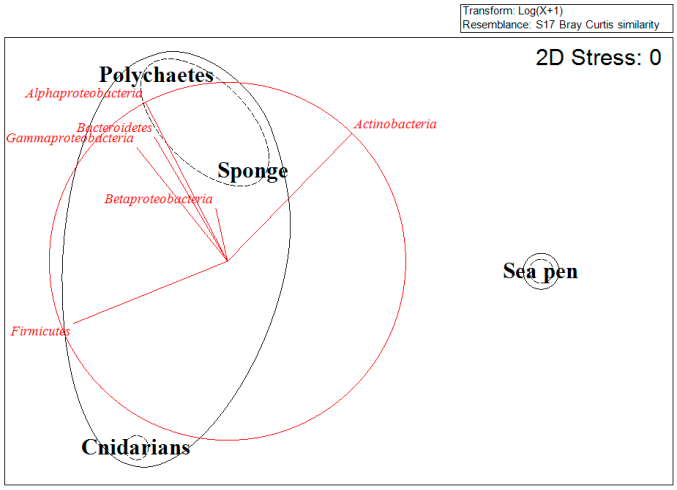

Figure 1. Non-metric multidimensional scaling (nMDS) showing the spatial distribution of BS-producing associated bacteria in relation to organisms of isolation.

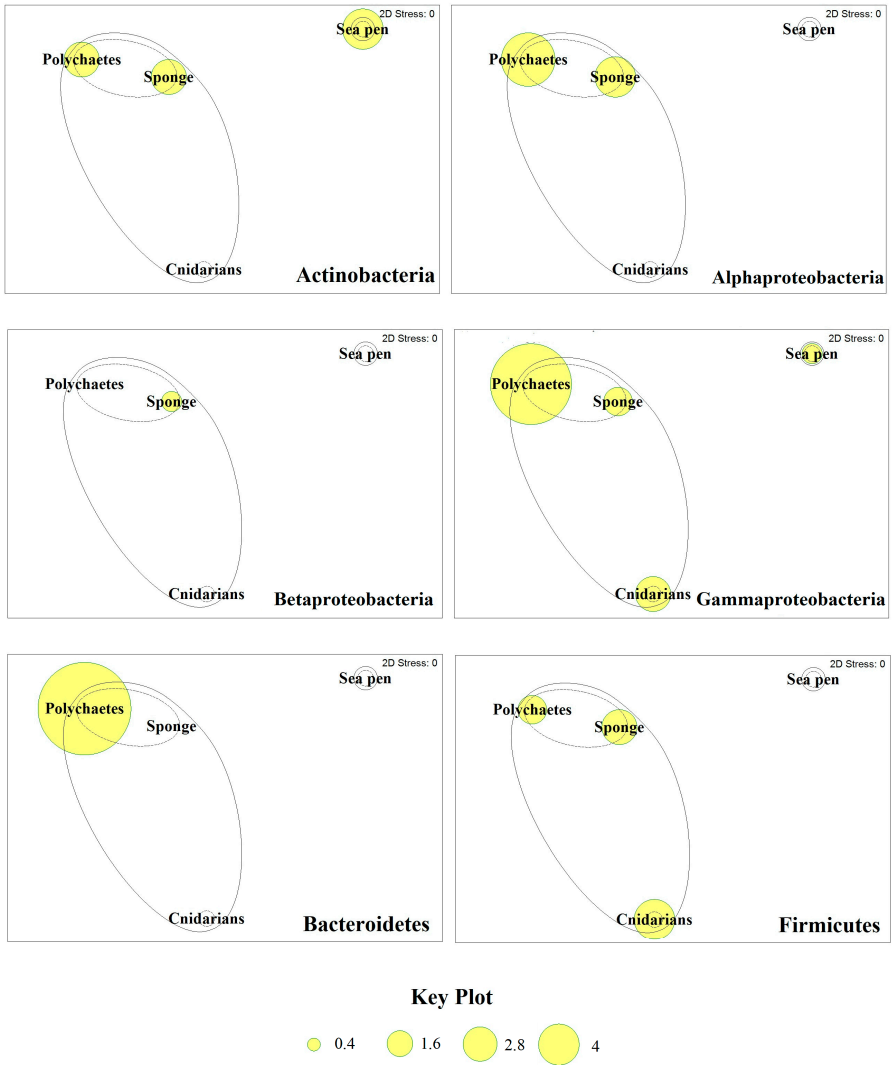

Figure 2. Non-metric multidimensional scaling (nMDS) showing the distribution of each Phylum of BSs bacterial producers in relation to higher organisms.

4.1.3. Applications of BSs Produced by Bacteria Associated with Marine Invertebrates

On the base of a literature that is still expanding about the BSs that are produced from marine associated bacteria, chemical characterization groups them mainly within the glycolipidic and lipopeptidic classes. The use of biological matrices as source of bacterial BS producers has revealed a successful tool for the detection of new biosurfactant compounds. This is the case of Kiran et al. [115], who proved the production of a new lipopeptide BS called *brevifactin* by *Brevibacterium aureum* MSA13 with a hydrophobic moiety of octadecanoic acid methyl ester and a peptidic moiety. Further, Padmavathi et al. [122] detected new BSs with antibiofilm activity produced by coral *Acropora digitifera* associated bacteria.

The activities that were exhibited by BSs from associated bacteria are various and involved in several fields. The possible applications in bioremediation strategies towards different types of contaminants are currently the most investigated. Rizzo et al. [113,124–126,147] and Graziano et al. [127] focused their works on hydrocarbons and heavy metals remediation. Starting by a selective selection procedure of potential producers among hydrocarbon-degrading bacteria, they demonstrated the occurrence of BS production in presence of such contaminants. The fact that BSs are generally not produced in a constitutive way by microorganisms supports this assessment. Specifically, diesel oil removal with percentages of 26.8, 38.2, and 52.7% was detected for the sabellid-associated strains *Joostella* sp. A8, *Pseudomonas* sp. A6, and *Alcanivorax* sp. A53, respectively. The biodegradation rate increased when the strains were cultured in co-cultures achieving rates of about 90% of removal [147]. In microcosm culture, *Joostella* sp. A8 confirmed its optimal performances by exhibiting competitive and encouraging hydrophobicity and emulsification rates in comparison with bacterial genera just known as hydrocarbon degraders and BS producers. The production of a BS of probable glycolipidic nature was highlighted for the pennatulid-associated *Vibrio* sp. PBN295 during growth in presence of diesel oil, by suggesting its bioremediating properties. Similarly, pennatulid-associated *Brevibacterium* spp. strains were reported as able to produce BSs in the presence of crude oil, in addition to diesel oil [127]. The glycolipidic biosurfactant that was obtained by a coral-associated *Bacillus* [119] was proposed as an optimal candidate for the bioremediation of spilled oils, with a removing capacity of about 45% of residual oil that was recovered using biosurfactant containing broth. The investigation about the use of BSs for heavy metal removal is poorer than that applied on hydrocarbon pollution, especially in the case of aqueous matrices. To date, the only case that reports bacterial producers that are associated with marine invertebrates is that of *Joostella* sp. A8, which is able to produce BSs in the presence of heavy metals (copper, zinc, cadmium), so supporting the possibility of a chelating activity, just suggested for BSs of different origin by other authors [136].

On the other hand, BSs that are produced by bacteria associated with marine invertebrates could be strongly correlated with antimicrobial effects, thus confirming the defensive role that the bacterial associated communities often act towards their hosts. Generally, it was assumed that lipopeptide surfactants possess more potent antimicrobial properties compared to glycolipids [148], but this result is sometimes contrasted for associated BS-producing bacteria. The lipopeptide BSs produced by the sponge-associated *Nocardiopsis alba* [111] was active against many human pathogens, i.e., *E. faecalis* and *B. subtilis*, and the pathogenic yeast *C. albicans*. A wider antimicrobial activity was proved for the new lipopeptide produced by the sponge-associated *Brevibacterium aureum* MSA13 [115], but also for the glycolipidic BS produced by the sponge-associated Actinobacterium *Brachybacterium paraconglomeratum* MSA21 [114]. Such activity was exhibited at different levels against *Streptococcus* sp., *S. aureus*, *B. subtilis*, *C. albicans*, *K. pneumonia*, *M. luteus*, *S. epidermidis*, *E. faecalis*, *P. aeruginosa*, *P. mirabilis*, and *E. coli* [114].

The investigations regarding the discovery of new natural drugs are really important, not only for their applicative resonance, but also for the ecological and evolutionary observations that could derive from them. Marine filter-feeding or benthic organisms that are are study object by time because considered direct producer of bioactive molecules, possibly employed in defensive functions. Indeed, their sessile nature imposes the acquirement of this type of survival strategies, but to this day, it is not

totally clear if the production of some bioactive metabolites is to be attributed to the hosts or to the associated bacteria. Finally, worthy of attention is the work on coral mucus associated strains [122], which opened a new avenue of possible applications, by reporting the inhibition of biofilm formation in *P. aeruginosa* ATCC10145 with percentages ranging from 85% to 89% and a strong thermal stability.

The detection of important BS activities that are expendable in some application fields, does not exclude the possible validity of the same molecules also for other uses. This means that the same biosurfactant, once discovered, could be applied and tested in several conditions and for numerous advantageous activities.

Although the discovery of new molecules remains the primary objective in the search for BSs, the second aim is the optimization of their production, which could lead to the production on large scale with the minimal cost and effort. Several attempts have been done in this sense, and the influence of a quantity of environmental parameters has been tested on BSs production [111,112,115,119,125,127,141]. The external parameters of major importance are surely temperature, pH value, and salinity. This is important, because for the industrial or biomedical application, the stability and specificity of the molecules are a determinant characteristic for their concrete employment and success.

4.2. Extracellular Polymeric Substances (EPSs)

EPSs are known as high-molecular weight (10–1000 kDa) homo/hetero-polymers that are constituted of sugars moieties and produced by microorganisms. Specifically, their structure is composed of monosaccharides and some non-carbohydrate substituents, such as proteins, lipids, and humic substances [149,150]. According to Delbarre-Latrat et al. [151], each EPS is composed of osidic monomers, on the base of which the structure could result in a homopolymeric—when is present a single type of monosaccharide- and heteropolymeric—if composed of different osidic residues and displays a ordinate structure with a repeating unit. This latter could contain organic or inorganic substituents, and it could be linear or branched, thus resulting in a great variety of possible molecular structures.

EPSs could be secreted into the environment or remain attached to the bacterial outer surface, so they are distinct in *intracellular* polysaccharides and *structural* and *extracellular* polysaccharides. Generally, extracellular polymers are grouped into four categories: polysaccharides, inorganic polyanhydrides, polyesters, polyamides [152], and have been collectively named extracellular polymeric substances, in slime and microcapsular forms [153]. While the capsular forms create polymeric reticules strongly adherent to the cells through interaction with the lipopolysaccharide or covalent bond, the slime forms are weakly attached to the cell. Thanks to their chemical composition, they are very versatile compounds for the employment in industrial and medical fields; therefore, they attracted interest in consideration of their compatibility, efficiency, and the absence of secondary pollution production [154].

EPSs are involved in several biological functions of fundamental importance for the cells, primarily as protective agents against environmental stressful conditions, such as extreme temperature or salinity, preserving the membrane integrity, allowing for the capturing of nutrients, or defending from xenobiotic substances [155–157].

Literature available on EPSs production is focused on bacteria that were isolated from extreme environments [158,159], such as deep-sea hydrothermal vents and polar environments. Indeed, several thermophilic strains that were reported as EPSs producers are affiliated to Gram-negative bacteria, such as *Pseudomonas*, *Vibrio*, and *Alteromonas* [160], and to *Geobacillus* sp., *Bacillus thermodenitrificans*, and *B. licheniformis* [158,159], *Sulfolobus*, *Thermococcus*, and *Thermotoga* [161–163].

Cold-adapted bacterial EPS producers have been also widely investigated [164–170], and are mainly affiliated to the genera *Pseudoalteromonas* and *Halomonas* [165,166,170,171]. Many authors clarified the significant contribute that exopolymer production gains in the organic carbon balance in the sea ice and in ice-water interface [172], and their biotechnological potential [171,173]. In addition, also reports on halophilic [174–176], alkaliphilic [177,178], and acidophilic [179–182]

bacteria are available. Thus, the biodiversity of bacterial EPS producers is strongly related to the environment of origin.

Anyway, a great part of the studies performed till date has used mostly abiotic sources for the isolation of the producers, while the use of biotic matrices is really scantly improved. Among marine invertebrates, the sponges have been the most used, with some first attempts performed also with corals and Polychaetes. Indeed, one of the first approaches to the use of biotic matrices is represented by Vincent et al. [58], who reported an EPS producer (assigned to the genus *Alteromonas*) isolated from the marine polychaete *Alvinella pompeiana*. The same organism was used by Raguénès et al. [183], who described for the first time a new microorganism, affiliated to the *Vibrio* genus, named *Vibrio diabolicus*, as being able to produce an innovative exopolysaccharide. Priyanka et al. [184] collected several marine organisms, and isolated three EPS-producing strains, i.e., *Alteromonas* sp. PRIM-21 from a marine seaweed, *Nitratireductor* sp. PRIM-24 from the gut content of a marine crab, and *Enterobacter* sp. strain PRIM-26 from the gastro-vascular contents of a sea anemone.

A *Spongia officinalis* sample, which was collected in Sicily from Mazara del Vallo, has been proved to host an associated *Bacillus licheniformis* strain that was able to produce a new polysaccharide (1800 kDa) composed of units of α-D-galactopyranosyl-(1→2)-glycerol-phosphate [185]. Similarly, even if from different environments, some Antarctic sponges (i.e., *Haliclonissa verrucosa* Burton 1932, *Hemigelliuspilosus* Kirkpatrick 1907, and *Tedaniacharcoti* Topsen 1908) were revealed as source of EPS-producing bacteria, i.e., *Shewanella* sp. CAL606, *Colwellia* sp. GW185, and *Winogradskyella* sp. CAL396 and CAL384. The optimization of culture conditions allowed for these producers to achieve an EPSs total amount ranging from 147 to 378 mg/L [117].

If we can consider the research of marine invertebrate as source of BS-producing bacteria at an initial phase, the exploitation of these matrices for isolation of EPS producers is located at a nascent phase, with still few reports. It is limited to the use of marine vegetable organisms as direct source of exopolysaccharides [186,187], or to the use of extremophilic strains that were isolated from natural samples, the issue more widely treated [117,157,160,188]. Contrastingly, the focusing in their associated bacterial communities with producing capacity was poorly improved.

When considering the scarcity of the works that were carried out on EPS-producing bacteria associated with invertebrates it is difficult to make assessments on the level of biodiversity, even because there are few references available for each exploited marine organism. In addition, the environments from which the organisms have been collected are different, with different features and, so and therefore, there is no way to make unique assessments at the current state. EPS producers that were isolated from the deep-sea polychaete annelids *Alvinella pompejana* and *A. caudata* tissues are affiliated to the Gammaproteobacteria, Alteromonadales or Vibrionales orders, and are able to produce different EPSs [58,189–191]. The EPS HE800 produced by *Vibrio diabolicus* [183,192] was analyzed and presented a hyaluronic acid-like chemical structure, with repeated units of hexosamines and uronic acids [193].

Several *Alteromonas* strains of different origin showed the production of EPSs with diverse chemical composition, as in the case of *A. macleodii* subsp. *fijiensis* biovar *medioatlantica* isolated from a deep-sea hydrothermal vent shrimp able to produce the EPS MS907 [194], whilst *Alteromonas macleodii* subsp. *fijiensis* produced a pyruvated EPSST716 [195,196], and *Alteromonas infernus* the sulfated EPS GY785 [197,198]. These observations suggest that a high level of biodiversity also correspond to a copious availability of different chemical molecules.

The selection of EPS producers is generally based on the observation of a mucous phenotype of colonies on solid media or of viscosity in liquid cultures [117,183,184,199], indicators of EPS production. A test described by Christensen et al., 1985 is useful to detect the production of slime correlated to EPS production, and it was employed by Caruso et al. [117] for the EPS producer *Pseudoalteromonas* sp. MER144 isolated from Antarctic water samples. The studies on EPS production were carried out with a great focusing on the chemical characterization of the molecules. The deep study and understanding of the chemical structure of a compound is of fundamental importance because it provides a wide

spectrum of evidences. First of all, it allows for the researcher to detect whether the compound under examination belongs to an already observed category or if it is a new biopolymer. Moreover, any chemical difference can correspond to important functional differences, such that the presence/absence of a functional group can determine a specificity of action. The chemical analysis mostly used includes several steps, which are carried out to gain the more possible details on EPS structure: *i*, molecular mass, *ii*, carbohydrates, protein, uronic acids, and sulphates content, *iii*, monosaccharidic composition, *iv*, functional groups. To achieve these results, the authors relied on different procedures and techniques, from the most common—i.e., gas permeation chromatography for the molecular weight, colorimetric assays for the molecular contents, FT-IR, and ^{1}H NMR [180,196]—to the most sophisticated—i.e., X-ray diffraction analysis and Energy dispersive X-ray spectroscopy to improve deeply the detection of the compound nature [200]. Table 3 summarizes the data currently available on EPSs produced by microorganisms associated with marine invertebrates. The molecular masses of bacterial EPSs that are generally are comprised between 20 and 2000 kDa with single and/or double fraction [174,201], but also achieve values that are higher of three or four order of magnitude [175,201]. All of the EPSs that are currently produced by associated bacteria presented a molecular mass, when it was detected, that filled within this range [185,200]. The EPS production occurs during the exponential phase [117,183,184], and it is generally accompanied by an increase of viscosity in the culture broth. The content in carbohydrates, proteins, and uronic acids is various, despite results that were obtained by Caruso et al. [117] revealed generally lower amounts of all chemical molecular categories. The presence of uronic acids and sulfates is commonly correlated to the emulsifying activities, as in the cases reported by Caruso et al. [117] and Singh et al. [200], whose EPSs emulsified several kinds of hydrocarbons and hydrophobic substrates, respectively. Despite a different sulfate content being reported by Caruso and coauthors than by Singh et al. [200], a stronger E_{24} index was highlighted by the former, with an average E_{24} value of about 82% for *Winogradskyella* sp. CAL384 versus a maximum average E_{24} of about 24% for *Bacillus licheniformis* strain. Conversely, Priyanka et al. [184] detected intermediate average sulfate values, but an emulsifying activity that was in line with those of cold adapted bacteria, as reported by Caruso et al. [117], towards hydrophobic substrates. The monosaccharidic composition of EPSs produced from associated bacteria commonly reveals the presence of glucose, mannose, galactose, but some authors also identified in the chemical structure the presence of glucuronic acid, galacturonic acid, glucosamine, ramnose [58], and arabinose [117,200]. Specifically, the arabinose presence was reported in bacterial EPSs for the first time by Singh et al. [200], who attributed it to the association of producer to seaweed.

On the base of this assumption, the knowledge of chemical structure of associated-bacterial EPSs is the source of precious informations about the interactions between microorganisms and their hosts, and led to suppose that the dynamic of the relationship and their functional and ecological role affect the type of bioactive molecules of bacterial origin that could be derived.

Table 3. Chemical characterization of extracellular polymeric substances (EPSs) produced by bacteria associated with marine invertebrates.

				Chemical Characterization					
Organism	**Species**	**Strain**	**Monosaccharides**	**CRB** ●	**PRT** ●●	**UA** □	**SULF** □□	**Properties**	**Ref.**
Sponge	*Haliclonissa verrucosa*	*Shewanella* sp. CAL606	glucose, galactose, mannose, galactosamine, glucuronic acid, galacturonic acid (1:1:0.9:0.6:0.3:0.1)	26 (mg/100 mg EPS)	3 (mg/100 mg EPS)	6.07 (mg/100 mg EPS)	2.4%	Emulsifying activities, cryoprotective effect, heavy metal tolerance	[117]
Sponge	*Hemigelliuspilosus*	*Colwellia* sp. GW185	glucose, mannose, galactose, galactosamine, glucuronic acid, galacturonic acid (1:1:0.7:0.7:0.3:0.04)	28 (mg/100 mg EPS)	2.08 (mg/100 mg EPS)	6.09 (mg/100 mg EPS)	3.8%	Emulsifying activities, cryoprotective effect, heavy metal tolerance	[117]
Sponge	*Tedaniacharcoti*	*Winogradskyella* sp. CAL396	mannose, arabinose, galacturonic acid, glucuronic acid, galactose, glucose, glucosamine (1:0.9:0.4:0.3:0.2:0.2:0.01)	21 (mg/100 mg EPS)	8.8 (mg/100 mg EPS)	3.2 (mg/100 mg EPS)	8.9%	Emulsifying activities, cryoprotective effect, heavy metal tolerance	[117]
Sponge	*Tedaniacharcoti*	*Winogradskyella* sp. CAL384	glucose, mannose, galacturonic acid, arabinose, galactose, glucosamine, glucuronic acid (1:0.5:0.3:0.25:0.1:0.1:0.1)	15 (mg/100 mg EPS)	2.4 (mg/100 mg EPS)	11.9 (mg/100 mg EPS)	7.7%	Emulsifying activities, cryoprotective effect, heavy metal tolerance	[117]
Crab	NS *	*Nitratireductor* sp. PRIM-24	-	390.7 (mg/g EPS)	119.9 (mg/g EPS)	218.7 (mg/L g EPS)	22 (mg/g EPS)	AntioxidantActivities, emulsifyingactivities	[184]
Sea anemone	NS *	*Enterobacter* sp. PRIM-26	-	625.2 (mg/g EPS)	31.7 (mg/g EPS)	253.3 (mg/L g EPS)	ND	AntioxidantActivities, emulsifyingactivities	[184]
Polychaete	*Alvinellapompejana*	*Vibriodiabolicus*	glucosamine, galactosamine, glucuronic acid, galacturonicacids	-	-	-	-	ND °	[197]
Sponge	*Spongiaofficinalis*	*Bacilluslicheniformis*	α-D-galactopyranosyl-(1→2)-glycerol-phosphate monomeric units	-	-	-	-	Antibacterial activity, Anti-biofilm effect	[185]
Polychaete	*Alvinellapompejana*	*Alteromonas* sp. strain HYD-154	Rhamnose, glucose, galactose, mannose, glucuronic acid, galacturonic acid	88.2/927 (g/100 g EPS)	0.2/3 (g/100 g EPS)	32.5/39, 33/36 (g/100g EPS)	-	ND °	[58]

● CRB, carbohydrates; ●● PRT, proteins; □ UA, uronic acids-, □□ SULF, sulfates; * NS, not specified; ° ND, not determined.

One important target in the EPSs study is the achievement of the optimal conditions for their production, because a good knowledge of them could allow a more suitable application for large-scale production. The most important parameter affecting the bacterial EPS production seems to be the carbon source and its amount. Caruso et al. [117] in a step-by-step approach detected the optimal conditions for the production by sponge-associated cold-adapted bacteria, revealing carbon source and temperature as the most affective parameters than pH and NaCl concentration. The procedure was successful, so that for each strain was possible to observe reduplication or the triplication of EPS amounts with respect to the starting growth levels. Indeed, in particular for the strain *Winogradskyella* sp. CAL396, an EPSs amount of 453.95 mg/L was achieved with the addition of sucrose (2%, *w*/*v*) in the culture medium at an incubation temperature of 4 °C, versus an initial amount of 94.9 mg/L. The higher EPSs production at a suboptimal incubation temperature is justified by the authors as a response to stressful conditions, as previously reported also for non-associated cold adapted bacteria [167,170,202–204]. A similar improvement of growth conditions for EPS production is not available in relation to associated bacteria, but several authors optimize the bacterial EPS synthesis, with experiment in batch culture by using fermenter [58], or assessing an optimization in relation to the incubation time [184].

Applications of EPSs Produced by Bacteria Associated with Marine Invertebrates

As described for the investigation on BS production, the search for EPSs needs some improvements about their properties and potential biotechnological applications, which are various. Keeping in mind that the research on EPSs produced by bacteria associated with marine invertebrates is still at its infancy, several applications have been investigated for the first time and need improvements. The bioremediation represents again one of the most important target fields, for which EPSs with optimal emulsifying activities are considered to be the most promising. The EPSs that are produced by associated bacteria are able to produce and stabilize emulsions with different insoluble compounds, i.e., hydrocarbons or mineral oils [117,184,200]. The EPS emulsifier capacity is strongly related to the possible removal of toxic compounds from polluted matrices, and more strength and stability are the emulsions more powerful could be the application. Anyway, the same properties could consist in the lucrative application of compounds in other industrial processes, as such as in food and oil industries, with the advantage of biodegradability and better specificity [200]. The EPS employment in bioremediation strategies is not only related to hydrocarbon removal, but also to the chelation activity on heavy metals, which are generally granted by the presence of uronic acids and sulfate groups that may act as ligands for cations [171]. Although the relevance of this aspect, it remains still unexplored in EPSs from associated bacteria, and the only investigation is relative to cold-adapted bacteria isolated from Antarctic sponges [117], which showed an increase in the tolerance toward zinc, copper, cadmium, iron, and mercury with the addition of sugar to the culture medium, as a stimulating agent for EPS synthesis. When the study on bacterial EPSs is approached with a final aim related to such bioremediation purposes, the use of filter-feeding organisms could be a powerful incentive for the discovery of new producers or new compounds, as favored by the accumulation of contaminants and the consequent possible establishment of bacterial communities that are specialized in their removal.

Bacterial EPSs are proved to also act by promoting or inhibiting the biofilm formation. The EPS produced by a sponge associated *Bacillus licheniformis* strain showed an interesting reductive action of the initial adhesion and biofilm development of *Escherichia coli* PHL628 and *Pseudomonas fluorescens*, with a quorum sensing independent mechanism [185]. The authors retained that the EPSs act as interfering agents on the cell-surface or cell-cell interactions, or with other processes at the base of biofilm formation, generally in force of a highly anionic structure (that in the sponge associated *Bacillus licheniformis* is granted by the presence of phosphate groups) that modulates these types of interactions. Many marine invertebrates have gained interest in the research of polymers with such capacities because they are able to maintain a clean surface, by developing several strategies to protect themselves from pathogens, predators or general competitors [205]. Very often, these strategies consist

in the production of bioactive metabolites, such as polysaccharides, and even if for a long time their production was attributed directly to the organisms, recently it was thought that it is to be attributed to associated microorganisms.

Priyanka et al. [184] investigated the role of EPSs from symbionts in antioxidant and radical scavenging activities, and highlighted the really promising performance for compounds produced by three associated bacteria, with regard for *Enterobacter* sp. PRIM-26, which showed higher antioxidant activities in terms of superoxide (IC_{50} 0.33 mg·mL^{-1}) and 1,1-diphenyl-2-picrylhydrazyl (DPPH, IC_{50} 0.44 mg·mL^{-1}) radical scavenging. This property falls in the defensive role of associated bacteria towards their host, as the free radicals' accumulation is source of damages for them, by causing many degenerative diseases, like atherosclerosis, cancer, inflammatory joint disease, asthma, diabetes, senile dementia, and degeneration of eyes [206]. In addition to this, the authors reported also a strong biofilm formation by the isolates, thus underlying their functional role in the survival of hosts, by increasing nutrient availability [207].

4.3. Biomedicals

The approaching to the study of interaction between microorganisms and marine invertebrates, and to the possible deriving bioactive molecules, was firstly stimulated by the need of new compounds with properties that are easily exploitable in medical fields. As previously specified here, marine invertebrates have been explored as direct source of bioactive metabolites, thus resulting in the discovery of many novel compounds, as in the case of bryostatins, halichondrins, and other antitumoral or anti-inflammatory active substances [208]. Antibacterial and antifouling activities have been found in several surface-associated bacteria, as suggested by previous studies on bacteria that are associated with sponges, corals, jellyfish, sea-anemones and seaweeds [209–211], and the new bioactive metabolites detected [212,213]. Even in the case of antimicrobial compounds, they are preferred to the synthetic counterparts because of their stability, activity at low-temperature and specificity of action. The promising results have led to increase more and more the interest towards such compounds, representing an important tool for the health care and industrial processing [214].

The most common pipeline in the investigations on biomedicals from marine associated bacteria usually includes the isolation of them from the organism and the phylogenetical characterization as a first step. After that, several screening tests are widely recognized and are used as reliable and safety tools for detection of drug-producing bacteria. This is particularly true for the agar overlay method [4] and the cross-streak method [118], which are the most common tests that are performed directly with the isolates of interest on solid medium. The assessment of a preliminary screening is a useful passage to focus on the subsequent analysis only on positive strains, allowing for an optimization of time and costs. Interestingly, some researchers hypothized the possible occurrence of volatile antimicrobial compounds (VOCs), and concentrated their attention on the improvement of the knowledge about them by devising a specific methodology for their detection and investigation [118]. Generally, the primary screening is followed by a secondary screening, dedicated to the extracts that were obtained from liquid cultures of promising strains. After extraction and purification steps, the extracts are generally assayed by the disc diffusion susceptibility test [120,121,123], and finally the minimal inhibitory concentration (MIC) detection is performed for a better comprehension of the extracts efficacy [123,214].

Shankar et al. [48] investigated the production of bioactive polymers by two epibiotic bacteria associated with the polychaetes *Platynereis dumerilii* and *Syllis* sp., and affiliated to the genera *Exiguobacterium* and *Actinobacterium*. The extracts exhibited important antibacterial and antibiofilm activity against bacterial pathogens, with inhibition zones ranging from 8 mm to 15 mm and higher activity against *Alteromonas* sp. target.

A contribution of undoubted scientific value is that of Papaleo et al. [118], who screened microorganisms that were associated to Antarctic sponges for the production of new natural polymers active against Cystic Fibrosis infections. They investigated a set of 132 bacterial strains that were

isolated from three sponge species (*Haliclonissa verrucosa*, *Anoxycalyx joubini*, and *Lissodendoryx nobilis*), and revealed that most of the isolates inhibited completely the growth of a pathogen strain, which is strongly involved in pathogenicity of cystic fibrosis, *Burkholderia cepacia*. The data presented by the authors are the result of an innovative approach in the research for molecules with antimicrobial activities, because it evidenced the volatile nature of the compounds, as revealed by the Solid Phase Micro Extraction gas chromatography mass spectrometric analysis. Moreover, the authors strongly suggested a specific activity against opportunistic pathogens belonging to the *Burkholderia cepacia* complex, with a higher activity than most of the commonly used antibiotics. The antibacterial compounds also revealed a good thermo-stability, suggesting their potential successful employement. The comparison between the efficacy of new compounds and that of common use antibiotics is a key parameter for the investigations in this field, because thus is the core aspect that could make them potential alternatives to the synthetic counterparts.

Nithyanand and coauthors investigated the bacterial communities associated to the mucus and tissue of the coral *Acropora digitifera*, and in 2009 [120] reported for the first time the antibacterial potential of its associated actinobacteria. After that, in 2010 [121], improved their investigations by screening coral-associated actinomycetes for antibiofilm activity against all of the biofilm forming M serotypes of *Streptococcus pyogenes*. The extracts were proved efficient in promoting the reduction of the cell surface hydrophobicity, an important parameter in the biofilm formation processes. Similar results have been provided for a coral associated *Bacillus horikoshii* [123], which inhibited biofilm formation of *Streptococcus pyogenes*, whose extracts resulted in really promising and qualitative, as active only against biofilm formation, without antibacterial action even at high concentrations. They reported a reduction up to 80% of biofilm formation at biofilm inhibitory concentration, and up to 40–60% at sub-biofilm inhibitory concentration, and also revealed an inhibition of the quorum-sensing, which is a base process for the biofilm formation. These findings are of scientific relevance, since bacterial adhesion on solid surfaces and the biofilm formation still represent problems of high resonance in aquatic environments. Indeed, the biofilm occurrence on abiotic and biotic surfaces is considered as a favorable condition for the development of infectious diseases, and more dangerously could be the causative agent in the appearance of new pathogens, trough horizontal transfer of genetic elements.

Many authors highlighted that microorganisms that were associated with marine organisms are a poorly explored matrix, but need to be more intensively screened because potent possible source of bioactive molecules, drugs, and antifouling compounds. Sponges are obviously the most employed source of bacterial producers, and led to many important discoveries. Antimicrobial activity has been reported for extracts from strains that are associated to *Dendrilla nigra* [215], *Axinella donnani*, and *Clathria gorgonoides* [216]. Moreover, also therapeutic applications have been improved, and many sponge endosymbionts have been proved able to produce cytotoxic compounds against several forms of carcinoma [217]. An interesting approach in the study of bioactive molecules from associated bacteria is the detection and amplification of specific genes, which are involved in the biosynthetic pathways, as recently reported by Brinkmann et al. [2], for the production of antimicrobial compounds by bacteria associated with the sponges *Candidaspongia flabellata* and *Rhopaloeides odorabile*.

The difficulties in the research for new drugs lies in in uncertainty about the attribution of the productive role and in the low quantity of biomolecules in the organism's tissues, so that obtaining enough amount of the polymers should need an invasive operation on the biotic benthic communities, with a destroying effect on them. Costa Leal et al. [218] suggested the *in toto* aquaculture strategy as possible innovative and resolutive approach, by culturing microorganisms-invertebrate assemblages in order to ensure a good availability of biomass that is suitable for the drug discovery. The authors retain that this could represent an optimal initial step for the drug discovery pipeline, and recommend a synergistic integration of auqaculture practices with culture-dependent and culture-independent methods.

Although the medical fields include the most important challenges in the research, also here there is the need of improvements and further analysis. Anyway, the use of associated bacteria seems to

be very promising also in this topic, especially to overcome the problem concerning the isolation and large-scale production of active metabolites.

5. Concluding Remarks

The interest in discovery new compounds of natural origin answers to an urgent need, dictated by the necessity to find adequate alternatives to synthetic molecules, widespreadly used during the last decades, sometimes in an excessive measure. Thus, the efforts of the researchers have been concentrated on bioprospecting, therefore the exploitation of biological material for searching commercially useful biologically active compounds. The marine habitats offer a strong potential in terms of biodiversity, which could correspond to a wide chemodiversity. The biotechnological use of resources that are offered from the marine environment is a relatively modern issue, also in comparison with terrestrial bioprospecting. It has been recognized that the chemical diversity of microbial compounds is the broadest one detected [219], even if the biotechnological development could be considered to be young [220].

The search for new metabolites started by exploiting several natural matrices for the isolation of bacterial producers, such as sediment, water, ice, snow, and so on. More peculiar, rich or extreme the habitats are, more easily their inhabiting microorganisms have been stimulated to produce active molecules. Anyway, the exploration for new bioactive metabolites has been focused not only on microbial production, but also investigated marine invertebrates, many of which are interesting because there are not their terrestrial counterparts, and whose adaptive strategies include the production of metabolites with defensive functions [221,222]. A lot of antimicrobial peptides have been isolated and detected from different taxa of invertebrates, i.e., porifera, anellida, mollusca, crustacea, chelicerata, Echinodermata, and so on (see references in Smith et al. [222].

In addition to the focusing on the sole microorganisms or invertebrates, a new perspective has gained interest during recent years. Indeed, the associations between different organisms represent currently a new potent resource. As it is here reviewed, the literature regarding marine invertebrates and their associated bacteria presents different levels of fragmentation in dependence of the taxa, because a lot of observations and investigations are available concerning some categories, i.e., sponge and cnidaria groups, while others are still unexplored, i.e., polychaetes. Many extracts that were obtained from marine invertebrates are proved to be the result of associated bacteria biosynthesis [223,224]. This finding constitutes an intriguing opportunity to obtain optimal amount of bioactive compounds by establishing symbionts in culture, or by manipulating genes that are involved in biosynthesis reactions [208]. The direct culturing of the microbial producers would provide a more suitable amount of bioactive compounds, and overcome the troubles deriving from harvesting marine organisms, by threatening their biodiversity. In this review, we outline the currently available improvements about the BS and EPS production from marine invertebrate associated bacteria, and pinpoint that each biological matrix could be suitable and efficient to achieve the goal, by treating the marine benthic members most used in more recent researches. The searching for these polymers could grant the possible exploitation for more than one purpose, if supported by an appropriate chemical analysis of the structure and the detection of active fractions.

Moreover, the present review represents an attempt to highlight future research directions, in order to enrich the existing available observations and to answer to the still remaining unresolved questions. On the base of actual knowledge, there is not the base to designate a marine invertebrate as model of study. Or better, surely benthic organisms which hosts complex microbial communities are more indicated for these types of investigations because should provide a higher level of biodiversity and thus a major possibility to obtain bacterial producers and new metabolites. Moreover, if extreme habitats have been proved as suitable sources of specialized microorganisms doted of complex enzymatic equipments, the benthic communities inhabiting them should host as such as complex associated bacterial communities with also a greater biotechnological potential. In conclusion, bacteria associated with marine invertebrates should be deeply explored as an optimal source for the discovery

of new and more functional compounds. As extensively treated, we can assume now that each microorganism could produce a molecule with its own peculiarities, which is different from all the others and in dependence of many external variables. Therefore, the optimization of culture conditions could enhance the yield and the economical practicability of large-scale production.

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