Plant-based intervention strategies for *Listeria monocytogenes* control in foods

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Listeriosis is a severe human disease caused by two potentially pathogenic listerial species: *Listeria monocytogenes* and *Listeria ivanovii*. In particular, the risk of contamination can be significantly high in refrigerated ready-to-eat foods, due to the limited use of traditional antimicrobial barriers. Moreover, *Listeria* spp. is widely disseminated in the manufacturing environment and can contaminate foods even after listericide treatments.

This chapter reviews the applications of plant-based biopreservation strategies for effective *Listeria monocytogenes* control in foods. In recent years, plant extracts and essential oils have attracted considerable interest for use as biopreservatives in food products, although industrial applications are still limited. The aim of the chapter is to analyse the possibile impact of published research in the manufacturing environment, as well as to assess future scientific needs.

Keywords plant-based; biopreservation; antilisterial; *Listeria monocytogenes*; control.

1. *Listeria monocytogenes* in foods

Listeria monocytogenes is a ubiquitous foodborne microorganism that has caused numerous outbreaks, especially in high-risk groups (YOPIs: Young, Old, Pregnant, Immunocompromised) with a fatality rate up to 30% [1]. This species can grow over a wide range of temperatures (-0.4 to 45°C), in a wide range of pH (4.3 to 9.6), at a high concentration of NaCl (up to 10%), in presence of nitrates and reduced water activity (a_w down to 0.90) [2-4]. Moreover, it is able to adhere to food contact surfaces such as glassware, stainless steel, rubber, and polystyrene, where it can survive for a long time due its ability to form biofilms [5]. Such versatility requires that the organism can assimilate information about its environment, and process this information quickly to adapt to changing conditions [6]. For these reasons, it can survive and grow in contaminated food systems and create substantial risk for human health.

The persistence of *L. monocytogenes* in both food and food processing environments has been frequently reported in the literature, in particular in meats and meat products [7-8], ready-to-eat products [9-10], fermented products [11], cold smoked fish [12-13], dairy products [14-15], fruits as melon and fresh vegetables [16].

Different food technologies are applied to prevent *L. monocytogenes* proliferation and survival in foods, as well as to reduce the count number in fermented, smoked and dried products [17-18]. In some fermented products, microenvironmental conditions are not suitable for *L. monocytogenes* survival, e.g. combination of a_w below 0.90, pH and air flow in meat products [19-20] or combination of starter culture, water activity (a_w), pH and heat treatments in dairy products [21]. Moreover, emerging non-thermal technologies, such as natural antimicrobial compounds, bacteriocins, pulsed electric fields, high pressures, irradiation, and pulsed light have been proposed to control *L. monocytogenes* in foods [22-24], with different effectiveness depending on the level of contamination.

Thermal treatment above 70°C is the most common intervention strategy against *L. monocytogenes* [25]. However, it is well known that the microorganism may develop an adaptive response during exposure to sublethal food processing interventions [26], preserving its pathogenic potential [27]. Several factors such as pH, NaCl, a_w, chemical composition, processing conditions, bacterial strain characteristics contribute to increase response variability. Moreover, environmental stresses such as starvation may increase its resistance to other lethal stresses [28]. Thermotolerance of *L. monocytogenes* can be affected by different factors: culture forms [29], recovering medium, heat shock, and thermal history of a cell population. The latter seems to be more pronounced when cells are subjected to chilling or freezing temperatures before treatment [30-31]. An extensive regulatory genes repertoire (7% of all predicted genes) has been reported for *L. monocytogenes* [32].

Other non-thermal treatments such as **High Hydrostatic Pressure** (HHP) have been applied to decrease the count of *L. monocytogenes* in foods. Recently, it was reported that treatment at 600 MPa for 3 min resulted in an immediate 3.9–4.3 Log CFU g^{-1} reduction of *L. monocytogenes* count in ready-to-eat sliced ham [24]. On the other hand, when inoculated carrot juice was pressure treated (500 MPa/1 min/20°C), *L. monocytogenes* was not detectable immediately after pressure treatment or during storage at 4, 8 and 12°C (>6 Log inactivation) [33]. Moreover, the strong cooperative effect of HHP with other antimicrobial compounds such as the lactoperoxidase system on foodborne bacteria inactivation was demonstrated in refrigerated cold-smoked salmon [34].

In addition, **High-Pressure Homogenization** (HPH) has been proposed to obtain microbial load reduction in liquid foods [35]. Although several studies demonstrated a major impact of HPH on Gram negatives, Iucci et al. [36] reported that the activity of lactoferrin and lysozyme against *L. monocytogenes* was enhanced and/or accelerated by HPH

treatment, particularly when cells were added to HPH-treated lactoferrin, processed simultaneously or separately with the target microorganism.

Pulsed Electric Fields (PEF), which causes electroporation-induced death of microorganisms, has been applied alone or in combination with other antimicrobials to reduce *L. monocytogenes* count. In PEF-treated raw whole milk, the proportion of sublethally injured microbial cells increased from 18.98% to 43.64% with the increasing electric field strength from 15 to 30 kV/cm [22]. On the other hand, Ait-Ouazzou et al. [37] reported the synergistic lethal effects of mild heat (54°C, 10 min) and PEF (30 kV/cm, 25 pulses) combined with 0.2 µl ml^{-1} of *Thymus algeriensis* L. and *Mentha pulegium* L. essential oils (EOs) in apple juice inoculated with *L. monocytogenes*.

Among the **antimicrobial compounds produced by lactic acid bacteria** (LAB), nisin, produced by *Lactococcus lactis,* has been proposed as a food preservative because of its lack of toxicity and residues in humans [38]. Pediocin, produced from *Lactobacillus pentosus*, was able to reduce *L. monocytogenes* in chilled, tray-packaged pork and recently amysin, produced by *Bacillus amyloliquefaciens*, showed antibacterial activity against a wide range of bacterial species such as *L. monocytogenes* [39]. The use of bacteriocins in combination with modified atmosphere, sodium benzoate, sodium chloride, sodium acetate, or sodium trypolyphosphate to reduce *L. monocytogenes* has been also reported [40].

Finally, **electrolyzed water and ozone** have been proposed recently to obtain 5 Log CFU ml^{-1} decrease of *L. monocytogenes* in liquid culture [41]. In the last years, essential oils have received increasing attention from both researchers and industry for use as alternative antimicrobials in foods [42]. In fact, in vitro and in situ studies have demonstrated antibacterial activity of essential oils (EOs) against *L. monocytogenes*. This topic will be analysed further in this chapter.

2. Classification of plant-based antimicrobials

Secondary metabolites produced by plants constitute a major source of bioactive substances that have been the focus of numerous researches in the last decades. They are traditionally considered to be non-essential for the basic metabolic processes of the plant [43], and some of these metabolites, either as pure compounds or as standardized plant extracts, are stated as GRAS (Generally Regarded As Safe). They can be used to control the growth of specific microorganisms in foods, either delaying or inhibiting their growth or killing them. Concentration and biogenesis of plant secondary metabolites is regulated by a wide variety of factors (Table 1).

Table 1 Factors that influence secondary metabolites biogenesis in plants (adapted from [44]).

Factors		
Plant related	Environmental	Agronomic
Plant species, variety, eco- and chemo-type, anatomical part of the plants, phenology (growth and life cycle), stage of maturity of the plant and ecological interactions.	Season and climate, temperature, humidity, duration of daylight, radiation, wind, geographic and geological location, altitude, soil fertility.	Conditions of cultivation and harvesting, irrigation, fertilization and methods of harvest, post-harvest processing factors such as storage and preservation.

The presence and concentration of plant secondary metabolites is also influenced by the procedures used for extraction, particularly by solvent, temperature, pressure and time. Extraction can be carried out either by conventional methods that are often time-consuming and require relatively large quantities of solvents, or by non-conventional methods that are more environmental-friendly, due to decreased use of synthetic and organic chemicals, reduced operational time, and better yield and quality of extract [45].

The main plant biocompounds have been divided into three main molecule families: (a) terpenes and terpenoids (approximately 25,000 types), (b) alkaloids (approximately 12,000 types), and (c) phenolic compounds (approximately 8000 types) [46].

Terpenes are hydrocarbons (alkenes) derived from isoprene units (C5). All types of terpenes and terpenoids (oxygenated alkene derivatives) are derived by repetitive fusion of branched five carbon units based on an isopentane skeleton, and most of the chemical intermediates in their biosynthetic pathway are known. Mono- and sesquiterpenes and their derivatives are the principal constituents of the essential oils, while the other class of terpenes are constituents of balsams, resins, waxes, and rubber. The antibacterial activity of some monoterpenes (C10), diterpenoids, sesquiterpenes (C15), triterpenoids and their derivatives showed strong structure–function influence of the antibacterial potential of terpenes [47]. In particular, terpenes and their derivatives exert a stronger antibacterial activity towards Gram-positives than Gram-negatives [48]. Such activity may be due, at least partially, to perturbation of the lipid fraction of bacterial plasma membranes, resulting in alterations of membrane permeability and leakage of intracellular materials [49].

Alkaloids are heterocyclic nitrogen compounds characterized by different antimicrobial activities. More than 10,000 different alkaloids have been discovered in species from over 300 plant families [50]. They often contain one or more

rings of carbon atoms, usually with a nitrogen atom in the ring, and are subclassified on the basis of the chemical type of their nitrogen-containing ring. The position of the nitrogen atom in the carbon ring varies with different alkaloids and with different plant families. There are two broad divisions based on chemical structures of the alkaloids:

I. Non-heterocyclic or atypical alkaloids, sometimes called 'protoalkaloids' or biological amines;

II. Heterocyclic or typical alkaloids, divided into 14 groups according to their ring structure.

More recent classifications are based on similarity of the carbon skeleton (e.g., indole, isoquinoline and pyridine-like) or biogenetic precursor (ornithine, lysine, tyrosine, tryptophan, etc). Alkaloid distribution in the angiosperms is uneven. The dicotyledon orders Salicales, Fagales, Cucurbitales and Oleales at present appear to be alkaloid-free. Alkaloids are commonly found in the orders Centrospermae (Chenopodiaceae), Magnoliales (Lauraceae, Magnoliaceae), Ranunculales (Berberidaceae, Menispermaceae, Ranunculaceae), Papaverales (Papaveraceae, Fumariaceae), Rosales (Leguminosae, subfamily Papilionaceae), Rutales (Rutaceae), Gentiales (Apocynaceae, Loganiaceae, Rubiaceae), Tubiflorae (Boraginaceae, Convolvulaceae, Solanaceae), and Campanulales (Campanulaceae, sub-family Lobelioideae; Compositae, subfamily Senecioneae) [51].

Phenolic compounds are commonly found in both edible and inedible vegetable tissues, where they protect plants from microbial infections. In plants, they are the most abundant secondary metabolites, which possess various biological effects as free radicals scavengers, as well as antimutagen and anticarcinogen compounds. They are classified into insoluble compounds such as condensed tannins, lignins, cell wall bound hydrocynammic acids, and soluble compounds as phenolic acids, phenyl propanoides, flavonoids and quinones. Various phenolic compounds have received attention during recent years as potential preservatives, disinfectants, and therapeutic agents [52-53].

In particular, flavonoids occur as aglycones, glycosides and methylated derivatives, and are widely distributed in fruits, vegetables, grains, bark, roots, nuts, seeds, stems, flowers, tea, wine, propolis and honey, where they may be synthesized in response to microbial infection [54]. Their basic structural feature is composed of two aromatic rings linked through a three-carbon bridge with a carbonyl functional group located at one end of the bridge [47]. Plant extracts generally contain flavonoids in glicosidic form, and this may be one of the reasons why extracts and pure compounds do not exert comparable inhibition [53]. The antibacterial activity of flavonoids has been correlated to the ability to form complexes with both extracellular and soluble proteins, as well as with bacterial membranes [55-56].

3. Extraction methods

3.1. Conventional methods

Traditionally, target compounds in natural products are determined after exhaustive extraction of the sample, by using solid–liquid extraction techniques. In particular, conventional extraction methods include maceration, hydrodistillation, solvent extraction, and percolation. Extraction efficiency mainly depends on the choice of solvents [55]; the polarity of the targeted compound is the most important factor for solvent choice.

Maceration does not produce pure EOs but EOs carried by oil. Plant materials, ground into small particles and added with hot oil in a closed vessel, are allowed to sit for some days, shaking occasionally. Then, the macerated plant material is removed and the oil is filtered and strained of any remaining solid residues (mark). The resulting oil is infused and contains the EO.

In Hydrodistillation or **Steam Distillation**, the plant material is almost entirely covered with water, which is brought to boil. In this case, EOs are carried over to the condenser along with steam. Not all of the produced EOs can be captured from steam, and the mix of water and oil that remains after the process (hydrosol) can be considered a byproduct of hydrodistillation. This method is not advisable for heat-sensitive compounds extraction, because some volatile components may be lost at high extraction temperature.

Solvent extraction is the most common method used to extract EOs from plants, by repeated washing (percolation) with an organic solvent, usually hexane or petroleum, under reflux in a special glassware. Plant materials are added with a chemical solvent in a rotating drum container. Drum rotation allows mixing and extraction, and finally the solvent is allowed to evaporate and leaves a substance (concrete), which contains EOs and plant materials. Then, alcohol is used to remove plant waxes from concrete, and is distilled away, yielding a high concentration of EOs.

In **Percolation**, steam is produced above the plant material, percolates downwards and is collected into a pipe, which passes through a series of cooling tanks to be distilled. This method is not widely used but can be interesting for woody plant materials. The shorter time required for percolation in comparison with hydrodistillation (4 hours instead of 12) allows obtaining a better quality EO. The efficiency of the overall process is not high, but given the large amount of solid used the extract is rich enough of extractable compounds.

3.2. Non-conventional methods

Conventional methods are time consuming and use high amounts of solvents, and therefore there is a growing demand of alternative methods [57]. In the last years, a number of novel extraction techniques have been used to optimize extraction of bioactive compounds from plants, such as: supercritical CO_2 extraction (SC-CO_2), pressurised liquid

extraction (PLE), pulsed electric fields (PEF), ultrasound assisted extraction (UAE), and microwaves assisted extraction (MAE). The major non-conventional methods and the corresponding plant compounds are summarized in Table 2.

Table 2 Non-conventional methods used to obtain plant extracts.

Method	Plant Compounds	Plant	Reference
Pressurised Liquid Extraction (PSE)	Flavonoids	*Hylocereus undatus*	[58]
	Antioxidants	*Picea abies*	[59]
Ultrasound-Assisted Extraction (USE)	Oil and polyphenols	*Vitis vitifera*	[60]
	Baicalin	Scutellariae (radix)	[61]
	Flavonoids compounds	Hawthorn seed	[62]
	Polyphenols (flavanone glycosides)	*Citrus sinensis* L. (peel)	[63]
	Polyphenols and antioxidants	*Punica granatum* L.	[64]
Pulsed Electric Fields (PEF)	Polyphenols (Resveratrol)	Grape skin	[65]
	Anthocyanin	Red cabbage	[66]
	Isoflavonoids phytosterols	Soybeans, maize germ oil	[67]
Microwave-Assisted Extraction (MAE)	Essential oils	*Rosmarinus officinalis* L. and *Mentha piperita* L.	[68]
	Carotenoids	Paprika powder	[69]
Supercritical fluids	Anthocyanins	Liquid grape seed extract	[70]
	Homoplantaginin, Rosmarinic acid, Scutellarein, Tryhydroxycinnamic acid derivative, Cirsimaritin, Rosmanol, Epiisorosmanol, Genkwanin, Carnosol, Methyl carnosate, Carnosic acid, Gallocatechin, Rosmarinic acid methyl ester.	*Rosmarinus officinalis* L.	[71]
	Terpenes, fatty acids and Vitamin E	*Piper gaudichaudianum* Kunth	[72]
	Lycopene and β-carotene	*Solanum lycopersicum* L.	[73]

Pressurised Solvent Extraction (PSE) uses organic solvents at high temperature and pressure to increase the efficiency of extraction. PSE operates at high pressures and temperature above the boiling point of the organic solvent. High pressure forces the solvent into the matrix pores and facilitates extraction, whereas the higher temperature promotes analyte solubility by increasing both solubility and mass transfer rate and decreasing viscosity and surface tension of solvents, thus improving extraction rate [74]. The use of pressurised solvent techniques offers the advantage of enhanced target molecule specificity and speed, due to physicochemical properties of the solvent, including density, diffusivity, viscosity and dielectric constant, which can be controlled by varying pressure and temperature of the extraction system [75].

In **Pulsed Electric Fields Extraction** (PEF), an external electric field induces critical electrical potential across the cell membrane, leading to an electrical breakdown with an increase of permeability and metabolites extraction. PEF can be operated continuously at room temperature and performed in a few seconds, minimizing deterioration of bioactive materials [76]. As a result, a number of different phenomena, such as intracellular liquid release, diffusion of solutes, and membrane releasing processes, develop inside the cellular structure after treatment.

Ultrasound-Assisted Extraction (UAE) is a valuable and effective method for extraction of bioactive compounds from plant materials [60-63]. Extraction efficiency is high, due to intensification of mass transfer, improved solvent penetration into the plant tissue, and capillary effects, in addition to cavitation (a nucleated process) produced in the solvent by the passage of an ultrasonic wave. During cavitation, bubbles are formed and their collapse near the cell wall produces cell disruption together with a good penetration of the solvent into the cells, through the ultrasonic jet [77]. High temperatures, which increase solubility and diffusivity, and pressures, which favour penetration and transport, result in high extractive power [64]. Other advantages include drastically reduced processing time, less consumption of energy and solvents, and reduced thermal degradation effects [78].

Microwave-Assisted Extraction (MAE) is the process of heating solvents in contact with a plant material, using microwave energy to partition compounds of analytical interest from the sample matrix into the solvent. Heating results in evaporation and generates a pressure on the cell wall, cell rupture, and increase in the yield of the plant metabolites. According with Alupului et al. [79], MAE involves three sequential steps described by: 1) separation of solutes from

active sites of sample under increased temperature and pressure; 2) diffusion of solvent across sample; 3) release of solutes from sample to solvent.

4. In vitro antimicrobial activity of plant-based biopreservatives against *Listeria monocytogenes*

A universal and standardized testing method for antimicrobial activity of biopreservatives is not available yet, although CLSI (Clinical and Laboratory Standard Institute, previously NCCLS) method for antibacterial susceptibility testing, principally designed for antibiotics, has been modified for testing EOs [80]. Therefore, the comparison of published data is complicated, since the outcome of a test can be affected by many factors, such as harvest season, plant component and EOs extraction from plant material, volume of inoculum, microbial growth phase, culture medium and its pH, incubation time and temperature, choice of emulsifier [81-82]. Moreover, the definition of MIC (Minimal Inhibitory Concentration) differs among publications, and different parameters may be chosen, such as Minimum Bactericidal Concentration (MBC) or bacteriostatic concentration. A list of the most frequently used terms in antibacterial activity testing of EOs is presented in Table 3.

Table 3 Terms used in antimicrobial activity testing.

Term	Definition, with reference to EO concentration	Ref.
Minimum Inhibitory Concentration (MIC)	Lowest concentration, expressed in mg l^{-1} (numerically equal to μg ml^{-1}, but we do not recommend the use of such units) that, under defined in vitro conditions, prevents the growth of bacteria within a defined period of time	[83]
	Lowest concentration resulting in maintenance or reduction of inoculum viability	[84]
	Lowest concentration required for complete inhibition of the test organism up to 48 h incubation	[85]
	Lowest concentration inhibiting visible growth of the test organism	[86]
	Lowest concentration resulting in a significant decrease in inoculum viability (>90%)	[87]
Minimum Bactericidal Concentration (MBC)	Lowest concentration, expressed in mg l^{-1}, that under defined in vitro conditions reduces by 99.9% (3 logarithms) the number of organisms in a medium containing a defined inoculum of bacteria, within a defined period of time	[83]
	Concentration where 99.9% or more of the initial inoculum is killed	[84-87]
Optimal Bactericidal Concentration (OBC)	Concentration that results in the maximum proportionate kill within a given time	[83]
	Concentration at which there is the greatest degree of killing in 3 hours	[88]
Minimum Antibacterial Concentration (MAC)	Concentration, below the MIC as defined above, that can exert specified biological effects on bacteria	[83]

Antimicrobial activity assays can be classified technically as diffusion, dilution, and bioautographic methods [89], or functionally as susceptibility testing methods, killing kinetics methods, and interaction test methods. Functional classification will be used in this chapter. Table 4 gathers a selection of MICs for EOs tested against *L. monocytogenes* in vitro, together with EOs principal components and their mechanisms of action. As shown, *Origanum vulgare, Thymus vulgaris, Cinnamomum verum*, and *Syzygium aromaticum* EOs showed antilisterial activity at lower concentration compared with *Thymus* x *porlok*, *Thymus euriocalyx* and *Cinnamomum cassia*, likely due to differences in chemical composition.

4.1. Susceptibility testing

The **disk diffusion method** is the most commonly used, especially in preliminary studies. A paper disk soaked with the EO is placed on the inoculated surface of an agar plate, and the zone of microbial inhibition is measured. Different parameters could affect the result, such as the volume of EO on the paper disks, the thickness of the agar layer and the solvent. Several solvents are reported in literature, such as ethanol, methanol, Tween-20, Tween-80, acetone in combination with Tween-80, polyethylene glycol, propylene glycol, n-hexane and dimethyl sulfoxide, which can create difficulties when comparing different studies [90].

Different dilution methods can be used to evaluate the level of antimicrobial activity in agar or broth. In published studies using dilution methods, varying solvents have been used to incorporate EOs in the medium [91-92], as well as different volumes of inoculum (1-100 μl) [91], which may be spotted [92], or streaked [93] onto to agar surface.

The **agar well test** is useful when EOs and/or large numbers of bacterial isolates have to be screened [48]. To make bacterial growth easily visualized, triphenyl tetrazolium chloride may be added to the growth medium [94-95].

Different techniques are available for **broth dilution**. The most common methods are: optical density (OD), also called turbidity measurement, and viable count. The former method can be automated, while the latter is labour intensive. Measurements of conductance/conductivity and endpoint determination by visual monitoring are less used.

Microdiluition method uses the redox indicator resazurin as a visual marker of the MIC. The results compare favourably with those obtained by viable count and OD measurement, and the method is more sensitive than the agar dilution method [96]. A patented color indicator based on resazurin has been used to determine the MICs for methanolic extracts of plant materials [97] and EOs [98] and the method can be automated by measuring the endpoint by fluorescence instead of visual means. Triphenyl tetrazolium chloride has been used for visual endpoint determination in the evaluation of tea tree oil in broth but the colour change did not fully correlate with MIC [84].

Table 4 MICs of selected essential oils, tested against *Listeria monocytogenes* in vitro.

Plant	Major constituents of essential oils	MIC approximate range µl ml^{-1}	Mechanism of action	Reference
Allium sativum	Allicin (70%)	88 µl ml^{-1}	Induced leakage	[99]
Origanum vulgare	Carvacrol (6 %), thymol (27 %), *r*-cymene (24 %), *g*-terpinene (1.1 %)	0.2 µl ml^{-1}	Released cellular content; permeabilized membranes; leaked potassium and phosphate; dissipated pH gradients	[100]
Satureja montana	Thymol (29 %), *r*-cymene (12 %), linalool (11 %), carvacrol (10.7 %)	0.5 µl ml^{-1}	Structural damages	[101]
Thymus vulgaris	Thymol (31%), *r*-cymene(17 %), carvacrol (12.4%), *g*-terpinene (11.1%)	0.2 µl ml^{-1}	Permeabilized membrane	[100]
Thymus x *porlok*	α-Phellandrene (13.7 %), thymol (31 %), cis sabinene hydroxide (9.6%)	125 µl ml^{-1}	Structural damages; clumping of intracellular material	[102]
Thymus euriocalyx	α-Phellandrene (38.7 %), thymol (31 %), cis sabinene hydroxide (8.1 %)	125 µl ml^{-1}	Structural damages	[102]
Cinnamomum verum	E-cinnamaldehyde (73.35 %), β-caryophyllene (4.09 %), linalool (3.55 %), cinnamyl acetate (2.9 %), eugenol (2.68 %)	0.3 µl ml^{-1}	Released cellular content; permeabilized membranes; leakage and coagulation of cytoplasmatic content	[100]
Cinnamomum cassia	Cinnamaldehyde (73.3 %), cumarin (10.6 %), cinnamic alcohol (2 %)	2640 µg ml^{-1}	Released cellular content; reduced intracellular pH; affected membrane integrity	[100]
Syzygium aromaticum	Eugenol (64 %), eugenyl acetate (16.3 %), caryophyllene (14.5 %)	0.3 µl ml^{-1}	Leaked ATP and potassium ions; released cellular content; affected membrane integrity	[100, 103]

4.2. Killing kinetics

The rapidity of a bactericidal effect or the duration of a bacteriostatic effect can be determined by **time-kill analysis** (survival curve plot) whereby the number of viable cells remaining in broth after the addition of EO is plotted against time, by measuring OD or viable count. The OD of the test suspension and control may be used to calculate an inhibition index [104]. Measurements of conductance can be used to calculate the period elapsing before growth can be detected or Detection Time (DT), after cells treatment with EOs [42, 105].

Comparison of the maximum specific growth rate values (μ_{max}), based on data from viable counts or absorbance measurements, has also been made in several studies [106-107].

4.3. Interaction testing

The **broth dilution checkerboard method** is a valuable tool to determine the antibacterial effects of EOs combinations. The assay is arranged as follows [108]: EO_A is diluted two-fold in vertical orientation, while EO_B is diluted two-fold in horizontal orientation. Different concentrations of EO_A and EO_B are prepared corresponding to 1/2,

1/4, and 1/8 of the MIC values, respectively. The checkerboard method is often combined with calculation of fractional inhibitory concentration indexes (FICI). FICI is calculated as FIC_A+FIC_B, were FIC_A = (MIC_A of the combination /MIC_A alone) and $FIC_{A\ B}$= (MIC_B of the combination /MIC_B alone). The results are interpreted as synergy (FICI<0.5), addition (0.5<FICI≤1), indifference (1<FICI≤4) or antagonism (FICI>4).

5. Mechanism of action of plant-based antimicrobials

Plant extracts are characterized by a very complex and rich composition. Therefore, several mechanisms, acting on specific targets simultaneously, have been proposed to explain their antimicrobial and particularly anti-listerial action. Indeed, particular attention must be payed to phenolic compounds, as they seem to be the principal responsible of antimicrobial action of plant extracts and EOs. In fact, EOs possessing the strongest antibacterial properties against food pathogens contain a high percentage of phenolic compounds, such as carvacrol, thymol and eugenol [90].

Antimicrobial activity of plant extracts is attributed to the presence of different compounds, and particularly of low molecular weight phenols, terpenes and ketones, which also show antimicrobial activity in pure form [109]. Some authors attributed the extent of inhibitory effects of the extracts to their total phenolic concentration and composition [110], although the chemical structure of individual plant extract components affects their particular mode of action and therefore their activity [90]. Specifically, number and position of substitutions in the benzene ring of phenolic acids and the saturated side-chain length influence antimicrobial potential of phenolic acids against several microorganisms. Phenolic acids seem to show greater antimicrobial potential with respect to their corresponding precursors such as catechin and epicatechin. Phenolic compounds appear to disturb the cytoplasmic membrane, and particularly proton motive force and active transport [90].

Tannins and anthocyanins also show antimicrobial activity; the antilisterial activity of tannins, abundant in Ribier grape skin, may be determined by the content and the spatial configuration of *ortho*-dihydroxyphenol groups in the polymer structure [111-112]. In addition, the activity of Ribier juice and skin polymers was found to be pH-dependent, suggesting that the molecular structure or charge could be essential for inhibitory activity. For instance, anthocyanin moiety, stabilized as flavylium cation form, is able to interact with the negatively charged bacterial cell [112].

Furthermore, organic acids are other common constituents of plant extracts. In the non-dissociated form, they can pass through the bacterial cell wall and enter into the cell, where they dissociate, lowering the cytoplasmic pH and disrupting the normal physiology of pH-sensitive bacteria, including *L. monocytogenes*. Organic acids in the anionic form cannot pass through the cell membrane, and therefore they accumulate within the cell, increasing osmotic pressure and disrupting some metabolic functions.

Many of the natural aromas of fruits and vegetables, such as hexanal, 2-(*E*)-hexenal and hexyl acetate exert antilisterial activity. For example, 2-(*E*)- hexenal seems to permeate by passive diffusion across cell membrane. Once inside the cell, its α,β-unsaturated aldehyde moiety reacts with biologically important nucleophilic groups, such as sulphydryl groups in proteins that play a key role in living cells [113].

Ultimately, the solvent employed for EOs extraction and/or the mode of extraction can affect antimicrobial activity; Raudsepp et al [114] recently demonstrated that ethanol infusions of several plants, including siberian rhubarb, tomato, blackcurrant and blueberry, have higher antibacterial activity than water infusions. The cytoplasmic membrane is the principal target of plant extracts, although with different effects: membrane permeability variation, membrane disruption, loss of intracellular constituents, depletion of the proton motive force, damage of enzymes involved in the synthesis of structural components, destruction or inactivation of genetic material [115] and even cell death. Inactivation of cellular enzymes and consequent loss of functionality are also reported in literature. It was suggested that the action of EOs is concentration-dependent, showing how low concentrations inhibit enzymes associated with energy production, whereas higher amounts may precipitate proteins [116].

While aqueous plant extracts need to be in contact with cells to exert antimicrobial activity, EOs are more volatile. Hydrophilic compounds are less volatile, whereas equilibrium is attained among volatile compounds in the headspace [117]. Chemical constituents of essential oils are mainly hydrophobic and can accumulate in the lipid-rich cell environment; thus, vapour pressure plays a key role in EOs toxicity and can be considered an indirect measure of their hydrophobicity. Factors able to increase vapour pressure can enhance their antimicrobial activity increasing their solubility in cell membranes [113]. Lipophilic molecules contained in EOs can accumulate in the lipid bilayer and distort the lipid-protein interaction [118], increasing membrane permeability and therefore causing structural and functional damages. Leakage of intracellular constituents and impairment of microbial enzyme systems can occur [109, 119], and the extensive loss of cell content will cause cell death. Finally, direct interaction of the lipophilic compounds with hydrophobic parts of cell proteins is also possible [120]. On balance, toxicity is related to an optimum range of hydrophobicity [117], whereas aqueous solubility might limit hydrophobic compounds accumulation up to lethal levels in cell membrane [121].

5.1. Analytical approaches

Different analytical approaches have been developed and exploited by many authors to evaluate specific effects of antimicrobial action. By means of **transmission electron microscopy**, Rasooli and colleagues [102] were able to study *L. monocytogenes* cells modification after treatment with two thyme EOs: *Thymus eriocalyx* and *Thymus* x-porlock. Untreated cells showed a continuous thin smooth cell wall, cell membrane and nuclear material, while cells exposed to MIC dilution of *T. x*-porlock had decreased size and were closed together, presumably in an attempt to manage their survival. Moreover, the cell wall underwent degenerative changes, showing splitting of the wall layers. With increasing oil concentrations, the cell wall progressively lost smoothness and uniformity, leading to wall rupture. Cell membrane disruption and lack of cytoplasm was also evident at lower thyme oil concentrations. Cytoplasm lost its even distribution and showed clumping of intracellular materials.

Leakage of intracellular content after exposure to antibacterial agents could be revealed by **measuring potassium leakage**, as an early indicator of membrane damage [122]. Potassium ions can be measured by means of an atomic absorption spectrophotometer [123]. More commonly, leakage is measured as loss of 260-nm absorbing material, after treatment with plant extracts and cells filtration. Results are generally expressed as proportion of the initial OD_{260} [119]. Potassium ions are the principal cytoplasmic cations for bacterial cells, being involved in key function for their vitality, such as activation of cytoplasmic enzymes, regulation of internal pH and mantainance of turgor pressure. Marked leakage of the cytoplasm is considerate a consequence of irreversible damage of the cytoplasmic membrane [124].

Membrane damage can also be simply revealed as **bacteriolysis**, measuring Optical Density at 620 nm, after cell treatment with plant extracts [119]. Although lysis can be due to the activation of specific lytic enzymes, it may be also be indicative of cell wall weakening and subsequent osmotic rupture of cytoplasmic membrane.

In this connection, **flow cytometry** allows evaluating membrane integrity, membrane potential and intracellular enzymes activity by means of a suitable fluorescent probe. Flow cytometry can be considered as a new approach to study the antibacterial activity of plant extracts and EOs [125]. Nguefack et al. [126] applied flow cytometry to estimate cell membrane permeability in *Listeria innocua* treated with three EOs at two concentrations, by using cFDA (carboxyfluorescein diacetate) as fluorochrome. This probe diffuses across the membrane and is converted by unspecific esterases into a membrane-impermeant fluorescent compound, carboxyfluorescein (CF), retained in viable cells with functional cytoplasmic enzymes and intact membrane. Paparella and colleagues [127] used flow cytometry to evaluate the physiological behaviour of *L. monocytogenes* after exposure to cinnamon, thyme and oregano essential oils, applied alone and in combination with NaCl. After treatment with different concentrations, the cells were double stained with cFDA and PI (propidium iodide). Propidium iodide is a membrane-impermeant nucleotide-binding probe, which penetrates cells and stains them only when membrane integrity is lost. Membrane disintegration seemed to be the primary inactivation mechanism of oregano and thyme EOs, while a different mechanism of action was apparently involved in cinnamon EOs treatments, with a lower activity and a minimal membrane damage. Interestingly, NaCl addition boosted membrane disintegration, probably by increasing vapour pressure, thus promoting the interaction between cell membrane and EOs, with a higher impact on membrane integrity. Comparable results were obtained [128], after treatment of *L. monocytogenes* with oregano EO, by means of dual staining with PI and SYTO 9.

Electron Paramagnetic Resonance was applied by Serio et al. [129] to investigate the effect of *Origanum vulgare* EO on *L. monocytogenes*. Embedding a nitroxide free-radical into the membrane and studying the change on EPR signal, it was possible to evaluate changes on microenvironmental order and fluidity. The authors studied living cells and were therefore able to register cytoplasmic membrane modification in response to EO application during time. After treatment up to 0.50% EO, the cells appeared able to modify membrane organization and to increase its order, as adaptative cell response, presumably to block, or at least to obstruct active molecules entrance and partition into the membrane. At higher concentrations, the cells could not hamper the oil effect and therefore the oil passed through the membrane, progressively disturbing its order and increasing its permeability. When cells were treated with sodium azide, then exposed to EO, their energy metabolism was impaired and therefore cells could not use energy to modulate membrane fluidity and to obstacolate the oil entrance; thus even low oil quantities had a large effect.

Carvacrol and thymol are the major components of oregano and thyme EOs, which explains the high activity of both oils on *L. monocytogenes.* Among substituted aromatic molecules, also eugenol and cinnamaldehyde are included, and their mechanism of action on *L. monocytogenes* seems to be different from carvacrol and thymol, being attributed to a reduction of energy generation inside the cell. **ATP pool measure** is a very useful tool to investigate energetic metabolism. Gill and Holley [103], measured extracellular and intracellular ATP levels in previously energized cells with glucose, and in cells treated with several antimicrobial agents before glucose addition. Also intracellular and external ATP levels were assayed by a continuous light output luciferase reaction. Cinnamaldehyde was less effective that eugenol, which had a dose-depending bactericidal effect on *L. monocytogenes* cells. After treatment, eugenol reduced intracellular ATP, without any corresponding extracellular increase, and therefore lethality could not be explained as a consequence of cytoplasmic membrane disruption. On the contrary, results suggested inhibition of glucose utilization, probably as a consequence of the inhibition of enzymes involved in glycolysis. As a consequence of a possible membrane damage caused by eugenol, ATP pools could be mantained by fermentative metabolism but treated cells were severely injured and spent most of the energy attempting to restore membrane gradient.

According to Raybaudi-Massilia and colleagues [116], cinnamon oil and cinnamaldehyde seem to produce a decrease in the intracellular ATP by ATPase activity, without apparent changes on *Listeria* cell membrane. This event could be attributed to the interaction of cinnamaldehyde with the cell membrane, which causes a disruption sufficient to disperse the proton motive force by leakage of small ions and not of larger molecules such as ATP.

5.2. Synergism

Antimicrobial interaction can produce synergism by sequential inhibition of common bacterial pathways, inhibition of protective enzymes, and cell wall active agents to enhance the uptake of other antimicrobials [130]. Synergism can explain the greater antimicrobial action of whole EOs with respect to the major components alone [95] or mixed together, as well as the best results obtained for EOs with richness and variety in volatile compounds, including precursors and final metabolites [131]. This observation suggests that also minor compounds may be critical to the activity. Specifically, some authors [131-132] observed a synergism between carvacrol and its precursor *p*-cymene. *p*-cymene alone has a low antibacterial activity but swells bacterial cells and probably enables carvacrol to be more easily transported into cells, expanding the membrane. This hypothesis supports the role of carvacrol as an exchanger of cations [132].

Mechanisms of antagonism are less known but they seem to include chemical interactions among compounds that have the same target. For instance, non-oxygenated monoterpene hydrocarbons, such as γ-terpinene and *p*-cymene, produce antagonistic effects, reducing the aqueous terpene solubility and therefore microbial availability of active compounds [121].

Although EOs have good antibacterial performances in vitro, greater concentrations are usually necessary to achieve the same effect in foods. Therefore a number of potential synergists have been suggested to be used with EOs: low pH, low water activity, chelators, low oxygen tension, mild heat, and raised pressure, although not all of these have been investigated in foodstuffs. Synergism between NaCl and mint oil against *L. monocytogenes* was found in taramosalata [105]. The combined effect of carvone (5 mmol l^{-1}) and mild heat treatment (45°C, 30 min) on exponentially growing cells of *L. monocytogenes* grown at 8°C has been studied [133]. Separately, the two treatments demonstrated no loss in viability but a decrease of 1.3 Log units in viable cell numbers was observed when they were combined. The authors hypothesized that the phospholipids composition of the cytoplasmic membrane of cells grown at 8°C had a higher degree of unsaturation to maintain fluidity and function at low temperatures. This increased fluidity would enable carvone to dissolve more easily into the lipid bilayer of cells grown at 8°C than into the bilayer of cells grown at 45°C.

Thymol and carvacrol have been shown to have a synergistic effect with HHP [134]. The viable numbers of mid-exponential phase *L. monocytogenes* cells were reduced more by combined treatment with 300 MPa HHP and 3 mmol l^{-1} thymol or carvacrol than by the separate treatments. Since HHP is believed to cause damage to the cell membrane, it is suggested that this common target is the root of the observed synergism [133]. The combinations of 200 µl l^{-1} of several EOs with HHP were tested for their effectiveness in inactivation of *L. monocytogenes* (initial concentration: $3 \cdot 10^7$ CFU ml^{-1}) in pH 4.0 and pH 7.0 buffers. Some compounds, such as (+)-limonene, carvacrol, *Citrus reticulata* L. EO, *Thymus algeriensis* L. EO or *Citrus sinensis* L. EO inactivated about 4–5 Log of the initial population, showing a significant synergistic effect.

5.3. Sublethal injuries

An important issue for evaluating antimicrobials is the possibility to cause sublethal cell damages. Discrimination of sublethally stressed and injured cells is essential, as these subpopulations can be critical if cell recovery becomes possible [127]. Within the same cell population, a recognized heterogeneity exists in sensitivity against stressing factors [135],which has been well documented in *L. monocytogenes* [127,129]. Such heterogeneity implies different changes in the membrane and therefore a different physiological state after exposure to biopreservatives. Sublethal injury of microbial cell membrane may alter its permeability and affect osmoregulation [119]. Consequently, the loss of tolerance to salt or to other potentially toxic compounds could be an effect of membrane damage in sublethally injured bacteria.

Moreover, survivors to antimicrobial treatments show a different lag phase, which can be interpreted as a measure of cell stress and damages. In fact, after exposure to stressing conditions, lag phase is dependent on the time required to repair damages [136]. For this reason, growth medium composition in stressing conditions is very important, as the presence of osmoprotectants favours recovery of sublethally stressed cells.

6. Antilisterial activity in food systems

6.1. Essential oils in foods

Food composition and structure have a significant effect on the final outcome of EOs antibacterial activity. Reaction with proteins, carbohydrates, lipids and food additives can decrease the activity of antimicrobials. For example, a reaction between carvacrol and proteins has been put forward as a limiting factor [137]. Carbohydrates in foods do not

appear to protect bacteria from the action of EOs as much as fat and proteins [138]. Instead, fat exerts a greater influence on the antibacterial effect of EOs in comparison with pH; in fact, the susceptibility of bacteria to the antimicrobial effect of EOs increases with pH decrease [105]. At low pH, EOs show higher hydrophobicity and dissolve better in the lipids of cell membranes. Water solubility or hydrophilic properties are needed for EO activity in the water phase, where microbial growth occurs but lipophilic properties are important for membrane interaction. Thus, like emulsifiers, EOs require a specific hydrophile-lipophile balance for optimal activity.

Studies on the growth characteristics of *L. monocytogenes* in oil-in-water emulsions have shown that, depending on the mean droplet size, the organism can grow in films, colonies or as planktonic cells; within colonies, bacteria may be protected from the action of EOs. Bacterial sensitivity can be affected by intrinsic properties of foods (e.g. composition, additives, pH) but also by extrinsic determinants (e.g. temperature, packaging) [105,138]. A significant amount of studies has been focused on the addition or incorporation of food antimicrobials to packaging materials to inhibit spoilers or pathogens.

6.2. Meat and meat products

Oregano EO at 1% showed a significant antibacterial and preservative effect on chicken fillet samples [139]. In combination with N,O-carboxymethyl chitosan, a complete inhibition of *L. monocytogenes* was observed after 2 days in the low inoculum (10^3 CFU g^{-1}) experiment and 4 days in the high inoculum (10^5 CFU g^{-1}) experiment. Encapsulated rosemary EO showed better antimicrobial effect compared to standard rosemary EO against *L. monocytogenes* in pork liver sausage [140].

Combination of 1% clove and oregano EOs in broth culture showed inhibitory effect against *L. monocytogenes* in vitro, while it was not effective in meat slurry [141]. A high activity of eugenol and coriander, clove, oregano and thyme oils against *L. monocytogenes* and spoilage microbiota has been reported in meat products, while mustard, cilantro, mint and sage oils were less effective or ineffective [90]. In particular, a synergistic effect of *Satureja montana* and *Pistacia lentiscus* EOs was observed in minced beef inoculated with 10^3 CFU g^{-1} *L. monocytogenes* [142].

Combinations of EOs and nisin showed enhanced antimicrobial activity against *L. monocytogenes,* and chlorophyllin-gelatin films and coating applications successfully reduced *L. monocytogenes* in cooked frankfurters [143]. Individual extracts of clove, rosemary, cassia bark and liquorice demonstrated strong antimicrobial activity, and the mixture of rosemary and liquorice extracts was the best inhibitor of *L. monocytogenes* in modified atmosphere-packaged fresh pork and vacuum-packaged ham slices stored at 4°C [144].

6.3. Fish

Application of EOs on the surface of whole fish or as coating for shrimps has been proposed to inhibit *L. monocytogenes* and natural spoilage microbial populations [90,124]. Recently, Rabiei et al. [145] evaluated the antimicrobial effect of black zira (*Bunium persicum*) EO against *L. monocytogenes* (10^4 CFU ml^{-1}) in fish model systems for 12 days at 4°C. Black zira EO performance significantly increased in broth with 4% NaCl. The effect of NaCl on different strains of *L. monocytogenes* in fish model systems had already been observed by Serio et al. [13]; in this study, salt addition effect started at 4% addition and was evident at 7% in Salmon Broth, probably due to a synergistic effect of NaCl and sub-optimal nutrient concentration in the medium.

The combined effect of *Zataria multiflora* EO and nisin (N) on *L. monocytogenes* (10^3 CFU ml^{-1}) growth in salted fish fillets (brine salted at 4% NaCl) at 4°C was evaluated; the best inhibitory effects were obtained at combinations of EO = 0.405% and Nisin = 0.25 μg ml^{-1} [146]. Lin et al. [147] evaluated the combined effect of cranberry and oregano water-soluble extracts on *L. monocytogenes* in meat and fish samples; the effect was clearly synergistic in cod samples when extracts were mixed at a ratio of 75% oregano and 25% cranberry (wt/wt) and was further increased by lactic acid.

6.4. Dairy products

The antibacterial activity of oregano and thyme EOs added to feta cheese inoculated with *L. monocytogenes* was investigated during cheese storage under modified atmosphere packaging containing 50% CO_2 and 50% N_2 at 4°C [148]. After treatment of feta samples with oregano EO, *L. monocytogenes* survived up to 18 days at 0.1 ml 100 g^{-1} and 14 days at 0.2 ml 100 g^{-1}.

The influence of HHP on inactivation of *L. monocytogenes* in a yogurt drink (ayran) was quantified with or without addition of mint EO [149]. Treatment of ayran samples at 600 MPa for 300 s reduced *L. monocytogenes* by more than 5 Log at room temperature. Addition of mint EO further enhanced inactivation by more than 1 Log CFU l^{-1}. Combination of mint EO with HHP provided a reduction in pressure treatment severity by 100 and 300 MPa or by 210 s to achieve the same inactivation with respect to HHP alone.

6.5. Fruit and Vegetables

The antimicrobial effects of EOs vary depending on the EO used and the type of vegetable involved. Application of EOs in vegetables gave more successful results in washing water, due to the low fat content of the products. Thyme EO is one of the most effective against *L. monocytogenes* and has a wide spectrum of antimicrobial activity [48,100,150].
An edible coating contaning trans-cinnamaldehyde (0.5% w/w), coupled with γ-irradiation, was investigated against *Listeria* spp. inoculated in peeled mini-carrots [151]. A 1.29 Log reduction of *Listeria* spp. count was observed in carrots packed under air after 21 days of storage, but the combination of irradiation with antimicrobial coating seemed to play an important role in enhancing radiosensitization of *Listeria* spp. to γ-irradiation.

The anti-listerial effectiveness of different EOs and herbs on a range of modified atmosphere packaged fresh-cut vegetables was evaluated, obtaining the following effect order: thyme EO > oregano EO > rosemary herb > rosemary EO [152]. However, all EOs damaged product appearance, likely due to the dark colour of the oils and phytotoxic effects. Fresh rosemary herb was only effective when stomached with the product. In this study, EOs effectiveness was clearly dependent on food type, with greater effects on carrot discs and shredded cabbage than on shredded lettuce. However, the anti-listerial effects of rosemary were lost by the end of storage, possibly due to the higher levels of microorganisms consuming the antimicrobials.
By the same token, De Azeredo et al. [153] observed undesirable effects on the sensory quality of minimally processed vegetables, treated with rosemary and oregano EOs. Undesirable sensory effects can be limited by careful matching of EO and food type, by diluting EOs, or by using combinations of EOs. Recently, Espina et al. [134] investigated the potential of EOs and single EOs components, combined with HHP in the inactivation of foodborne pathogens in fruit juices. The most effective biopreservatives were (+)-limonene, carvacrol, *Citrus reticulata* L. EO, *Thymus algeriensis* L. EO and *Citrus sinensis* L. EO, which were capable of inactivating about 4-5 Log cycles of the initial cell populations in combination with HHP.

7. Conclusions

There is a growing interest in plant-based intervention strategies for *L. monocytogenes* control in foods, proved by the increasing number of published papers in recent years. The research literature indicates that several antimicrobials of plant origin can effectively reduce or inhibit this organism, and thus have a potential to become a good alternative to synthetic antimicrobials. Different technologies can be applied to obtain plant-based biopreservatives, although in many cases valuable bioactive compounds get lost during the process. The development of new cost effective extraction procedures, coupled with purification and standardization, will avoid loss of functional properties of active compounds and reduce the cost of biopreservatives, which is currently a limit for food applications.

Recent literature clearly indicates that plant extracts and EOs must be considered as natural phytocomplexes, whose activity mostly results from additive or synergistic effects of pure components. Therefore, to study and compare plant-based biopreservatives, it is of paramount importance to standardize methods for evaluating their antimicrobial activity. Furthermore, the use of natural antimicrobials in combination with other technologies in a multi-hurdle preservation approach can significantly increase performance and open up new perspectives for industrial applications. Specifically, this strategy can be particularly valuable to increase food safety and extend product shelf-life. In this respect, recent trends in food regulations worldwide stress the need to control *L. monocytogenes* thoughout the food chain, while reducing the addition of traditional preservatives.

As a result, research on biopreservatives will allow obtaining effective results at low doses, with minimal impact on sensory quality and cost of food products. Moreover, it is expected that plant-based antimicrobials showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. However, phytocomplexes should be considered as natural drugs, to be used after careful toxicology evaluation. Indeed, it is true that many plant extracts have been consumed by humans for thousands of years; however, typical toxicological information such as acceptable daily intake (ADI) or no observed adverse effect level (NOEL) are not available for them. In fact, batch-wise compositional variability and lack of standardization in extraction hamper identification of ADI or NOEL for plant-based biopreservatives.

In conclusion, the applications of plant-based strategies for *L. monocytogenes* control in foods are likely to grow, due to the consumer demand for natural products (green label) and the industry interest for additive decrease (clean label). Research has the important task of providing knowledge to increase effectiveness and safety of biopreservatives, at the same time reducing their cost and variability.

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