Chapter

Essential Oils and Microbial Communication

Filomena Nazzaro, Florinda Fratianni, Antonio d'Acierno, Raffaele Coppola, Fernando Jesus Ayala-Zavala, Adriano Gomez da Cruz and Vincenzo De Feo

Abstract

The World Health Organization highlighted the increase in the resistance to conventional antibiotics for most pathogens and observed also a decrease of the threshold for all mechanisms of cell-cell microbial communication, leading to the formation of biofilms and to the increase of microbial pathogenicity. Scientific community is therefore oriented to the identification and study of alternative substances to antibiotics. In such context, substances of vegetal source, such as essential oils (EOs), always used in traditional medicine, stimulated—particularly in recent decades—the scientific world to discover and identify substances, intended as a mixture or single components capable to fight pathogenic microorganisms. From this point of view, the study of plants is very interesting and offers many interesting ideas and results. This brief chapter describes the basis of the microbial communication, until the formation of biofilm, and some mechanisms through which essential oils, or some of their main components, may decrease or inactivate the complex mechanisms that lead to pathogenicity, both of prokaryotes and eukaryotes.

Keywords: bacterial resistance, quorum sensing, biofilm, essential oils

1. Bacterial resistance

In recent years, the World Health Organization repeatedly highlighted with alarm the problem of an increase in the resistance of most pathogens to conventional antibiotics. Several causes determined such alarming picture, not least the lack of availability on the market of "new" molecules (given the low economic appealing that such a study raises on the pharmaceutical industries). Wrong behaviors on the part of man are also included among the recurring causes, such as an unjustified abuse of antibiotics [1], as well as possible incorrect medical prescriptions of the drug or the duration of antibiotic treatment. An extensive and indiscriminate use of antibiotics also in agriculture and livestock breeding can cause an indirect contribution to antibiotic resistance also in humans indeed. The often inconsiderate use of broad-spectrum antibiotics could indiscriminately reduce also the number of the so-called "commensal" microorganisms, favoring the onset of diseases more serious than those for which the use of the drug was initially required, varying and consequently altering the relationship between

microorganism and host. Microorganisms that generally do not cause diseases in their natural habitats, due to this new environmental situation, can become highly pathogenic. Normal constituents of the intestinal flora, such as *Escherichia coli*, may therefore become harmful in other districts, such as the urinary bladder, spinal cord, lungs, etc. Other microorganisms can become highly pathogenic under certain conditions: for instance, *Streptococcus viridans* physiologically present in the oropharyngeal tract, in some circumstances can invade different organs through the bloodstream, causing serious diseases (e.g., bacterial endocarditis). Today, there is much talk about the so-called "multidrug-resistant" (MDR), "extensively drug-resistant" (XDR), and "pan-drug-resistant" (PDR) strains. Such microorganisms can be figuratively enclosed in a cluster comprising pathogens of infections that are today intractable [2]. Unfortunately, it is also difficult to fight those pathogens belonging to the so-called "ESKAPE" group, an acronym comprising the microbial species *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. It is widely believed by the scientific community that the study of alternative strategies to the use of conventional antibiotics could represent an important way to be taken into consideration, to combat this dangerous situation. The use of bacteriophages in phage therapies, known for their high specificity, the development of new vaccines against *P. aeruginosa* [3] and *A. baumannii* [4], and the use of strategies to inhibit the bacterial virulence factors can be considered some of the solutions. Recently, research also focused on the exploitation and identification of new microorganisms, isolated, for example, from the ground, enabling to block the microbial growth of one or more species belonging to the ESKAPE group [5].

2. Mechanisms of cell-cell communication

Multicellular organisms are composed by a rigidly regulated society of individual cells, organized into tissues and organs, which all together collaborate for the functioning of the individual and whose final "purpose," from the biological point of view, is to reproduce (or to allow to the reproduction of a similar genome). The coordinated work of the different cell types that leads to the formation of an adult individual, as well as the cell growth, differentiation, and organogenesis giving rise from a single fertilized cell, requires sophisticated signaling mechanisms. Thus, in the course of evolution, molecular messengers were generated, synthesized, and released in some part of the organism and then specifically recognized by the respective receptors expressed in the target cells. Complex molecular machines were simultaneously selected to transduce the activated receptor signal. While generally the term "extracellular messengers" involves those intercellular communication mechanisms taking place within a multicellular organism, it should be emphasized that even unicellular microorganisms are capable of "social" behaviors that require a coordinated response. This sophisticated cell-to-cell process of communication between microorganisms, the so-called quorum sensing (QS), consists in the synthesis by bacteria, both Gram-positive and Gram-negative, of specific molecules, which are called "autoinducers" or "bacterial pheromones." After production of such molecules, bacteria release them into the extracellular medium to be detected by specific receptors/transducers. Quorum sensing is an extremely important communication system for microorganisms. Through this system, bacteria are in fact capable to measure their concentration and to modulate gene expression in response to population density, which lead to the secretion of virulence factors, biofilm formation, competence, and bioluminescence [6, 7]. When bacteria that generate signals are in close proximity to each other, the concentration of their

QS signal amplifies. This event leads to a boost of the binding of the QS signal to specific receptor proteins, to a consequent activation of the specific receptor, and to the enhanced gene transcription with appropriate promoter sequences. QSs give to bacteria a great evolutionary advantage, allowing them to adapt to the change of the environment. Some authors propose these as neo-Darwinian mechanisms of evolution, which had an important function in the arrival of the first multicellular organisms [8]. The result of this "bacterial communication" can be represented by an increase in virulence (e.g., *Staphylococcus aureus*), by the formation of a biofilm (e.g., *Pseudomonas aeruginosa*), by sporulation, etc. To date, more than 100 different autoinducers are known for bacteria, archaea, and fungi.

Bacteria exhibit two main QS mechanisms, based on distinct signaling pathways, which present a certain analogy with the mechanisms found also in multicellular organisms. The first, generally used by Gram-negative bacteria, is based on the synthesis of a family of small molecules, the so-called AHL (acylated homoserine lactones). These molecules have a similar central structure and differ only in the length of a side chain, which specificity is determined by the length of the acyl chain and the substitution (—H,—OH or =O) on carbon. Generally, every type of bacterium can produce at least one AHL type; however, it can happen that bacteria produce more than one of them. Due to their chemical characteristics, AHL are capable to cross the bacterial membrane and spread outside; in addition, from the extracellular medium, they can freely enter within the cell and bind to specific receptors, called LuxR because the QS phenomenon was described for the first time in a microorganism, *Aliivibrio fischeri*, which is able to emit light *in vitro* only when its concentration exceeds a certain threshold [9]. In this microorganism, the AHL autoinducer is at low concentration and does not induce bioluminescence; when the bacterium is in the luminous organ of the giant squid, its cell density is high, so the transcriptional activator reaches the DNA, binds to the recognition sequence (LuxBox), and activates the transcription of genes for the enzyme luciferase, which produces bioluminescence. The advantage of this mechanism is to save energy, to ensure that bacteria become luminescent only when they are present in large numbers, and to prevent them from wasting energy when the population is toosmall to emit a visible signal. The complex AHL-LuxR in turn binds to the bacterial DNA, thus regulating the transcription of specific genes. In a situation where the concentration of bacteria is low, the level of synthesized AHL is below the threshold required for the LuxR bond. However, as the concentration of bacteria increases, the amount of AHL also increases, and the AHL-LuxR complex is formed accordingly. A gene encoding for the enzyme catalysing the AHL synthesis is present within the genes induced by the AHL-LuxR complex. This gives rise to a positive feedback leading to a rapid and synchronous answer from the whole microbial population. Signal transduction through the AHL-LuxR system, based on an extracellular messenger able to cross the membrane and on a receptor that is also a transcription factor, can be thought to obey the same logic of signaling through steroid hormones. On the other hand, Gram-positive bacteria use autoinducer molecules formed by peptides with a variable length ranging between 5 and 17 amino acids. Such molecules are produced by the processing of precursors and are often subjected to posttranslational modifications. These peptides require special transporters to be secreted in the extracellular environment that, in turn, is detected by a sensor histidine kinase [10] and transduces the signal through phosphorylation of intracellular targets. This mechanism of action is therefore similar to that used by growth factors in multicellular organisms. Not always, the nature of quorum sensing molecules (QSMs) is peptidic: for instance, some Gram-positive bacteria, such as *Streptomyces*, produce ɣ-butyrolactones as QSMs [11]. Finally, different researches report the autoinducer 2 (AI-2), with a rather unusual cyclic boronic ester, as a QS

system common to both Gram-positive and Gram-negative bacteria, although its role as a true QSM has been doubted for some microorganisms [12–15]. This system might give rise to a family of molecules that are supposed to operate as a common language for most bacteria.

The production of the AHL involved in the QS mechanism was recently discovered also in several Gram-negative bacteria, such as *Roseobacter* sp. TB60 and *Psychrobacter* sp. TB67 associated with the Antarctic sponge, *Anoxycalyx joubini* [16], indicating this a certain "universality" of the system. Dong and Zhang [17] and McDougald and co-workers [18] demonstrated the existence of other two novel signaling pathways: hydroxyl-palmitic acid methyl ester and methyl-dodecanoic acid.

2.1 QS in eukaryotes

Some eukaryotic microorganisms monitor their population density through QS mechanisms [19, 20] too. This is not so surprising, taking into account that many bacteria and eukaryotic microorganisms inhabit in common ecological niches and often play similar challenges. In fungi, QS mechanisms are in charge to check and regulate processes such as sporulation and production of some molecules, such as secondary metabolites, as well as to those events giving rise to the morphological transition and enzyme secretion by the cells. Considering this and starting from the assumption that even this type of organisms is extremely varied, we can undoubtedly affirm that fungi exhibit different cell-cell communication mechanisms, using a wide variety of signal molecules [19]. Furthermore, fungi can communicate with bacteria and even with their plant or mammalian hosts. In yeasts and dimorphic fungi, aromatic alcohols originating from amino acids mediate the QS type of regulation [21]. Therefore, yeasts, through the production of tryptophol and phenylethyl alcohol, can manage the formation of pseudohyphae and biofilm [22] and probably trigger the virulence process toward some plants such as *Vitis vinifera* [23]. QSMs stimulate the exit from lag phase inducing germ-tube formation and hyphal development [24]. *Candida albicans* remains the most studied species from this point of view. It produces some QSMs, such as tyrosol, described also in other fungal species, such as *Saccharomyces cerevisiae* [25]. QSMs of *C. albicans* influence the formation and structure of biofilms [26, 27] as well as the dispersal of cells from a biofilm; hence, it, as well as other molecules, plays important roles in pathogenesis. E-farnesol, the other most known QSM produced by *C. albicans* [28], is an exogenous molecule that, on the contrary, inhibits biofilm formation when provided early during adherence; furthermore, it acts as an inhibitor of hyphal formation indeed [29]. Therefore, this organism can modulate its morphology (vegetative/hyphal) and, consequently, all events related, including the pathogenicity, through the modulating production mainly of these two QSMs. Dodecanol and γ-butyrolactone are other molecules identified as mediators of QS processes present in other eukaryotic organisms, such as the filamentous fungi, *Aspergillus* and *Penicillium* spp. Some species of *Penicillium*, such as *P. sclerotinum*, produce sclerotiorin, a secondary metabolite with antibiotic properties, and γ-butyrolactone-containing molecules such as multicolic acid, which act as QSMs [30]. Taking into account that Gram-negative bacteria produced lactones (AHLs) as QSMs and that filamentous fungi produce butyrolactone I [31], γ-heptalactone [32], and γ-butyrolactones [33], the discovery that γ-butyrolactones are produced also by the filamentous bacterium *Streptomyces* [11] suggests a convergent evolution or a horizontal gene transfer occurring during the evolution [30]. At the same time, different fungi, such as basidiomycete *Cryptococcus neoformans*, produce as QSMs some peptides, similar to how Gram-positive bacteria do. This means that fungi use a language "analogous" in some way to that exhibited by other phyla [10, 30].

In some species of *Aspergillus*, such as *A. flavus*, oxylipins were identified as QSMs: these molecules modulate both the morphological differentiation and the production of either asexual spores or sclerotia. Furthermore, oxylipins regulate a QS-dependent pathway controlling development and mycotoxin production [34]. Fungi produce also other QSMs: terpenes, such as farnesol, are produced, for instance, by the dimorphic fungi *C. albicans* [28] and *Ophiostoma piceae* [35]; cyclic sesquiterpenes act as QSMs for the dimorphic fungus *Ophiostoma floccosum* [36]; QS alcohols, including tryptophol and phenylethyl alcohol, are produced by *S. cerevisiae* [21]. It is important to underline that the higher organisms evolved mechanisms with which they are capable to interfere with the quorum sensing process of the bacteria. These mechanisms could play an important role both for peaceful cohabitation of human and microorganisms, such as the case of the intestinal flora, and in processes of resistance to pathogens.

2.2 Biofilm

The term "biofilm" is referred to a structure, enough complex, formed by microbial cells, associated with each other that attach to a surface, which are in a certain sense kept isolated from the external environment (although they exert an important influence on this) through the formation of a sort of "dome" of polysaccharide nature [37]. Biofilm has generally a three-dimensional structure: it contains more or less channels and pores, used as a sort of intercellular communication channel and for the maintenance of the entire bacterial community [38, 39]. Biofilm is thus one of the subsequent mechanisms giving rise from the communication among bacteria, which precisely through the formation of biofilm and other microbial behaviors and social exchange (not only bioluminescence, conjugation, and virulence but also motility, sporulation, competence, etc.) form the social microbial system of interaction, the QS (**Figure 1**) [40, 41], that prokaryotes and some eukaryotes developed many millions of years before the actual human social media, which is certainly more organized and complex. The system is so organized and evolved that allows microorganisms to easily adapt to adverse environmental conditions and to use them even to switch to counterattack, with an action of growth, proliferation, and change in their metabolic pathways and morphology. Biofilms allow the survival of bacterial cells in a hostile environment; the extremely

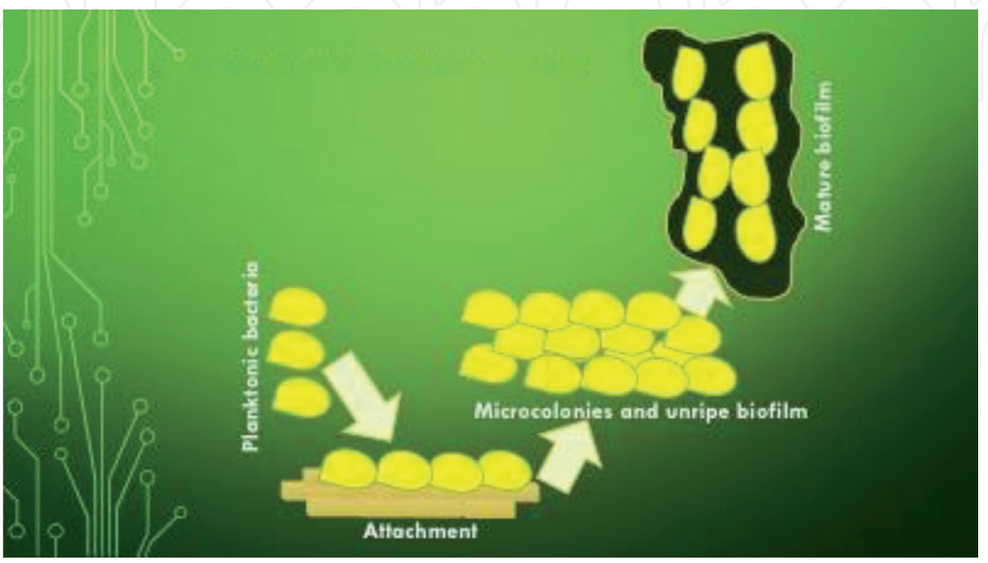

Figure 1.
Mechanism of biofilm formation.

complex structure and the metabolic and physiological heterogeneity that characterize them suggest an analogy between these communities and the tissues of higher organisms. Bacterial biofilms, not easily eradicated with the conventional antibiotic therapies, affect a large number of chronic bacterial infections. Biofilms represent a cohesive matrix of microorganisms and other cellular constituents that can be present in any natural environment; they are also characterized two pints by the ability to adhere to surfaces; by a structural heterogeneity; by a genetic diversity of the components; by complex interactions of communities, even mixed; and by an extracellular matrix of polymeric substances. At the end of the 1990s, it was ascertained that the so called planktonic growthis an artifact and that the type of growth prevalent in natural environments is sessile (fixed to a substrate). When nutrient intake is limited, biofilms tend to adhere to solid supports and remain stable at the solid/liquid interface, where nutrients are concentrated. Once adhered, the biofilms secrete exopolysaccharides that surround them, guaranteeing their cohesion to the support and between them. This creates biofilms, which in most cases are polymicrobial. Bacteria grow slowly inside the biofilm, forming microcolonies. The biofilms are mature when the growth reaches the point where most external cells come off, returning to the planktonic life and then starting the formation of new biofilms. The whole process takes from a few days to a few weeks. In mature biofilms, bacteria are present in different states, depending on the location: the innermost ones are metabolically less active, and the more external have metabolic characteristics similar to those of planktonic growth bacteria. At first, sessile growth attracted attention due to some negative effects of biofilm formation (corrosion of cables and submerged structures but also the dental plaque of many animals) and of the resistance of the bacteria included in the biofilm to the antimicrobials. Studies carried out with the confocal microscope have shown that the biofilm is highly organized within it, with channels through which the surrounding fluid circulates in the matrix carrying the nutrients and removing the toxic products. Maintaining a structure of this type requires complex mechanisms of cell-cell regulation and communication to prevent undifferentiated growth obstructing the canaliculi. The phenomenon of sessile growth has determined, in the last decade, the onset of new pathologies, linked to the colonization of prosthetic implants by bacterial biofilms [42]. Biofilms are present in the most diverse environments, e.g., in thermal springs or on the bottom of lakes and rivers, and can be used not only for the purification of water in an industrial environment but also for the removal of oil or other pollutants from contaminated marine areas. Moreover, it is now established that most bacterial species, when conditions allow it, modify their behavior to find true "microbial cities" in the form of biofilms. These include "fortification walls," consisting of a three-dimensional array of polymeric sugars, and "shipping channels" for the transport of nutrients and catabolites. Two main types of adhesion are involved in the formation of biofilm: *adhesion* of the bacterium to a solid substrate (supra inert) also by attacking host proteins and *intercellular adaption*, which determines the formation of multiple layers of the biofilm. In the biomedical field, biofilms are involved in a wide range of pathologies, involving cochlear, articular, orthopedic, etc. The sessile structures of which biofilms are endowed, the multi-species communities of which they are constituted, and the influence that the dynamics of fluid flows in which they are immersed exert on them are the factors that have contributed to considering biofilms as the core reefs of the microbial world. Obviously, the multicellularity of a biofilm translates into a better defense of microorganisms, contributing substantially to their survival. Nutrient depletion creates some areas of activity alteration; the outer cell layers of the biofilm contribute to the formation of a sort of barrier, capable to absorb external damage. The innermost microorganisms have the task, to some extent, to elaborate a response to

intrinsic stress. The biofilm bacteria are 10–10,000 times more resistant to antibiotic treatment than the planktonic phenotype. For this reason, biofilm infections show recurrent symptoms after cycles of antibiotic therapy. This persistence in the heart of the biofilm is linked to the presence of the so-called "sleeper" microorganisms, with low activity and which determine the phenomenon of persistence.

Biofilm adapts to environmental fluctuations, such as temperatures, pH variations, osmolarity, and nutrient availability through multiple gene expression; its resistance is not genotypic. Microbial cells contained within the biofilm are much more difficult to reach; moreover, they have the advantage, compared to the host organism, of being able to communicate outside the biofilm, through the previously indicated system of channels and pores. At the same time, it becomes more difficult for synthetic drugs to "break" the biofilm organization, just for how it is structured and how it is composed. After a certain threshold, bacteria change their life perspective, in the sense that they no longer act as a single cell, but as a component of a microbial team. Such community grows through the recruitment of other cells, which arrive there. In this manner, microbial colony can spread upward of the surface. At the beginning, this gives rise to the formation of small colonies and unripe biofilm. At the end of the process, the biofilm is ripe (**Figure 1**). The production of compounds such as exopolysaccharides determines the embedding of bacteria in a complex matter constituted also by nucleic acids, lipids, and proteins [43]. A so organized matrix supplies bacteria for several advantages: for instance, it can manage the flow of nutrients and protect bacteria against the action of antimicrobial substances and the host immune system, which encounter great difficulty in scratching the structure and organization of the biofilm matrix [44]. So, manipulation and inhibition of the QS system might open new scenarios and improve therapies for chronic bacterial diseases [45, 46], including even cancer [47, 48].

3. Essential oils

Several possible strategies could treat infections associated with biofilms: substances capable of destroying the biofilm matrix (e.g., dispersion B), substances capable of destroying resistant cells, quorum-quenching enzymes that interfere with the quorum sensing phenomenon, substances that cause self-destruction of the biofilm, and then, in particular, strategies to strengthen the action of antimicrobials. The treatment of biofilms with antibiotics often causes only partial killing, allowing the surviving bacteria, present in the depth of the biofilm, to act as a true nucleus of propulsion for the spread of the infection after the interruption of the antibiotic therapy. Antibiotics can be inactivated by the production of specific enzymes within the biofilm. In some extreme cases, even the sessile population must not be surgically removed from the body. Another aspect to take into consideration is the age of biofilm: the younger is the biofilm, the easier is its eradication.

The need to identify substances/active ingredients able to replace synthetic drugs in the fight against pathogens, in particular against those more resistant to conventional treatments, also directed research toward (or better to say, to the rediscovery of) the "natural world," source of bioactive compounds used by traditional medicine since ancient times. Moreover, these substances have always exhibited a great spectrum of action that can be considered of great benefit, also due to the chemical structural differences of the active compounds. In such context, substances of vegetal origin, such as essential oils, have always been successfully used in traditional medicine and stimulated, practically always but particularly in recent decades, the scientific world to discover and identify substances, intended as a mixture or as single components that are able to fight pathogenic microorganisms. From this point of view, the study of

plants is very interesting and offers many interesting ideas and results: the same kind of plant can provide a pool of substances with a wide and very diverse spectrum of action [49]. Within the same genus, in fact, there are species with a different chemical composition, which therefore can provide bioactive substances (hydroalcoholic fraction or essential oils) different for the qualitative and quantitative profile. Moreover, the same plant species can diversify and present a different chemical composition depending also on the environmental and climatic conditions in which it grows, the stage of maturity, and method of extraction. Essential oils are substances that appear liquid, aromatic, and limpid and are obtained from different portions of plants through different extraction procedures, such as crushing, distillation, fermentation, the so-called enfleurage, or the use of organic solvents. The International Organization for Standardization (ISO) (ISO/D1S9235.2) defines an essential oil as a product made by distillation with either water or steam or by mechanical processing or by dry distillation of natural materials. About 300 essential oils, within the more than 3000 known types, are available on the market. The antimicrobial and antifungal properties of essential oils have been known since ancient times; however, the first "scientific" demonstrations of this activity date back only to the 1950s, when both Guenther [50] and Boyle [51] described in detail the activity of natural preservatives exhibited by different essential oils derived from plants and spices. The increase in interest from the economic world has meant that, by increasing the research on these substances, other properties were discovered [52], among which, for example, those antivirals [53, 54]. Chemical characterization of essential oils, conducted through chromatographic approaches (GC and GC/MS), has allowed obtaining detailed information on their composition. Essential oils are generally formed by volatile substances, also called volatile organic compounds (VOCs), molecules characterized by a high lipophilicity and a high vapor pressure. Within each essential oil, one can identify one or more quantitatively more abundant molecules and a series (more or less numerous) of other molecules, sometimes present only in traces. Moreover, within the essential oils, other types of molecules can be identified, such as phenolic compounds [55], alkaloids, saponins, and sesquiterpenes, which contribute to the antimicrobial activity of the oil. Further than other properties, EOs protect the plant against some pathogenic microorganisms. Through their smell, they are capable of exercising repulsive action against insect or, concurrently, to attract others to favor the dispersion of pollens and seeds. The same smell can also negatively affect the appetite drive of some herbivores. Some essential oils are reported to be very effective allelopathic agents. Thus, EOs can play a role in mediating the interactions of plants with the environment in a way that, although improperly, we could almost define as similar (however in a certain way opposite) to that exhibited by QSMs that allow these last to communicate in microorganisms with each other and with the environment. Essential oils can be classified according to the chemical constituent contained in greater concentration. Following such criterion, we have, among others:

- EOs with a predominance of mono or sesquiterpene hydrocarbons (e.g., *Citrus*, *Juniperus* L)
- EOs with a prevalence of aldehydes (e.g., cinnamic aldehyde in *Cinnamomum verum*, benzoic aldehyde in *Prunus dulcis* var. *dulcis* and in var. *amara*)
- EOs containing predominantly alcohols (geraniol in *Geranium*, santalol in *Santalum album*, linalool in *Coriandrum sativum*)

- EOs with high content of ketones (carvone in *Carum carvi*, thujone in *Artemisia*, *Thuja*, and in *Salvia officinalis*)
- EOs with a predominant amount of phenols (eugenol in *Dianthus caryophyllus*, carvacrol in *Satureja* and in other Labiatae)
- EOs that have a prevalent content of sulfured compounds (bisulfide, allyl disulfide in *Allium*)
- EOs with a prevalent content in esters and alcohols (linalool and linalyl acetate in *Lavandula angustifolia*)
- EOs having predominantly peroxides (ascaridol in *Chenopodium*)

The composition and the relative differences among the EOs lead to different biological activities that EOs can exhibit [54]. This also means that some species of plants, which exhibit different chemotypes, are characterized by a different composition and rate among the EO components too, to lead a change in EO biological properties. Thus, the final effect of an EO against a specific pathogen can give rise from a synergistic mechanism of its components or from just a unique compound that, although present in less percentage, can enhance the antimicrobial activity of the entire EO. In general, plant EOs and their components have a broad spectrum of inhibitory activities both against Gram-positive and Gram-negative pathogens [56, 57]. Citronellol can exert a broad inhibitory activity against the formation of biofilm. In fact, it acts against the planktonic forms of different Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and Gram-negative species (*E.coli*, *Pseudomonas aeruginosa*), inhibiting their capability to form biofilm when used at percentage ranging between 3.5% and 7% wt [58, 59]. However, the antibacterial effectiveness can vary depending not only on the EO but also on the bacteria. Therefore, some EOs, such as those of sandalwood (*Santalum album*), manuka (*Leptospermum scoparium*), and vetiver (*Chrysopogon zizanioides*), can act against some Gram-positive bacteria, but result ineffective against Gram-negative [60, 61]. Concurrently, sa microorganism can be more or less to the activity of different EOs. Thus, *Cymbopogon citratus* (lemon grass), *Syzygium aromaticum* (clove), and *Laurus nobilis* (bay laurel) EOs as well as *Thymus vulgaris* (thyme), *Rosmarinus officinalis* (rosemary), and *Mentha piperita* (peppermint) ones are capable to act against *St. aureus* at concentrations of $\leq$0.05%. On the other hand, *Ocimum basilicum* (basil) and *Eucalyptus globulus* (eucalyptus) EOs exhibit the same level of activity against this microorganism only if used at 1% concentration [60–62]. Different types of cardamom EOs affect differently the growth, the Qs, and the capability to form biofilm of several bacteria [63]. Thyme, *Origanum vulgare* (oregano), *Melaleuca alternifolia* (tea tree), *Cinnamomum verum* (cinnamon), lemon grass, bay laurel, *Backhousia citriodora* (lemon myrtle), clove, and *Aniba rosaeodora* (rosewood) EOs result the most active antimicrobials, at concentration and MICs also less than 1% [60, 64]. Most of these EOs, in particular bay laurel, clove, lemon grass, oregano, and thyme oils, inhibit growth of *E.coli* at concentrations of 0.02, 0.04, 0.06, 0.05, and 0.05%, respectively. In few cases, a major constituent molecule could exhibit a more effective activity compared to the EO. For example, carvacrol and eugenol, present in clove EO or terpinen-4-ol, which is the main component of tea tree EO, can show greater efficacy than the relative oil. This highlights also that, in the study of a biological activity of an EO, it is important to provide also the chemical composition, so to best argue about [65, 66].

4. Mechanisms of essential oils on microorganisms

As above indicated, the mechanisms which allow EOs to damage bacteria are largely dependent on their composition. Usually, antimicrobial activity can originate from a flow of reactions implicating the total bacterial cell; this is essentially due to the fact that, since the EOs are composed of many groups of chemical compounds, these last act in different ways [30]. Generally, Gram-positive bacteria and Gram-negative bacteria are differently susceptible to the action of EOs, due to the structural differences of their cell wall of these two groups of bacteria. The higher susceptibility of Gram-positive bacteria is caused mainly by the presence of peptidoglycan within their cell wall, which allows more easily the hydrophobic molecules to have access within the cell, acting therein with cytoplasm [30]. The cell wall of Gram-negative bacteria shows an outer membrane, composed of a double layer of phospholipids linked to the peptidoglycan layer by lipopolysaccharides. This allows these bacteria to exhibit greater resistance to the penetration of essential oils and/or their components; in fact, some hydrophobic molecules can be capable to enter into the cell, only through the access given by the porins, proteins that form water-filled channels distributed all over the cell wall. The different compositions of cell wall let it that Gram-negative bacteria are even more resistant to hydrophobic antibiotics [30, 67]. The mechanism through which the EOs or their components act on microbial cell is well known: it includes one or more simultaneous actions, ranging from cell wall degradation, to the damage caused to the cytoplasmic membrane and membrane proteins, as well as to a reduction of the proton-motive force until to damage to the ATP synthesis mechanism. Lipophilic character of EO compounds allows them to penetrate the cell membrane and remain between the phospholipids and/or affect the synthesis of membrane lipids, with a consequent change of membrane structure and with an alteration of its permeability. In addition, EOs can affect directly also the morphology of bacterial cell, altering it even irreversibly, to cause the complete destruction of the entire microbial cell scaffolding [30, 68] (**Figure 2**).

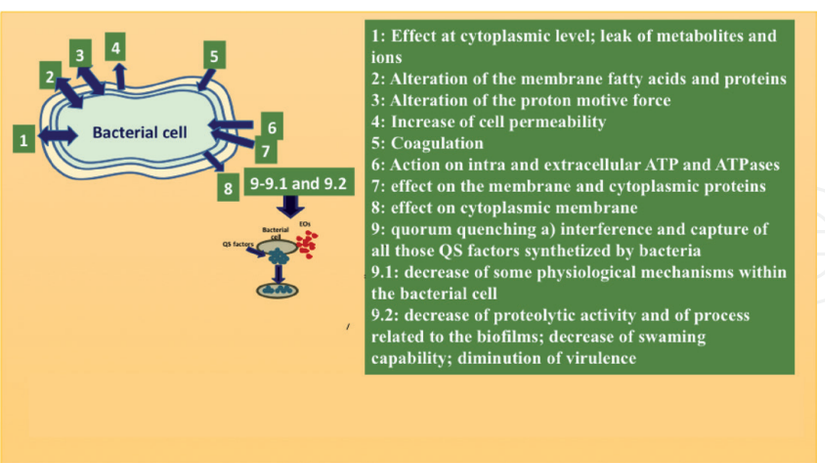

Figure 2.
Effect of essential oils on microbial cells (modified from [65]).

5. The action of the essential oils on quorum sensing system and biofilm formation

EOs can act also on QS systems that coordinate the whole system of pathogenicity of bacteria [30, 69] (**Figure 2**). This property is of noticeable interest, due to the continuous research for new therapeutic and antibacterial agents, which could

concurrently act in no toxic manner and without encouraging the development and emergence of resistant bacterial strains [45]. EOs can work on one or more events regulating the entire quorum sensing activity of microorganisms. Summarily, bacterial QS may be inhibited through different mechanisms. Their action against Gram-negative bacteria can be mainly expressed in three basic steps: a first step can block the "upstream" mechanism through the synthesis of AHL; a second mechanism may act further downstream, blocking the AHL transport and/or secretion. If bacteria still manage to produce AHLs and these molecules are still transported and secreted outside, other EO or their components could in any case be able to "capture" these molecules, effectively preventing cell-cell communication between bacteria. Other EOs can therefore act by exhibiting an antagonistic action with respect to AHLs or operating an inhibitory effect downstream of AHL receptor binding [49]. The versatility of action of EOs depends essentially on their chemical composition and the presence of functional groups. EOs containing largely terpenes (*p*-cymene, limonene, terpinene, sabinene, and pinenes) as well as some oxygenated components (for instance, camphor and camphone, borneol and bornyl acetate, 1,8 cineole, α-pinene, and verbenonone) generally do not exhibit a so strong antibacterial activity, which is just more manifest against Gram-positive bacteria. Further than the composition, the antimicrobial activity of EOs is also due to their concentration. In fact, depending on such element, EOs or their components can operate in a different manner on one or more factors that affect the mechanisms of cell-cell communication among bacteria. Thus, some EOs, also at low concentration, are capable to impede the chemical activity of those enzymes involved in the production of energy for the survival and growth of bacteria or, at higher concentration, even to disaggregate and denature microbial proteins [30, 70]. Subinhibitory concentrations of clove EO, tested on *P. aeruginosa* and *Aeromonas hydrophila*, were capable to significantly reduce the las- and rhl-regulated virulence factors such as LasB, total protease, chitinase and pyocyanin production, swimming motility, and exopolysaccharide production. The biofilm-forming capability of these two strains was also reduced in a concentration-dependent manner at all tested sub-MIC values [71]. Peppermint EO at sub-minimum inhibitory concentrations (sub-MICs) strongly can interfere with the production of AHL-regulated virulence factors and biofilm formation in *P. aeruginosa* and *A. hydrophila*. Such effect is mainly due to the presence of menthol, which interferes with QS systems of various Gram-negative pathogens, acting essentially on the las and pqs QS systems [72]. Different bacterial strains are used to test the potential inhibitory effect of essential oils on QS. Apart from the well-known models (*Vibrio harveyi*, *P. aeruginosa*, *S. aureus*, *E. coli*) more recently *Chromobacter violaceum*, in particular the mutant strain CV026, has been also used with this scope. This strain can provide, through the production or not of its purple pigment violacein, directly linked to QS, useful information about the capability of a substance to act or do not act as quorum-quenching agent, respectively. Through the use of such approach, Szabo and co-workers [73] studying several EOs ascertained that, among some EOs, rose, geranium, lavender, and rosemary EOs were the most potent QS inhibitors. Eucalyptus and citrus oils were moderately active, while the chamomile, orange, and juniper EOs which did not show any were ineffective. In several cases, the synergistic effect of more components can enhance the capability of an EO to inhibit the mechanism of communication among bacteria. Khan and co-workers evaluated the capability of different EOs and of their main components to act as quorum-quenching agents. Their study evidenced that clove essential oil showed promising anti-QS activity, followed in activity by cinnamon, lavender, and peppermint oils, and that eugenol, the major constituent of clove oil, could not exhibit anti-QS activity [74]. In other cases, the effectiveness of EOs is related both

to their composition and to the bacterium of reference; thus, an EO can act as mixture, better than a singular component on a specific bacterium; therefore, one or more components can act better than parent EOs against another bacterium. The effect of clary sage, juniper, lemon, and marjoram EOs and their major components on the formation of bacterial and yeast biofilms and on the inhibition of AHL-mediated QS, evaluated using *Bacillus cereus*, *Pichia anomala*, *Pseudomonas putida*, and a mix of bacteria containing also *E. coli*, demonstrated that marjoram EO inhibited all these tester strains. However, all components exhibited more strength in limiting the biofilm capacity of *B. cereus* than the parent EOs. Lemon EO was capable to inhibit *E. coli* and mixed-culture biofilms; on the other hand, cinnamon was effective against the mixed forms [75]. Conversely, the entire EO of tangerine (*Citrus reticulata*) is capable to inhibit the *P. aeruginosa* biofilm formation more than its main component limonene, by an inhibition of the QS autoinducer production and elastase activity [76]. This also highlights how, within a same genus, not all the species show the same biological activity. Thus, the EO of *C. reticulata* (tangerine) can be more active in inhibiting the QS system; on the other hand, the EO recovered from orange (*Citrus sinensis*) can be completely ineffective [73]. Some terpenoids, for example, thymol, carvacrol, linalool, menthol, geraniol, linalyl acetate, citronellal, and piperitone, have antibacterial activity mediated by their functional group. Carvacrol is one of the most active components present in different EOs, in particular from Labiatae. Its spectrum of activity is much wide. At sublethal concentrations (<0.5 mM), it is capable to inhibit the formation of biofilms of *C. violaceum*, *Salmonella enterica* subsp. *typhimurium*, and *S. aureus*, while it does not exhibit effects on the formation of *P. aeruginosa* biofilms. In all cases, this concentration seems to not have effects on total bacterial numbers, indicating that carvacrol bactericidal effect could not be also linked to its inhibitory effect on biofilm formation. Sub-MIC concentrations of carvacrol could reduce the expression of cviI (a gene coding for the N-acyl-L-homoserine lactone synthase) and decrease the production of violacein and the activity of chitinase (both regulated by quorum sensing) at concentrations coinciding with carvacrol's inhibiting effect on biofilm formation. These results indicate that carvacrol activity in inhibition of biofilm formation might be also related to the disruption of quorum sensing [77]. Thymol, one of the main constituents of *Thymus vulgaris* EO, can affect (at the same manner of the parent EO) not only the AHL production (acting thus in the blockage of the communication system among bacteria), but it also can suppress flagella gene transcription (reducing the mRNA level of flagella gene), the bacterial motility, and finally the formation of biofilm [78]. Cinnamaldehyde, another widely diffused component, present, for example, in cinnamon EO, can show different mechanisms of action. The use of 60 μM cinnamaldehyde can decrease down to 55% the bioluminescence of *V. harveyi BB886*, which is induced by *3*-hydroxy-C4-HSL, and from 60 to 100% that of *V. harvevi* BB170 (mediated by AI-2). This indicates, once again, that the activity of EOs, like all other phytochemicals, can be dependent even on the strain used within the same species [30, 65, 79, 80], further than on the QS molecule involved. Another study showed that cinnamaldehyde particularly directs its action toward the short-chain AHL synthase (RhlI) and inhibits AHL production by RhlI [81]. Also cinnamaldehyde analogs and derivatives are capable to inhibit AI-2-based QS system of *V. harveyi* in a dose-dependent manner [82] and are effective against AI-2-regulated QS of *Vibrio* spp. too [83]. Three other cinnamaldehyde analogs, *trans*-2-nonenal, *trans*-3-decen-2-one, and *trans*-3-nonen-2-one, can interfere with AI-2 QS in different manner. In *Vibrio* spp., *trans*-2-nonenal and *trans*-3-decen-2-one inhibit the AI-2-based QS system by reducing the DNA-binding ability of LuxR, causing a decrease in the production of QS-regulated virulence functions such as biofilm formation, matrix production, and protease

production [83]. Therefore, some compounds, such as *p*-anisaldehyde can act as AHL mimics, inhibiting the production of violacein by *C. violaceum* [84]. Eugenol inhibits QS in pathogenic bacteria; this was shown, for example, by Zhou and co-workers [85], evaluating the reduction of violacein production in *C. violaceum* after contact with eugenol. This molecule is also capable to affect lasB and pqsA in *E. coli*. This suggests an inhibitory action of eugenol on Las and pseudomonas quinolone signal (PQS)-controlled transcription. The action of eugenol on pathogenic bacteria at subinhibitory concentrations also considerably translates into a reduction in the QS-regulated production of some molecules/enzymes (elastase, protease, chitinase, pyocyanin, and exopolysaccharides) with a concurrently decreased formation of biofilm EPS in *P. aeruginosa* PAO1 [86]. In the Gram-positive pathogen, *S. aureus*, eugenol exhibited also antivirulence property acting on bacterial capability to produce exotoxin, through the repression of the agrA transcription [86]. Some EOs can effectively act both in preventing the biofilm formation and in disrupting the preformed biofilm. The EOs obtained from *Pogostemon heyneanus* and *Cinnamomum tamala* are capable to reduce the extracellular polymeric substance (EPS) and the synthesis of the two factors of the biofilm assemblage built by methicillin-resistant *S. aureus* (MRSA) strains. These EOs are also effective in reducing some virulence factors, such as staphyloxanthin and hemolysin. In silico docking studies demonstrated that (E)-nerolidol showed better binding affinity toward the enzyme dehydroxysqualene synthase of MRSA which is responsible for the synthesis of staphyloxanthin [87]. Different ratios between two components present in an EO can provide a different effectiveness of the EO as a QS inhibitor. Two among five EOs of *Lippia alba*, in particular one containing a greater prevalence of geranial/neral (the two isomers of the octa-2,6-dienal citral) and the other with an higher limonene/carvone content, were the most effective QS inhibitors and also had small effects on cell growth [88]. The activity of EOs on the cell-cell mechanism of communication could depend also on the chemical organization of one or some of their main components. The (+)-enantiomers of carvone, limonene, and borneol are potentially capable to increase the production of violacein and pyocyanin in *C. violaceum* and *P. aeruginosa*, respectively, while their levorotary analogs inhibit such production [84]. Among phenols present in the EOs, eugenol at subinhibitory concentrations is capable of inhibiting the production of virulence factors, involving production of violacein and pyocyanin, synthesis and expression of elastase, and finally the organization of the biofilm. In fact, using two *E. coli* biosensors, MG4/pKDT17 and pEAL08-2, Zhou and co-workers demonstrated that also this compound could act of one or more QS systems, in particular on las and pqs QS systems [85]. The process applied for the extraction of the EO may affect its biological activity indeed. The inhibitory effect of *Citrus medica* L. var. *sarcodactylis* EO obtained by hydrodistillation extraction (HE), microwave-assisted hydrodistillation extraction (MHE), and ultra-microwave-assisted hydro distillation extraction (UMHE) on biofilm formation by *S. aureus* and *S. typhimurium* was significantly higher than that of the essential oil obtained by standard extraction. This could also be related to the different chemical compositions of the different EOs, which elements can differ in terms of quality and rate [89]. Therefore, the diurnal variation can affect the chemistry of the essential oils, affecting their biological properties, including the capability to inhibit the biofilm [90]. New EOs are exhibiting interesting action against the formation of biofilm by microorganisms. *Cannabis sativa* EO is receiving particular attention because, further than other well-known properties, it showed a certain capability to attenuate the virulence of *Listeria monocytogenes* [91], with downregulation of flagella motility genes and of the regulatory gene prfA and a decreasing ability to form biofilm and to invade Caco-2 cells.

6. Action of EOs on biofilm of eukaryotes

More recently, the role of EOs and their components was studied for their potential capability to block the formation of biofilms in eukaryotes [64]. Terpenes are capable to inhibit the formation of biofilm through different mechanisms of action. Thymol, for instance, can affect the envelope of the planktonic form of *C. albicans*: it reaches to alter its membrane permeability [92] by infiltrating between the fatty acyl chains of the membrane lipid bilayers, with subsequent disruption of the lipid organization and damage to membrane fluidity. These events led to important alterations of yeast cell and can also reduce its adherence capability, which represents a major step in biofilm formation. Eugenol acts as a potent agent in blocking the biofilms associated with polystyrene too. Also in the case of *C. albicans*, the action of terpenes on the biofilm formation depends on the concentration of the compound used. Thus, a decrease of approximatively 50% of the metabolic pathway linked to biofilm is observed with 0.016% of carvacrol, geraniol, or thymol; however, a higher concentration is requested when we want to use citral, and even a percentage > or equal to 0.25% is necessary to decrease at 50% the biofilm of *C. albicans* if we want use 1,8-cineole, eugenol, farnesol, linalool, menthol, and α-terpinene [93]. Also using the same terpenes at the same concentration, the capability to inhibit the biofilm formation is related to the strain within the same species of the yeast [94], to the different species belonging to the same genus [95] or even to the genera considered [96]. It should not be overlooked the fact that much often, due to the different mechanisms of communication between bacteria, between fungi and among different bacteria and fungi, the own nature led to the formation of complex biofilm containing mixed population. Even, we can find such very frequent situation. In this case, it could be also more difficult to eradicate a biofilm, being the strength of more than a unique type of microorganisms against whom to combat. For instance, *C. albicans* may be associated with mixed infections of *Streptococcus mutans* to form plaque biofilms [97]. The chemical interaction between these two pathogens results in mixed biofilm development, mainly at oral level; therefore, there are no effective treatments in preventing or inhibiting the formation of mixed biofilms or in preventing inter-microbial communication. Eugenol, at sub-MICs, inhibits single and mixed biofilms of *C. albicans* and *S. mutans*. Also in this case, such capability cannot be expanded to every strain of *C. albicans* neither to all strains of *S. mutans*. In fact, Jafri and co-workers [98] ascertained that eugenol is effective against the biofilm formed by two of more than ten strains of *C. albicans* (in particular, the strain CAJ-01) and *S. mutans* (strain MTCC497), studied singularly on in mixed form, with a concurrent reduction of cell viability and a disruption of cell membrane.

7. New opportunities from the use of essential oils

In view of the importance of quorum sensing on the physiology regulation of microorganisms, research is also addressing toward the identification of new prospects for the use of essential oils in the blocking of cellular communication mechanisms. In recent years, the opportunity to associate essential oils or their main components with synthetic substances (drugs, enzymes, etc.) has been evaluated, to identify the associations that improve their performance in this sector. In particular, emerging resistance to last-resort antibiotics, such as carbapenems and polymixin B, led to theory of the so-called post-antibiotic era [99–101], and now research is moving to identify new compounds with stronger activity also against the most resistant pathogens. Different research groups are thus carrying a

screening of "no-drug compounds," including the EOs, in association with nonsteroidal anti-inflammatory drugs (the so-called NSAIDs) for inhibition of quorum sensing and biofilm formation in pathogens. Some NSAIDs, such as Z-phytol and lonazolac, can block the QS system of *Salmonella enteritidis*, acting as antagonists with respect to the AHLs [102]. The association of EOs with azolic drugs gives interesting challenges in the treatment of fungal infections determined by fungi particularly difficult to be eradicated [103]. Thymol in combination with the azolic drug, fluconazole, is capable to act against several species of *Candida* isolated from clinical specimens [104]. Chitosan nanoparticles containing miconazole and farnesol can inhibit *Candida* proliferation. The presence of farnesol is also capable of decreasing the pathogenicity of infection, demonstrated through the absence of inflammation [105]. The combination between the EO of *Cinnamomum tamala*, or its main component cinnamaldehyde, and linalool, with a commercial DNase I and marine bacterial DNase, might offer an alternative strategy to fight the biofilm formation and quorum sensing-mediated virulence factors in the aquaculture pathogen *Vibrio parahaemolyticus*, acting as effective food-preserving agent too [106]. The synergistic association between some EOs and DNase can decrease the biofilm-forming ability of *S. aureus* [87]. The association between EOs and/or their main components with conventional antibiotics can allow to eradicate also some particularly resistant biofilm, decreasing concurrently their resistance threshold. This allows also to minimize the antibiotic concentration, decreasing also its potential accompanying toxic side effects [107]. Synergistic interactions between EOs and their components with antibiotics are recognized, including several instances of antibiotic re-sensitization in resistant isolates, in support of this strategy to control antibiotic resistance, although synergistic effects are not well explored outside a preliminary identification of antibacterial interactions and mechanism of action is seldom defined, despite many hypotheses and recommendations for future studies [108]. The possibility of using essential oils has also been evaluated in the prevention of biofilm development by microorganisms (*Klebsiella*, *S. aureus*, *Staphylococcus haemolyticus*, *E. coli*, *Enterococcus cloacae*, *Enterococcus faecalis*), and the EO of *Matricaria chamomilla* (Asteraceae) could be considered as a good candidate, encouraging research related to the application of essential oils to fight diabetic complications [109]. The nanomaterials including EOs, at subinhibitory concentration, can inhibit QS and prevent biofilm formation and virulence development in pathogens [110]. The capability of blocking the quorum sensing mechanisms and biofilm formation by EOs can be combined with conventional drugs for a better treatment efficacy, as well as to design new more effective drugs, capable of acting also against those particularly resistant bacteria and fungi. The possibility to use nanotechnology, through the production of nano-vesicles containing EO, can result useful in a variety of applications including medical and pharmaceutical recipients and in home products for treating or preventing microbial colonization, as well as avoiding biofilm development [111, 112], also in food technology [113, 114]. Nano-coating with different inorganic and organic materials also supported by EOs proves to be particularly useful in the treatment of chronic wounds, such as venous or arterial ulcers, diabetic foot ulcers, pressure sores, and non-healing surgical wounds, all scenarios associated with chronic mono- or polymicrobial biofilm infections, formed by different bacteria, such as *S. aureus* and *P. aeruginosa*, followed by various species of *Enterobacteriaceae* such as *E. coli*, *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Morganella morganii*, *Citrobacter freundii*, *Serratia marcescens*, *Providencia* spp., *Enterococcus* spp., *Streptococcus* spp., and rarely *Corynebacterium* spp. or *Acinetobacter baumannii* [115]. In agro-food industry, the increasing demand for eco-friendly material stimulated the study of the elaboration of complex bio-nanocomposite films containing also EOs, such as that of rosemary,

which can act as effective eco-friendly nanocomposite films in packaging industries [116]. Algae are beginning the new frontier for the supplement of new bioactive compounds with antimicrobial activity and represent a unique opportunity for the science. Some investigations are exploring the therapeutic potential of algal extracts and their chemical groups, including terpenes, capable to have antimicrobial activity and to block the mechanism of communication between microorganisms. The alga *Pithophora oedogonium* targets *Staphylococcus* and *Salmonella*. The algae *Rivularia bullata*, *Nostoc spongiaeforme*, *Codium fragile*, *Colpomenia peregrina*, *Cystoseira barbata*, and *Zanardinia typus* have already demonstrated activity against many Gram-negative and Gram-positive bacteria [117, 118]. Finally, innovative techniques, such as the optical technique of bio-speckle [119] and the biofilm electrostatic test (BET) [120], will support the research in the near future to have a very fast scenario of EO biological activity. Speckle decorrelation can lead us to visualize the effect of essential oil on the decrease of the usual self-propelling movement of microorganisms taking place when they interact with coherent light. BET is as a simple, rapid, and highly reproducible tool for evaluating *in vitro* the ability of bacteria to form biofilms through electrostatic interaction with a pyro-electrified carrier and for ascertaining the impeding effect of an EO on the microbial capability to form biofilm in just 3 h.

8. Conclusion

Essential oils can represent a precious mine to fight pathogenic microorganisms, in particular to counteract the communication mechanisms that allow them to trigger those processes leading to their greater virulence and danger, in the hospital, environmental, and food sectors. However, *in vivo* studies remain crucial to evaluating the potential of this strategy as a mean to treat antibiotic-resistant infections, and profounder understanding of the mechanism of action is required. Finally, in the case of the association EOs-antibiotics, the preliminary evaluation of the *in vitro* toxicity of EO-antibiotic combinations is needed prior to in vivo studies indeed.

Conflict of interest

The authors declare no conflict of interest.

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Author details

Filomena Nazzaro[1*], Florinda Fratianni[1], Antonio d'Acierno[1], Raffaele Coppola[2], Fernando Jesus Ayala-Zavala[3], Adriano Gomez da Cruz[4] and Vincenzo De Feo[5]

1 Institute of Food Science, CNR-ISA, Avellino, Italy

2 DiAAA, Department of Agricultural, Environmental and Food Sciences, University of Molise, Campobasso, Italy

3 Center for Research in Nutrition and Development (CIAD AC), Hermosillo, Mexico

4 Department of Food Engineering, State University of Ponta Grossa (UEPG), Ponta Grossa, Brazil

5 Department of Pharmacy, University of Salerno, Fisciano (Salerno), Italy

*Address all correspondence to: filomena.nazzaro@cnr.it

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