The mode of antibacterial action of essential oils

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The antimicrobial activity of essential oils and their components has been recognized for a very long time. Essential oils (EOs) are made from a very complex mixture of volatile molecules that are produced by the secondary metabolism of aromatic and medicinal plants and can be obtained by distillation of different parts of plants. The large number of studies on the antimicrobial activity of EOs has allowed the scientific recognition of these compounds on the control of a wide range of microbial pathogens. The progresses made on the investigation of the mode of action of EOs against bacterial cell targets give us new perspectives to combat persistent and antimicrobial resistant bacterial pathogens. The recent investigation on the activity of EOs disruption of quorum sensing process is an excellent example. On the other hand proteomic analysis show that bacterial pathogens respond to EO sublethal doses using known mechanisms of adaptation to several environmental stress conditions.

Keywords essential oils; bacterial cell target

1. Introduction

The prevention of food borne disease has lead to the construction of sophisticated food safety and food control systems, mainly in developed countries, but world-wide, the majority of countries are aware that foodborne disease continues to be a major public health issue. Severe implications both on the health of individuals and on the development of modern society can arise from foodborne diseases. This fact is recognized by the World Health Organization (WHO) [1] and claims from each member state allow the development of systems to assure a real reduction in the burden of foodborne disease.

Since ancient times, the antimicrobial impact of essential oils and their components isolated from aromatic and medicinal plants, both on health and food preservation has been recognized. During the last decades these properties have been confirmed. The control of food spoilage and pathogenic microorganisms is achieved mainly by chemical control but the use of synthetic chemicals is limited due to a number of undesirable aspects which include carcinogenicity, teratogenicity, acute toxicity and the requirement of extended degradation periods with the consequent development of environmental pollution problems. The awareness of modern consumers about these problems has resulted in a "green" consumer profile that demands the absence of synthetic chemicals in food preservation accompanied by the requirement for extended shelf life in the majority of food products in conjunction with the scientific community and agro-industries and pharmaceutical industries to search for natural compounds that may satisfy consumer requests. Essential oils may be included in this group.

The use of essential oils (EOs) in food and food products, in addition to being recognized as GRAS practice (Generally Recognized As Safe) [2], must be applied with caution and firstly approved by food regulation organizations. This question is particularly sensitive due to different perceptions about toxic effects that most of the times are related with mass usage of the essential oils [3].

The usage of EO to combat hospital-acquired infections and to control epidemic multi-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* is promising [4,5]. The EO of eucalyptus, tea tree, thyme white, lavender, lemon, lemongrass, cinnamon, grapefruit, clove bud, sandalwood, peppermint, kunzea and sage oil were tested against several MRSA strains, *Streptococcus* and *Candida* strains and the EO of thyme white, lemon, lemongrass and cinnamon oil demonstrated to be effective against these problematic bacteria [4].

2. Essential oil composition

Essential oils are made from a very complex mixture of volatile molecules that are produced by the secondary metabolism of aromatic and medicinal plants and can be obtained by different methods, including the use of low or high pressure distillation of different parts of plants or the employment of liquid carbon dioxide or microwaves. Several factors influence the quality and quantity of the extracted product, in particular the soil composition, plant organ, vegetative cycle phase and climate [6-8]. EO composition can be differentiated in two component groups (examples in Fig 1). The main group has terpene and terpenoid origin and the second is constituted by aromatic and aliphatic components. Terpenes are the major group of plant natural products characterized by an extensive variety of structural types and the most valuable compounds [9]. Monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}) are the main terpenes, but hemiterpenes (C_5), triterpenes (C_{30}) and tetraterpenes (C40) also can be found. A terpene containing

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oxygen is designated terpenoid. The aromatic compounds result from phenylpropane and are less common than terpenes. Plants use different biosynthetic pathways to synthesize terpenes and the phenylpropane by-products, but may jointly occur in some, however one major pathway will prevail. See the example of fennel (*Foeniculum vulgare* Mill.) where *trans*-Anethole (31-36%), α-pinene (14-20%) and limonene (11-13%) are produced [10].

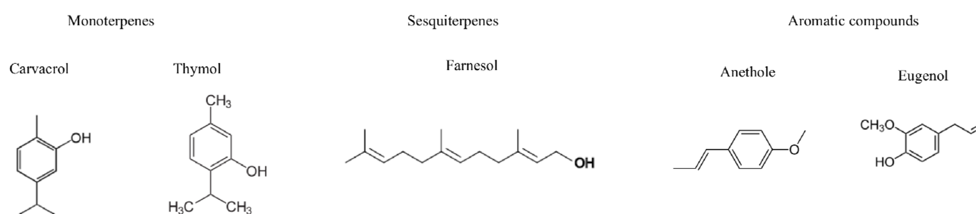

Fig. 1 Chemical structures of selected essential components

3. Antibacterial action kinetics

The range of the EOs action against bacteria may achieve values that i) only inhibit the bacterial growth (bacteriostatic) or ii) may be used at either high concentrations or are inherently more aggressive and their action results in a decline in the number of bacterial cells (bactericide). The bacteriostatic action has a reversible character since, after neutralization of the agent, the microbial cells will recover their reproductive capacity [11]. In contrast, the bactericidal effect has a permanent effect; as even after the neutralization of the agent, the microbial cells are not capable of growth and reproduction [11].

Usually, the antimicrobial action is determined by using microbial populations and not individual cells. In these circumstances, we are dealing with a dynamic situation: some cells are reproducing whereas others may already been dead and for this reason, sometimes the difference between the microbiostatic and microbiocidal values is difficult to establish.

The determination of these effects on microbial growth is based on the growth curve analysis done under standard conditions; this means that the agent is absent and the nutritional, temperature and atmospheric conditions are optimal for the microorganism under study. Generally, the discontinuous system of microbial growth is adopted. In Figure 2 (A and B) the characteristic growth curves under a (A) or microbiocide (B) effect are shown.

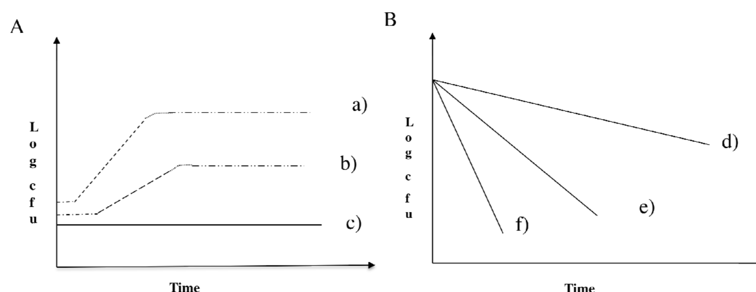

Fig. 2 A. Growth curves. a) normal growth curve, - - - exponential phase, - · - · · lag phase, — · · – stationary phase, b) and c) inhibitory effect (bacteriostatic); B. Survival curves, d, e and f) bactericidal effect with increasing concentrations from d to f (adapted from Bloomfield [11]).

The normal growth curve is represented under a) and can be divided in

_ _ _ _ the exponential phase during which cell division occurs according to a logarithmic or exponential relationship;

_ . . _ the stationary phase during which the total number of viable cells remains constant. This phase occurs due to several factors such as nutrient exhaustion, oxygen, decrease in pH value and toxic products accumulation.

_ . _ the bacteriostatic effect may be understood as an increase of the lag phase accompanied by a decline on the specific growth rate (μ) which may be partial, as in b) or total as in c).

The kinetics of the bactericidal effect is usually determined from the survival (dead) curves (Fig. 1 B). The characteristic survival curve is represented by d), the curves of the type e) and f) are associated with an increased death rate.

3.1 Factors that influence the determination of the antimicrobial activity

First, it is important to highlight that in addition to the need for the development and implementation of standardized tests for the evaluation of the antibacterial activity of the essential oils [12], the majority of the applied techniques on the determination of the antibacterial activity of the essential oils in vitro have been adapted from the evaluation of antibiotic activities [13-15]. The difficulties on the determination of the antibacterial activity of essential oils are well recognized and this is mainly due to its volatile properties as well as their insolubility in water. Of particular relevance is their hydrophobic nature and high viscosity, which causes an irregular distribution throughout the culture medium as

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well as an unequal dilution and because of this is required the use of a dispersing agent in order to prevent the irregular distribution of the EO in the culture medium.

Several features are distinguished as key factors in essential oil activity evaluation; the culture medium composition, assay technique, microbial species under study, extraction method, pH value, solubility of the essential oil in culture medium and temperature among others. Some of these factors will be examined.

3.1.1 The assay technique

Different types of tests for evaluation of the antimicrobial activity of essential oils *in vitro* are in use and the selection of each technique seems to be done according to several characteristics, namely technical demand and cost. Usually three types of methods are distinguished: diffusion, dilution and the bioautographic techniques [12,16,17]. The agar diffusion is one of most frequently used and is characterized by a great simplicity and cost-effectiveness. In this technique, several reservoirs of the essential oil can be employed. The most common technique uses filter paper disks [18-24] or stainless steel cylinders which are distributed on the agar medium surface, and also holes punched into the agar medium may be used as reservoirs of the essential oil [25-27]. In any case, the oil quantity and the reservoir diameter are crucial parameters.

The reservoir containing the essential oil to be evaluated, after being in contact with the inoculated medium and the required incubation period, the diameter of transparent zone around the reservoir (inhibition zone) is measured. This method was first designed to evaluate the antibiotic properties from crude extracts.

Whereas the agar diffusion method may be considered very precise on the essential oil antimicrobial activity determination, several less favourable aspects can be pointed out, such as the volatile characteristics of the essential oil components will result, on their loss, simultaneously with the solvent during incubation, whereas the less soluble compounds may not diffuse appropriately across the culture medium [12,16,17,28]. The parameters to consider include the disk or cylinder/hole diameter, the quantity of the oil and the solvent or emulsifier used. This last factor seems to differ significantly between studies and several substances have been used, including ethanol [29-36]; Tween-20 [13,37-40]; Tween-80 [41-44], methanol [45,46], dimethyl sulfoxide (DMSO) [47-50]. Particular importance must be given to the use of safe concentrations of solvent or emulsifier in order to not disturb microbial growth. Another aspect to take in account is to have a negative control (such as sterile water or solvent) and a positive control (usually a reference antibiotic) in each assay.

The degree of the essential oil activity is revealed by the size of inhibition zone that is expressed by the diameter of the referred inhibition zone (in mm or cm) and usually the diameter of the disc/hole/cylinder is included.

Due to the simple nature of this assay and the reduced amount of essential oil required, the use of this technique is generally recommended for the evaluation of numerous essential oils, and highlight the ones that present the highest activity allowing them to be subjected to more in depth characterization. This technique is also used to determine the susceptibility of a significant range of microbial species to a particular essential oil. However, this technique is less suitable for quantification purposes, such as the determination of the MIC and MBC values.

The techniques that require a homogeneous dispersal in water (dilution method; in agar or in liquid medium) are usually applied in order to determine the values of Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) through growth curve analyses by comparison with the culture grown in the absence of the essential oil (control culture). The MIC and MBC parameters are largely used in the evaluation of the essential oil antimicrobial activity but significantly differences have been found on their precise definition [12, 51, 52]. Other than this controversial aspect constituting an obstacle to the appreciation of the different studies, it seems that standardization is required.

The dilution method in agar or liquid medium is used for both bacteria and fungi [46, 53,54]. The volumes of culture broth supplemented with different essential oil concentrations vary substantially but presently the tendency is to use reduced volumes varying from 1-5ml [35, 45, 55] to 10ml [56]. The use of methods based on microdilutions is more intense [20,24,37,51,57,58] and appear to be very appropriate for the determination of MIC and CMB values. The efficacy of the antimicrobial activity when this method is applied both using tubes or microplates is verified by the change on optical density (OD) [16], by colorimetry [13, 52-55] or by viable determination [63], the latter being a very demanding technique. A combination of agar dilution and viable counts using the drop method [64, 65] may constitute a good alternative technique for bacteria that have a reduced growth in broth. Because growth is measured by change in OD is crucial to assure that no changes in the OD are due to the EO itself or the dispersing agent, in use. In case that this is not possible the viable count must be done.

The use of methods based on microdilutions is mainly recorded in the determination of the susceptibility to antibiotics [66] and they supply an important amount of information principally at present where the need for new and effective antimicrobial agents is very challenging, especially for natural products such as essential oils and plant extracts. Additionally, these methods can rapidly discriminate the resistant strains that emerge at very high frequency even among the foodborne pathogens [67, 68].

Other non-conventional methods such as the microatmosphere, bioautographic and impedance or conductance measurements can also used in the evaluation of antimicrobial activity of the essential oils [12,16].

The essential oils activity in vapour phase has been less explored but the consumer demand for new means of preservation replacing the use of chemicals and the knowledge regarding the potential inhibition activity by volatile components of EOs [69-71] had forced the search for new control agents and new methods to evaluate the volatile components, especially for elimination of resistant and persistent bacterial species such as methicillin resistant *Staphylococcus aureus* (MRSA) and *Legionella pneumophila* [72,73]. Assurance of microbial quality of food and pharmaceutical processing environments can be attained by the use of essential oils in the vapour phase [64-83].

The microatmosphere method is the result of a slight modification of the agar diffusion and is more appropriate to the determination of the EO activity in the vapour phase. This method is applied to the EOs that are intended for atmospheric control. The procedure for this method consisted of the inoculation of the solidified medium with the test microorganism as for the solid diffusion assay but the disc with the EO is placed on the medium-free cover of a Petri dish that is inverted and after the proper incubation time the activity is determined, as for agar diffusion method, the inhibition zone of the microbial growth is measured [52, 79, 84,85].

The bioautographic method is particularly applied on the evaluation of plant extracts [86-89]. The plant extracts are constituted by diverse components that can be separated throughout a thin layer or paper chromatography technique. When the solvent evaporates the inoculated culture medium can be distributed through the paper or the chromatographic plaques and after the adequate incubation period the microbial growth is measured. If the activity is positive no growth is observed and the extract components eluted and subsequently identified.

The bioautographic method is rarely used on the EOs activity assessment [86,90]. Nevertheless a version of this method that couples thin-layer chromatography and the bioautographic method (TLC-bioautographic) was recently developed and applied to test the antibacterial activity of the EOs of thyme, lavander, eucalyptus, spearmint and cinnamon [91].

The impedance or conductance method is characterized by being very expedite, having similarities with the most rapid methods. This technique is based on the correlation between the change of the electric parameters of microbial growth that are connected, in a restricted way, with the metabolic activity of the test microorganism and equally to the number of viable cells. The results of this evaluation are expressed by the Time of Detection (TD). This parameter is defined as the time required for a microbial culture to reach a threshold quantity that in general is equal to 10^6 cfu.mL-1. Regarding this, the antimicrobial activity of the EO can be expressed either by the time that the microbial culture in the presence of the oil takes to reach the TD in comparison to the control microbial culture (no EO present) or by the decline rate of the microbial cells [92-96].

3.1.2 The test microorganism

Overall, the activity of EOs against the microbial cells of the same genera and species determined under the same conditions seems to be similar [24,42,97]. However, some bacterial isolates may show a different response in comparison to the type strains [79]. It is important to use a significant number of strains from different origins in order to simulate a more realistic situation instead of just using laboratory strains that may not reflect the behaviour of the strains that can be found in nature. The antimicrobial activity may increase when the inoculum size is reduced, for this reason it is crucial to use a standard microbial density.

3.1.3 Culture conditions

The utilization of specific culture media on the determination of the susceptibility to antimicrobial agents such as antibiotics is standardized and the use of Mueller-Hinton medium is recommended [15] and was adopted in the determination of the antibacterial activity of EOs [13,45]. The culture medium not only can activate or decrease the EOs activity but also can protect the microbial cells from their action as the nutritional rich media can do. It is therefore necessary for uniformity among the several studies as this factor is vital for comparison of the different antimicrobial action between different studies.

Regarding the effect of the pH value on the EO activity, it is known that phenolic compounds and carboxylic acids only can cross the microbial cell membrane when they are in a non-charged form and the activity of the EO components eugenol, l-carvone, d-carvone and menthol are superior when the pH value of the culture medium increases from 6 to 8 [11,28]. The pH value also influences the modifications of the charge on the cell surface and because of this, the binding of the charged component of the EO to the microbial cell can be compromised.

It was demonstrated that at pH 8.7 the EO of the conifer, *Picea excelsa* was more efficient against *L. monocytogenes* and this action was related with a possible repulsion between negative charges of amino compounds and the cell surface [97].

4. Interactions between the components of the essential oils

Essential oils are complex mixtures of a wide diversity of components and their antimicrobial activity is therefore related to their composition, configuration, amount and their possible interaction [98]. Three effects can be highlighted:

additive, antagonist and synergetic. The additive effect occurs when the combined effect of the components is equal to the sum of the individual effects. Synergism is registered when the activity of the combined substances is higher than the sum of the individual activities. In contrast the antagonistic effect is registered when the activity of components in combination is inferior in comparison when they are applied separately.

For quantitative purposes the concept of fractional inhibitory concentrations (FIC) is frequently used. The calculation of FIC for each component in a mixture is based on the minimum growth inhibitory concentration (MIC) value and is the ratio of the MIC for the component in the mixture to that of the individual component. The FIC index is the sum of the FIC values for the components of the most efficient mixture and indicates the nature of their interaction. The value of FIC index accepted, as indicative of synergism is 0.5 and for the additive effect is 1.0. If the FIC index exceeds this last value, it means that an antagonistic effect had occurred [99]. Some EO combinations can be ineffective and the high potential of an EO will be eliminated or greatly reduced in the combination and there for it is of great importance to determine the possible interactions between EOs and their components [26].

Oregano EO is constituted mainly by two major components, thymol and carvacrol varying in their proportions [24,51] and their combination produces an additive effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [51].

The identification of additive, antagonistic and synergistic effects of fractions of cilantro, coriander (seeds and leaves), dill and eucalyptus EOs was investigated in the study of Delaquis et al. [95]. The fractions of the EOs each one including several components) were mixed in different combinations and applied against Gram positive and Gram negative bacteria and the yeast *Saccharomyces cerevisae*. The combination of a cilantro fraction (fraction 9) and a eucalyptus fraction (fraction 2) resulted in an additive antibacterial action against all the tested Gram-positive bacteria and *S. cerevisae*. However when the two fractions were tested against Gram-negative bacteria an antagonistic effect, except for *Yersinia enterocolitica* was evidenced. A synergistic effect was observed only when the combination of the two fractions was used against *Y. enterocolitica*. This synergistic effect resulted in a reduction of 75 times of the MIC value of the cilantro fraction which was 1.5% when used alone and in the combination with 1% of the eucalyptus fraction 2 resulted the MIC value of the cilantro fraction was 0.020% [95].

The combination of clove and rosemary essential oils produced an additive effect against the Gram-positive and Gram-negative bacteria, namely *Staph. aureus*, *Staph. epidermidis*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris* and *Ps. aeruginosa* [100]. The synergetic effect of this combination was observed when the mixture was applied against the yeast *Candida albicans* but when this mixture was applied against the fungi *Aspergillus niger* an antagonism effect was produced [100].

The EO of *Thymus vulgaris* or the EO of *Pimpinella anisum* used alone against *Ps. aeruginosa* were not active at the highest concentration (500.0 µg/ml) used whereas their combination (1:1) inhibited the growth of this bacterium [101].
Additionally, to the interactions that may occur between EOs and their components, another combination that has attracted the attention of many investigators is the synergism that can be released from the mixture of different EOs or their components in conjunction with other antimicrobial agents (eg. organic acids, or antibiotics) [55,102,103].
The type of interaction (synergistic, additive and/or antagonistic) between the EO of *Melaleuca alternifolia*, *Thymus vulgaris*, *Mentha piperita* and *Rosmarinus officinalis* when combined with ciprofloxacin demonstrated to be dependent on which proportion the two components are used and due to this variance on the type of interaction the use of EO in combination with antibiotics must be subjected to a previous evaluation [102]

The exploitation of the synergetic action resulting from the combination of EO with other biological antimicrobial substances such as bacteriocins has been investigated [56, 104-105]. The use of oregano or savory EO showed a synergistic activity with a combination of cell-adsorbed bacteriocin of a *Lactobacillus curvatus* strain when applied to control *Listeria monocytogenes* in pork meat storage at 4ºC [56]. The association of the EO components, thymol or cinnamaldehyde with low temperatures (≤8ºC) also produces a synergistic effect on the control of the foodborne bacteria *B. cereus* [106]

The combined treatment with electrolyzed NaCl solutions and 1% solution of essential oils consisting of 0.5% carvacrol and 0.5% thymol was applied to prevent the microbial and chemical spoilage of carp fillets during drying and the treatment demonstrated to be effective both in the control of microbial populations and chemical deterioration as antioxidant effects were registered [107].

The incorporation of essential oil vapours with negative air ions results on a very effective bacterial removal [108,109]. The combination of *Cympobogon citratus* oil vapour and negative air ions exposure during 4h lead to a high *Pseudomonas flourescens* elimination (91.8%) in comparison to a much lower elimination rate achieved with *C. citratus* oil vapour alone (72%) or reached by negative air ions alone (50.9%) [109].

5. Bacterial cell targets

The number of studies about the mechanisms of action of plant essential oils has been increasing [27, 36, 45, 51, 110-115]. Nevertheless, in comparison the high number of studies on the EOs characteristics and their components to the number of studies performed on the investigation of the specific target(s) of the antimicrobial action of EO and their

components an enormous difference still remains. The knowledge about the cell target(s) of the EOs and their components is crucial to understand which cell target(s) is affected, consequently the survival of the pathogen in a food matrix, in a living tissue or the host infection process can be impaired. Ultimately a proper application system can be elaborated based on more accurate information.

The antimicrobial actions of EO are linked to one of the most important EOs characteristics, its hydrophobicity resulting in increased cell permeability and consequent leaking of cell constituents [27,51,115-121]. It is important to comprehend that a disturbed cell structure may affect others cellular structures in a cascade type of action [122].

5.1 Cell wall and membrane disturbance

The most elucidated action concerns the components of oregano and thyme EOs, carvacrol and thymol. It is recognized that their action results in the release of the lipopolysaccharides from Gram negative bacteria with the consequent cell membrane permeability increase and ATP loss [51,108,111,123,124].

The evaluation of the loss of cell constituents contributes to elucidate the severity of the cell membrane damage and a significant number of studies had used this approach to clarify the antibacterial action of EOs [114,119,122] and those indicate that the tested EOs affect the bacterial cell on the same target, the cytoplasmic membrane.

Bouhdid et al [114] investigated the cellular damage by *Origanum compactum* EO on *Pseudomonas aeruginosa* ATCC 27853 and *Staph. aureus* ATCC29213 by evaluating the cell viability, potassium leakage using flow cytometry and transmission electron microscopy. The treatment of *Ps. aeruginosa* at the MIC value and at 1.5x of the MIC value resulted in a series of physiological injuries, namely the growth was totally inhibited, the respiratory activity significantly diminished, the cell membrane permeability was affected and the membrane potential fails. By contrast the cell damage in *Staph. aureus* was not so pronounced at the MIC value, in particular both the membrane potential and the permeability were not significantly affected, but when the MIC value was increased to 1.5x the viability and the membrane potential go through a significant reduction. The differences registered between the two bacteria are mainly due to differences in the membrane and cell wall composition and structure [114].

The observation done by scanning electron microscopy (SEM) of the two Gram negative food borne bacteria, *E. coli* 0157:H7 strain EDL 933 and *Salmonella enterica* subsp *enterica* serovar Typhi strain ATCC 19430 when exposed to mustard EO (allyl isothiocyanate is the main component) evidence an imperfect and unfinished cell shape [115]. The treatment of *E. coli* 0157:H7 with Spanish oregano causes alterations on the cell wall, the presence of white spots or holes on the cell wall were observed [108]. However the use of Spanish oregano against *L. monocytogenes* cells did not caused the production of white spots or holes, but the production of an imperfect *L. monocytogenes* cell [124]. It seems evident that *E. coli* 0157:H7 cells treated with Chinese cinnamon EO were able to keep the energy sufficiently high to repair and maintain the cell surface apparently not damaged. This type of injury can be related with the differences on the cell wall structure. Such differences on cell damage were also verified with other bacterial pathogens, namely *Bacillus subtilis* (strain APL 87/35) and *E. coli* (strain APL 87/1) cells treated with oregano and clove EOs [110]. *E. coli* cells treated with both EOs showed a more evident damage: holes at cell surface whereas in *B. subtilis* the damage just resulted on cell surface malformation [110].

Enriched thymol EO such as *Thymus erocalyx* and *T. x-porlock* (thymol content ranged from 63.8 to 31.7%, respectively) can cause injury to *L. monocytogenes* by disrupting or induce the formation of a very thick and rough cell wall and even at lowest thyme oil concentration used the disruption of the cell membrane and lack of cytoplasm was observed [45].

The treatment of *Staph. aureus* with *Inula graveolens* (rich in bornyl acetate (43.3%) and borneol (26.2%)) and *Santolina corsica* (rich in myrcene (34.6%) and santolina triene (13.5%)) EOs at MIC values (5 mg.ml^{-1}) for both EOs) produces invaginations of the cytoplasmic membrane accompanied by the formation of a thicker cell wall and aggregation of the cytoplasmic contents [125]

A possible indirect action of EOs on the membrane is the secretion of toxins. This aspect is particularly important to the control of *Staph. aureus* and *B. cereus*. The exposure of *B. cereus* to carvacrol resulted on inhibition of diarrheal toxin production [126] and the use of Oregano at 0.3 and 15 µl/ml completely abolish the enterotoxin production of *S. aureus* [127]. Structural modifications and energy limitation may explain the inhibition of toxin production. The secretion of toxins may be prevented by modifications in the bacterial membrane due to the attachment of the essential oil that may disturb the phospholipid bilayer with consequences to the trans-membrane transport process limiting in this way the release of toxins to the contiguous environment [128]. By other hand the limitation of intracellular ATP and proton motive force will restrict the secretion of toxins.

Combined treatments may also act synergistically degenerating the bacterial cells. The cells of *Ps. flourescens* treated with *C. citratus* oil vapour and negative air ions experience a completely relapse, the cytoplasmic material is spilled out of the cells whereas the cells treated with negative air ions alone only experience a restricted cell surface deformation [109].

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5.2 ATP production

The disruption of the cell membrane by any antimicrobial agent, including the EOs will compromise a series of vital functions, namely the energy for conversion processes, nutrient processing, synthesis of structural macromolecules, and secretion of many growth key enzymes. The production of ATP in prokaryotes occurs both in the cell wall and in the cytosol by glycolysis. So is expected that alterations on intracellular and external ATP balance will be affected due to the action of the EO on the cell membrane. The correlation between the intracellular and extracellular ATP concentration has been found [115,117,124]. ATP losses are supposed to occur through the disturbed membrane [115,124]. The treatment of *E. coli* 0157:H7 strain EDL 933 and *Salmonella enterica* subsp *enterica* serovar Typhi strain ATCC 19430 with mustard EO causes a significant loss of intracellular ATP, in particular when the essential oil is used at the determined MIC value (0.2%, v/v) [115]. The addition of carvacrol at 2mM or 1mM results on a decrease of intracellular ATP to 0 after 10 and 14 min., respectively [129]. The use of oregano EO at 0.020 and 0.025% (w/v) against *L. monocytogenes* cause a decrease on intracellular ATP [130] and at 0.010% and 0.013% (w/v) also produces a decline on the intracellular ATP content of *Staph. aureus* [131]. In both *L. monocytogenes* and *Staph. aureus* a combined treatment with oregano EO and irradiation caused a more significant reduction on the intracellular ATP quantity [130,131].

Other intracellular events may contribute to the intracellular ATP decrease accomplished with a minor ATP release, namely the intracellular ATP may suffer a significant reduction by hydrolysis which can be due to the loss of inorganic phosphate across the compromised high permeable membrane [115,124,132] or in virtue of the efforts made by the cell to recover the electrochemical gradient by proton extrusion driven by the ATPase an increased hydrolysis is established. This last mechanism was verified when *L. monocytogenes* cells were eliminated by the treatment with the bacteriocin pediocin PA-1 [133].

5.3 Protein synthesis

Burt et al. [36] first reported the action of EO components on protein synthesis. The EO components, carvacrol and p-cymene induced the synthesis of heat shock proteins (HSPs) when bacterial cells were treated with these two EO components [36]. The HSPs are molecular chaperones involved in the different processes of assembly and release of newly synthesized polypeptides that, in general, increases when bacterial cells contact with toxic substances or other stress conditions. The cells of *E. coli* O157:H7 incubated overnight in the presence of carvacrol at 1mM produced significant amounts of heat shock protein 60 (HSP60) (GroEL) and the synthesis of flagellin is inhibited resulting in non-motile cells. In contrast, p-cymene at 1mM or 10mM did not affect the production of HSP60 or flagellar synthesis [36]. The approach to evaluate the effect of EO or its components on protein synthesis that can contribute with a more comprehensive view is the proteomic approach, namely the use of two-dimensional electrophoresis (2-DE) coupled with MALDI-TOF MS and this type of approach as used in the study of Di Pascua et al. [134] to evaluate the modifications on the protein expression of *Salmonella enterica* ser. Thompson treated with a sub lethal concentration of thymol. Di Pascua et al. [134] found that *Salmonella* cells treated with 0.01% over expressed a set of molecular chaperone proteins, namely DnaK, GroEL, HTpG and the Trigger factor Tf, outer membrane associated proteins (OmpX and two OmpA) and proteins involved directly or indirectly in the citrate metabolism and ATP synthesis were also affected evidencing the action of thymol as a large-scale stressor and acting in different pathways.

5.4 pH disturbance

The pH_{in} in bacterial cells exposed to EOs has been monitored and a significant reduction has been found [115,124]. The pH homeostasis may be impaired by the action of EOs on the membrane that loses its capacity to block protons [51,115,124]. In the study of Turgis et al. [115] a significant decrease on the intracellular pH (pH_{in}), the initial pH_{in} (no EO) changed from 6.23 to 5.20 for *E. coli* 0157:H7 and from 6.59 to 5.44 for *S. Typhi* when the bacterial cells were treated with the MIC value of the mustard EO.

The pH_{in} of *E. coli* 0157:H7 was affected by the use of the Spanish oregano at 0.025% (v/v), Chinese cinnamon at 0.025% (v/v) and savory EOs at 0.05% but the E. coli 0157:H7 pH_{in} was more affected by Chinese cinnamon EO. At concentration 0.025% (v/v) the Chinese cinnamon EO caused a decrease of the pHin from 7.25± 0.20 to 5.16±0.05 whereas the Spanish organo at this same concentration origin a decrease from 7.25± 0.20 to 6.68±0.37[124]. This effect is similar to the mustard EO described by Turgis et al. [115].

The addition of oregano EO to *Staphylococcus aureus* and *Pseudomonas aeruginosa* cultures caused a rapidly pH gradient dissipation [44]. In *B. cereus* the addition of 0.25mM to 0.5mM of carvacrol causes a decrease in the pH gradient and at 1mM the pH gradient is completely dissipated [129]

The maintenance of the pH_{in} at appropriate levels to achieve the various crucial cellular processes (DNA transcription, protein synthesis and enzymatic activity) is critical when the cell is exposed to severe injury [135-137]. Even the neutrophile *E. coli* is capable to overcome acid stress and do this by activating four different acid resistance mechanisms [138,139]. These systems function on the basis of amino acid decarboxylases (glutamate, arginine and

lysine) and antiporters[1]. The reduction of the pH_{in} by the EOs treated cells is compared to the action of weak organic acids, namely benzoic in the yeast *Saccharomyces cerevisae*, as the weak acids also EOs are lipophilic and as weak acids is possible that due to their lipid permeability a subsequent release of protons occurs [115,140-142]. If the intracellular proton release goes over cytoplasmic buffering capacity or the capacity of proton efflux systems, the intracellular pH value starts to decrease and vital cellular functions may be broken [143,144].

5.5 Intracytoplasmic changes

In the study conducted by Becerril et al. [145] *E. coli* cells treated with oregano EO exhibited intracytoplasmic changes, where coagulated material appeared in specific areas located to the cell wall and apical ends. When *E. coli* cells were treated with cinnamon EO, the periplasmic space showed significant changes, in particular they became larger and irregular. The investigators also noted the absence of fimbriae in the altered (larger) periplasmic space. *Staph. aureus* cells treated either with oregano or cinnamon EO exhibited the same cell malformations as *E. coli* but in a less pronounced manner.

5.6 DNA

Once the bacterial DNA is physically attached to the bacterial cell membrane is expected that EO may act on DNA and this fact has been used to measure the genotoxicity and antimutagenic effects of EOs and other agents [146-153]. The most used tests are the Ames test [146] and the SOS-Chromotest [147]. The Amest test is based on use of different sets of *Salmonella enterica* subspecies *enteric*a serovar Typhimurium strains (four strains) that have different mutations in the histidine operon, becoming auxotrophic for histidine. The strains sets have a deletion on the *uvrB* region of the chromosome removing the DNA repair system [154]. The other mutations are on *gal*[2] and *rfa*[3] that affect, to different levels, the polysaccharide side chain of the lipopolysscharide (LPS) that covers the bacterial surface. These mutation confer a high rough appearance to the bacterial cells and such bacteria are high permeable and fully nonpathogenic. The TA1535 set (TA 1535, TA1536, TA1537 and TA1538) which have mutation on *rfa* and *uvrB* is the most susceptible to mutagenesis, so is suggested for regular testing for mutagens and carcinogens, *in vitro*. The TA1975 set (TA 1975, TA1976, TA1977, TA1978) only have the *rfa* mutation, so is suggested to analyze the effect of the repair system on mutagenesis and eradication. Quillardet et al. [147] mounted a colorimetric assay based in the SOS response [154] and called it SOS-chromotest. This test is based on an operon fusion that putted the lacZ[4] under the control of the *sfiA* gene[5] (*sfiA*::*lacZ*) in *E. coli* K-12 (denominated PQ37 *uvrA* strain) . The mutagenic activity of an agent at given concentration C(R(C)) can be expressed by the ration of β-galactosidase activity to alkaline phosphatase activity. In this strain alkaline phosphatase synthesis is constitutive and is not inducible by DNA damaging agents being determined in simultaneously with β-galactosidase. The SOS induction factor I(F)=R (C)/R(0) in which R(0) is the mutagenic activity calculated in the absence of the agent. The mutagenic and anti-mutagenic effects are useful whenever the safety aspect of the EO use is required and several studies are covering this aspect [150,151,153].

5.7 Quorum sensing

Bacteria produce and use small signalling molecules to evaluate their external environment and their internal physiological status i.e. to cell-cell communication (quorum sensing) modulating their populations. These molecules are in general known by autoinducers. The Gram negative bacteria use acyl homoserine lactones (HSLs) whereas the Gram positive bacteria use modified oligopeptides [155]. Quorum sensing (QS) is involved in biofilm production, motility, swarming, stress resistance and virulence [156]. The participation of QS on so many essential aspects of the bacterial life makes this process an interesting target to control infections, diminish antimicrobial resistance and food spoilage [157]. The investigation of the anti-QS activity of EOs or its components is in progress [158-162].

The effect of cinnamaldehyde on transcription of two HSLs, the 3-oxo-C6 and the 3-oxo-C12-HSL was evaluated by using a green fluorescent protein bioreporter system and the effect on the bioluminescence mediated by the 3-hydroxy-C4-HSL and the autoinducer-2 (AI-2) of *Vibrio harveyi* was followed by using two bioluminescent reporter V. *harveyi* strains (BB886 and BB170) [158]. At 200 $\mu mol.l^{-1}$ cinnamaldehyde reduced in 70% the transcription of LuxR6[6] led by the P_{luxI} promotor, which is induced by the 3-oxo-C6-HSL. In contrast the effect of cinnamaldehyde on LasR[6], which transcription is lead by P_{lasR} promoter and induced by 3-oxo-C12-HSL was not significant [158]. The exposure of *V.*

[1] Antiporters are proteins that belong to the transport system and function simultaneously by drive out one compound present inside the cell and import another compound from the outside.
[2] The *gal* (now denominated *galE*) gene codifies to UDP-galactose-4-epimerase.
[3] *rfa* is a cluster of genes encoding several glycosyltransferases for the lipopolysaccharide central biosynthesis and connection of O antigen.
[4] lacZ is the structural gene of β-galactosidase.
[5] *sfiA* (now denominated *sulA*) gene is a SOS cell division inhibitor which expression is induced to significant levels when DNA replication is disrupted.
[6] LuxR and LasR are activator proteins. Bind to specific sites on DNA and induce transcription.

harveyi BB886 (the bioluminescence of this strain is led by 3-hydroxy-C4-HSL) to 60μmol.l^{-1} of cinnamaldehyde resulted on a 55 % reduction of bioluminescence and the near 60% of the bioluminescence of the strain BB170 (mediated by AI-2) was reduced at 100 μmol.l^{-1} [158]. Virulence of *V. harveyi* to *Artemia* shrimp can be reduced by the use of cinnamaldehyde and its derivative 2-NO_2-cinnamaldehyde when used in combination [159]. Using the nematode model *Caenorhabditis elegans* Brackman and colleagues [162] demonstrated the efficacy of 3,4-dichloro-cinnamaldehyde on the reduction of the virulence of *V. anguillarum*, *V. harveyi*, *V. vulnificus* by mainly affecting the DNA ligand ability of LuxR.

The EO of rose, geranium, lavander, rosemary and clove seem to be very effective on as QS inhibitors whereas orange and juniper EO seem to have no anti-QS properties [160,161].

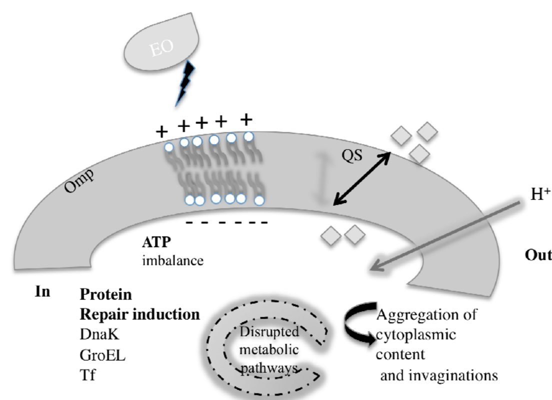

Fig. 3 Identified bacterial cell structures and cellular processes disrupted by the action of EOs or their components. Omp (Outer membrane protein), QS (quorum sensing). EO treated cells are more permeable to protons, experience an ATP imbalance and induce the synthesis of chaperones. Metabolic pathways can be injured.

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