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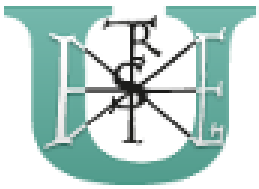

SZENT ISTVÁN UNIVERSITY

FACULTY OF FOOD SCIENCE, BUDAPEST

The antimicrobial and resistance modifying activities of *Nigella sativa* oil

Doctoral (Ph.D.) dissertation by

Ahmad Mouwakeh

Under the supervision of

Professor Csilla Mohácsi-Farkas and

Dr. Gabriella Kiskó

Szent István University

Faculty of Food Science

Department of Microbiology and Biotechnology

Budapest, 2018

PhD school

Name: PhD School of Foood Science

Field: Food Science

head: **Dr. Gyula Vatai**

Professor, Dsc

Szent István University, Faculty of Food Science

Department of Food Engineering,

Supervisors: **Dr. Csilla Mohácsi-Farkas**

Professor, PhD

Szent István University, Faculty of Food Science

Department of Micobiology and Biotechnology

Dr. Kiskó Gabriella

Associate professor, PhD

Szent István University, Faculty of Food Science

Department of Micobiology and Biotechnology

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Signature of Head of PhD School Signature of Supervisor

……..... Signature of Supervisor

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LIST OF ABBREVIATIONS

ABC	Adenosine Triphosphate (ATP)-Binding Cassette
B.	*Bacillus*
C.	*Chlamydia*
Car	Carvacrol
CCCP	Carbonyle Cyanide m-ChloroPhenylhydrazone
CDC	Disease Control and Prevention
CF	Cystic Fibrosis
CFU	Colony Forming Unit
Cip	Ciprofloxacin
CLSM	Confocal Laser Scanning Microscopy
CO	Crude Oil
COM	Chronic Otitis Media
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl Sulfoxide
E.	*Escherichia*
ECDC	European Center for Disease Prevention and Control
EO	Essential Oil
EP	Efflux Pump
EPI	Efflux Pumps Inhibitor
EPS	Extracellular Polymeric Substances
Ery	Erythromycin
EtBr	Ethidium Bromide
EtOH	Ethanol
FDA	Food and Drug Administrations
GC-MS	Gas Chromatography-Mass Spectrometry
GLC	Gas-Liquid chromatography
GRAS	Generally Recognized As Safe
HPP	High-Pressure Processing
IC50	Inhibitory Concentration
IFU	Inclusion Forming Units
L.	*Listeria*
L.	Linnaeus

LGV	*Lymphogranuloma venereum*
LRI	Linear Retention Indices
MATE	Multidrug And Toxic Compound Extrusion
MDR	Multidrug Resistance
MFS	Major Facilitator Superfamily
MH	Monoterpenes Hydrocarbon
MIC	Minimal Inhibitory Concentration
MID	Minimal Infection Dose
MP	Minced Pork
MP-CO+Lm	Minced Pork with 2% *Nigella sative* Crude Oil and inoculated with *Listeria monocytogenes,*
MP-Lm	Minced Pork inoculated with *Listeria monocytogenes,*
MTT	Thiazolyl Blue Tetrazolium Bromide
N.	*Nigella*
NHI	National Health Institute
OD	Optical Density
OM	Oxygenated Monoterpenes
P.	*Pseudomonas*
PA*β*N	Phenylalanine Arginylβ-Naphthylamide
PBS	Phosphate Buffered Saline
p-cy	p-cymene
PDR	Pandrug-resistant
PEF	Pulse Electric Field
PUFA	Polyunsaturated Fatty Acid
QS	Quorum Sensing
Res	reserpine
RFU	Relative Fluorescence Units
RND	Resistance-Nodulation-cell Division
RT-Qpcr	Real-Time Reverse Transcriptase Quantitative Polymerase
S.	*Staphylococcus*
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SH	Sesquiterpene Hydrocarbon

SMR	Small Multidrug Resistance
SPG	Sucrose-Phosphate-Glutamic
STI	Sexually Transmitted Infection
Tet	Tetracycline
TFA	Total Saturated Fatty Acid
TGE	Tryptic Glucose Extract
Thq	Thymoquinone
TIC	Total Ion Current
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WHO	World Health Organization
XDR	extensively drug-resistant
YOPI	Young, Old, Pregnant, Immunosupressed

1. INTRODUCTION

More than 200 known diseases are transmitted through food by a variety of agents that include bacteria, fungi, viruses, and parasites. Food quality and safety is becoming an important public health issue due to the increased risk of foodborne illness over the last 20 years and the rapid spread of multidrug resistant bacteria. This has necessitated the discovery of new antibacterial and resistance modifying agents.

Microbial resistance to antibiotics was predicted in 1945 due to the mass-production of penicillin, and has now become a major concern in medicine throughout the world. Bacterial resistance targets a single class of antibiotics as well as multiple classes. It is essential to point out that two categories of resistance can be distinguished: innate resistance, which microorganisms acquire by heredity and acquired resistance, which is caused by different factors such as mutation and horizontal gene transfer.

Bacteria form so-called biofilm communities that constitute protection against adverse environmental conditions, allow better access to nutrients and increase the genetic diversity. Compared to the free-floating cells, these communities show increased resistance to disinfectants and antibiotics. Biofilm formation is an important issue in food industries due to the potential contamination of food products with pathogenic and spoilage microorganisms. Food spoilage and deterioration by biofilm contamination results in huge economic losses and deficiency of food quality and safety. It is also an increasing problem in the medical field. A significant proportion of nosocomial infections are linked to biofilms. Treatment of this infections is limited by high antibiotic resistance of biofilm bacteria. In recent years, the resistance of biofilms to antimicrobials has led to the search of novel antimicrobial compounds.

The interest in plant products as alternative antimicrobial agents to control pathogenic microorganisms has been increased due to the big number of antibiotic resistant bacteria. A major group of plants' antimicrobial compounds is represented by essential oils, which are complex mixtures of volatile secondary metabolites. They are used in the food industry because of their preservative potency and their antimicrobial effect against foodborne pathogens. Essential oils and their bioactive compounds, besides their antimicrobial effect, can increase antimicrobial activity of some antibiotics.

Antibacterial constituents in plant extracts have a wide range of mechanisms of actions: inhibiting bacterial virulence, direct bactericidal action, improving the wound-healing response, and improving the activity of antibiotics through synergy.

On the other hand, the use of plant extracts as pharmaceuticals presents some limitations, such as the variation in the soil quality, stages of maturity of the plants, seasonal variations, and different climates, and extraction method can all have an impact on the concentrations of bioactive constituents present in the final extract. Importantly, toxicity such as skin irritation, allergy and respiratory depression in infants must be thoroughly studied before plant extracts can be widely used against clinical infections. Finally, bacteria might as well develop resistance to the bioactive compounds present in plant extracts.

In the present study, different organisms were chosen to model: foodborne spoilage, foodborne pathogens, multidrug resistant, and intracellular pathogen bacteria.

Objectives

Foodborne pathogenic bacteria and antibiotic resistant organisms represent an increasing risk to the consumer. The overall objective of my study was to study *Nigella sativa* oils (crude oil and essential oil) as active antimicrobial and resistance modifying agents.

The specific objectives of my research were:

1- to determine the fatty acids composition of the crude oil;

2- to determine the active compounds of the essential oil;

3- to study the antibacterial effect of the crude oil and essential oil against food spoilage organisms, foodborne and non-foodborne pathogens;

4- to investigate the synergistic activity between the crude oil and nisin against *Bacillus* spores;

5- to assess the resistance modifying activity of the essential oil, thymoquinone, carvacrol and p-cymene against *Listeria monocytogenes* and *Staphylococcus aureus*;

6- to assess the anti-biofilm activity of the essential oil, thymoquinone, carvacrol and p-cymene.

2. LITERATURE REVIEW

2.1. *Nigella sativa* and its active compounds

2.1.1. *N. sativa* oils

N. sativa L. is an annual flowering plant. It grows to 20–30 cm (7.9–11.8 inch) tall and has linear lanceolate leaves, commonly known as black seed or black cumin, that has been traditionally used in the Arabian countries (Sayed, 1980), as an herb, pressed seed oil and as a spice. Many healing effects related to respiratory, stomach and intestinal diseases, circulatory and immune system support and for the general well-being are attributed to it. The seed oil of *N. sativa* has been reported to have antioxidant, cytostatic, antibacterial and anti-inflammatory activity (Toma et al., 2010). The nutritional compositions of *N. sativa* are vitamins, carbohydrates, mineral elements, fats and proteins that include eight or nine essential amino acids (Forouzanfar et al., 2014).

N. sativa essential oil contains significant amounts of phenolic compounds such as p-cymene, thymoquinone, and carvacrol which might be the reason of their potential antimicrobial activity. The strength of inhibition and the spectrum of antimicrobial activity of *N. sativa* essential oil suggest that interactions between individual components led to the overall activity (Hassanien et al., 2015). Different extracts of *N. sativa* have a broad antimicrobial spectrum including Gram-negative, Gram-positive bacteria, viruses, parasites and fungi. The effectiveness of *N. sativa* seeds is variable and depends on species of target microorganisms (Forouzanfar et al., 2014).

Over the last few years, increased interest in cold-pressed oils has been observed as these oils have better quality than those after refining (Parry et al., 2006). Cold-pressing involves no heat or chemical treatment. Cold-pressed black cumin seed oil may contain a higher level of hydrophilic phytochemicals including natural antioxidants. Furthermore, antimicrobial activity of natural extracts is closely linked with their phenolic content. Therefore, oils were rich in phenolics and other bioactive compounds may serve as potential natural antimicrobial agents (Ramadan, 2013).

Before antibiotics, diseases were treated, if at all, by plants. The interest in plant products as alternative antimicrobial agents to control pathogenic microorganisms has increased due to the big number of antibiotic resistant bacteria (Hemaiswarya et al., 2008). Plants and their essential oils are potentially useful sources of antimicrobial compounds. Numerous studies have been published on the antimicrobial activities of plant compounds against different microbes, including food-borne pathogens (Friedman et al., 2002). As a source of natural microbial agents, essential oils (EOs), which are complex mixtures of volatile secondary metabolites, are becoming more popular due to their wide applications as food preservative, complementary medicine and therapeutic agents (Rowsni et al., 2014).

EO is a concentrated hydrophobic liquid containing volatile aroma compounds from the plant, extracted from aromatic plant materials and known as fragrant oils, steam volatile oils, aromatic oils, ethereal oils. EOs are used in perfumery, cosmetics, medicine, aromatherapy, incense, household cleaning products and for flavoring food and drink. The majorities are stable and contain natural antioxidants and natural antimicrobial compounds as on cinnamon (Nanasombat and Wimuttigosol, 2011). EOs are isolated from plants by hydrodistillation, steam distillation, solvent extraction, soxhelt extraction, supercritical fluid extraction, microwave-assisted hydrodistillation, ultrasound-assisted extraction, solvent-free microwave extraction and microwave hydro diffusion and gravity (Rassem et al., 2016).

While individual bioactive compounds have been found to have effective antimicrobial activity, very often, the whole EO has greater antibacterial activity than their major component alone, suggesting synergy amongst the components of the EO (Tisserand and Young, 2014). Essential oils and their bioactive compounds, besides their antimicrobial effect, can increase antimicrobial activity of some antibiotics (Langveld et al., 2014).

The main constituents of essential oils, mono- and sesquiterpenes, phenols, alcohols, ethers, aldehydes and ketones, are responsible for the biological activity of aromatic and medicinal plants as well as for their fragrance. Due to these properties, spices and herbs have been added to food since ancient time, not only as flavoring agents but also as preservatives (Kalemba et al., 2003). Some well-known plant-derived bioactive compounds are summarized in Table 1.

The wide and various range of action of each individual component of the EOs could be considered as the defining characteristic of the antibacterial activity of EOs. While current

antibiotics tend to target one specific molecule in bacteria, any given active ingredient in EOs might have a whole range of different actions, primarily focused in membrane destabilization, but many other mechanisms both intra- and extra-cellular have been described.

Table 1: Antimicrobial constituents of plants

Common name	**Latin name**	**Antimicrobial constituents**	**Reference**
Aloe vera	*Aloe vera*	p-Coumaric acid	(Lawrence et al., 2009)
		Cinnamic accid	
		Pyrocatechol	
		Ascorbic acid	
Black tea	*Camellia sinensis*	Tannic acid	(Payne et al., 2013)
Cinnamon bark	*Cinnamomum cassia*	Cinnamaldehyde	(Lang et al., 2016)
Eucalyptus	*Eucalyptus citriodora*	1,8-cineole (Eucalyptol)	(Charles et al., 2004)
		α-pinene	
Garlic	*Allium sativum*	Allicin	(Kumar et al., 2016)
Oregano	*Origanum vulgare*	Carvacrol	(Di Pasqua et al., 2006)
Peppermint	*Mentha piperita*	Menthol	(Charles et al., 2004)
Black pepper	*Piper nigrum*	Piperine	(Srinivasan, 2009)
Nutmeg	*Myristica fragrans*	Sabinen	(Gupta et al., 2013)
Tea tree	*Melaleuca alternifolia*	Terpinen-4-ol	(Hammer et al., 2006
		1,8-cineole (Eucalyptol)	
Thyme	*Thymus vulgaris*	Thymol	(Di Pasqua et al., 2006)
Rose	*Rosa canina*	Tellimagrandin I	(Shiota et al., 2004)
		Rugosin	
Wintergreen	*Gaultheria procumbens*	Methyl salicylate	(Charles et al., 2004)

Mechanisms of effective antibacterial constituents in EOs range from being directly bactericidal (Horne et al., 2001), to decrease bacterial virulence (Burt et al., 2007), to improve the immune response (Maenthaisong et al., 2007), and improving the activity of antibiotics through synergy (Shiota et al., 2004).

The major characteristic of EOs components regarding antibacterial activity is their hydrophobic nature (Burt et al., 2007). Many components of EOs have one or more phenol group, allowing them to be lipophilic and act on membranes. This can lead to increased membrane permeability, interruption of cellular respiration, alteration of membrane transport and enzyme function (Cox et al., 2000), action potential disruption (Burt et al.,

2007), flagellin disruption (Burt et al., 2007), and decreased toxin release (Turchi et al., 2017).

Because of their predominant effect on cell membranes, the antibacterial effect of EOs is more effective on Gram-positive bacteria, given their comparably thick cell wall as compared to Gram-negative bacteria. However, small, hydrophilic solutes might be able to pass through the outer membrane of Gram-negative bacteria via porins, and this leads to effectiveness of some EO constituents against Gram-negative bacteria. In addition to the effect that EOs have on the bacterial membrane, effects on the cytoplasm e.g. DNA synthesis (Lou et al., 2012) and effects on bacterial communication or quorum sensing have also been described (Szabó et al., 2010).

2.1.2. Thymoquinone

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) (Fig. 1) is one of the most active constituent of *Nigella sativa* and has different beneficial properties. Focus on antimicrobial effects, different extracts of *N. sativa* as well as thymoquinone, have a broad antimicrobial spectrum including Gram-negative, Gram-positive bacteria, viruses, parasites and fungi. The effectiveness of *N. sativa* seeds and thymoquinone is variable and depends on species of target microorganisms (Forounzafar et al., 2014).

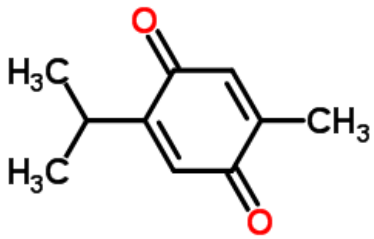


Figure 1: Structure of thymoquinone (Internet 1)

2.1.3. Carvacrol

The phenolic monoterpene carvacrol (2-methyl-5-(1-methylethyl) (Fig. 2) phenil, isomeric with thymol) is a major component of the essential oils of plants of the Labiatae family, including *Origanum* and *Thymus*, which are commonly used as seasoning and in traditional medicine since ancient times (Nostro and Papalia, 2012). Carvacrol has been

classified as GRAS (Generally Recognized as Safe) and approved for food use (Hyldgaard et al., 2012). Beside anti-inflammatory, antioxidant, antitumor, analgesic, anti-hepatotoxic, and insecticidal properties, several studies have demonstrated that carvacrol has antimicrobial properties (Hyldgaard et al., 2012).

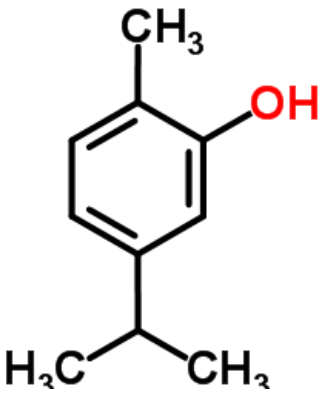


Figure 2: Structure of carvacrol (Internet 2)

Carvacrol is a volatile molecule which evaporates easily, and its vapor phase has shown antimicrobial activity (Nostro and Papalia, 2012; Magi et al., 2015; Suntres et al., 2015). Obaidat et al. (2009) have reported the efficacy of carvacrol vapor against food-borne bacteria *Escherichia coli* O157:H7 and *Salmonella* on the surface of freshly produced vegetables such as lettuce, spinach and tomatoes. Also, Inouye et al. (2001) investigated the potential role of essential oils in the inhalation therapy. Carvacrol was effective against respiratory tract pathogens such as *Hemophilus infuenzae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*.

The activity of carvacrol is extended to drug-resistant microorganisms. For example, the activity of this molecule against pathogens such as *Staphylococcus aureus* and *S. epidermidis* methicillin-resistance is well-known (Nostro et al., 2004). Carvacrol is also effective in reducing the resistance of *Salmonella Typhimurium* SGI 1 (tet A), *Streptococcus pyogenes* ermB, *S. aureus* blaZ, *E. coli* N00 666 to many antibiotics (Palaniappan and Holley, 2010). Moreover, its antimicrobial activity against resistant bacteria can be also potentiated when combined with antibiotics (Magi et al., 2015).

2.1.4. p-cymene

The carvacrol precursor p-cymene (1-methyl-4-(1-methylethyl)-benzene) (Fig. 3) is found in more than 100 plant species. It is a monoterpene that has a benzene ring without

any functional groups on its side chains. p-cymene is not an efficient antimicrobial compound when used alone (Bagamboula et al., 2004), but it potentiates activity of compounds like carvacrol. It has a high affinity for membranes and causes membrane expansions and affects the membrane potential of intact cells (Ultee et al., 2002).

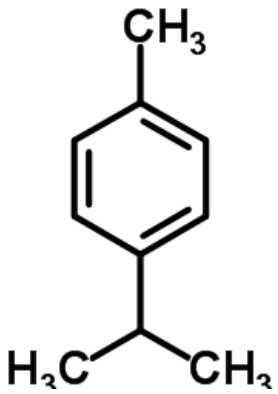


Figure 3: Structure of p-cymene (Internet 3)

2.2. Organisms included in the study

2.2.1. *Staphylococcus aureus*

Staphylococcus aureus is found to colonize several animal species, including humans. In 2016, 311 samples tested in Hungary at farm level, 165 (53%) were positive for *S. aureus*. In the same year in Italy from the monitoring of e.g. milk from 110 out of 165 samples (23.8%) were positive for *S. aureus*. Consumption of raw milk or recontaminated milk increases the chances of infections with foodborne pathogens or toxins i.e., *S. aureus* enterotoxins (EFSA, 2017). Enterotoxigenic strains of *S. aureus* have been reported to cause a number of diseases or food poisoning outbreaks, the most recent large scale outbreak occurred during June 2000 in Japan caused by consumption of low-fat milk produced from skim milk powder contaminated with *S. aureus* enterotoxin A (Asao et al., 2003). The frequencies of hospital acquired staphylococcal infections have increased during the last decades. Treatment of these infections is frequently difficoult because of the emergence of multidrug resistance strains. Both food safety and medical side motivated to choose this bacterium to study.

S. aureus is Gram-positive bacterium that is cocci-shaped (0.5-1.5 µm) and tend to be arranged in cluster that are described as “grape-like”. The cells are non-motile and non-spore-forming. These organisms can grow aerobically or anaerobically (facultative) and at

temperatures between 18-40°C. On media, the organism is resistant to dry conditions and can tolerate up to 10% salt concentrations. Colonies are often golden or yellow (aureus means golden or yellow). Typical biochemical properties include catalase positivity (all pathogenic *Staphylococcus* species), coagulase positivity (to differentiate *Staphylococcus aureus* from other *Staphylococcus* species and to differentiate potentially pathogenic *Staphylococcus aureus* from other non-pathogenic *Staphylococcus aureus* strains).

There are more than 50 species and sub-species of staphylococci of which *S. aureus* is the most human pathogenic staphylococcal species, and the most common cause of fatal skin and soft-tissue infection (Kakarla et al., 2017). Even with clinical availability of antimicrobials against *S. aureus* infections, the death rate associated with infection is about 20-25% because of the development of drug-resistant *S. aureus* strain like methicillin resistant *S. aureus* (MRSA) (Fridkin et al., 2005). It has very resistant properties to which the bacterial efflux pump mechanism is contributed. Table 2 summarizes the efflux pump families in *S. aureus* as examples of some of their transport proteins.

Table 2: Efflux pump families in *S. aureus*

Efflux pump family	**Proteins**	**Source**
MF	NorA, QacA	Nishino and Yamaguchi, 2001
SMR	QacC	
ABC	MsrA	
MATE	mepA	Couto et al., 2008

S. aureus is both a commensal and an extremely versatile pathogen in humans, causing different syndromes: (i) superficial lesions such as skin abscesses and wound infections; (ii) deep-seated and systemic infections such as osteomyelitis, endocarditis, pneumonia, and bacteremia; and (iii) toxemic syndromes such as toxic shock syndrome. Furthermore, it causes staphylococcal scarlet fever (both due to toxic shock syndrome toxin 1 and staphylococcal enterotoxins, staphylococcal scalded-skin syndrome (due to exfoliatins), and staphylococcal food poisoning (due to staphylococcal enterotoxins) (Jarraud et al., 2002). During the past 60 years, the number of incidence of *S. aureus* disease increased from 3 per 100 000 person-years, up to 30 per 100 000 person-years (Tong et al., 2015).

Around 20-40% of the human population are carriers and some humans are intermediate carriers whereas others are persistent carries (Foster, 2009). *S. aureus* has also been isolated from the surfaces of live poultry (Devriese et al., 1975). Small numbers of staphylococci have been found in air, dust, soil and water, and on inanimate surfaces or fomites, molluscs, insects and plants in areas frequented by mammals and birds (Elek, 1959; Kloos and Schleifer, 1981).

2.2.2. *Listeria monocytogenes*

Nowadays, *Listeria monocytogenes* is one of the most important foodborne pathogen. It is a leading cause of severe foodborne illness in the developed world. Although it is an unusual cause of food-borne illnes, however it is very important because of its exceptionally high levels of mortality. *L. monocytogenes* is capable of causing severe diseases in susceptible human populations. While disease in healthy individuals is usually a self-limiting gastroenteritis, susceptible individuals can develop very sirious symptomps and diseases. The organism is highly salt tolerant and additionally is able to grow at low tempratures and can outcompete other bacteria and multiply in most RTE food environments. Over the years, *L. monocytogenes* has become a model system of intracellular pathogenesis. According to EFSA, 2536 confirmed invasive human cases of listeriosis was recorded for the year 2016, which was an increase of 9.3% compared with 2015 (EFSA, 2017). Based on these facts there would be many additional benefit of further *L. monocytogenes* research.

L. monocytogenes is Gram-positive (0.5 µm wide and 1-2 µm long), rod shaped bacterium, isolated or arranged in small chains. They are motile at 20-25°C and non-spore-forming. These organisms can grow aerobically and facultatively anaerobically at a temperature between -1.5-45 °C. It can tolerate high salt concentration up to 16% and low pH around 4.3. Typical biochemical properties in identification include catalase-positivity, oxidase negativity and positive esculin hydrolyses.

L. monocytogenes is an intracellular bacterial pathogen able to grow at -1.5 °C, and thus may grow well at refrigeration temperatures and cause serious infection, namely listeriosis in humans. Occurrence of listeriosis is rare, but the lethality rate is quite high, usually up to 20-30% and hospitalization rates >92%. The most sensitive groups are the newborn infants, the elderly, pregnant women and immunosuppressed individuals (Mead et al., 1999). The infectious dose depends on several factors, such as the food matrices,

virulence factors. According to the FDA (2014) the minimal infectious dose (MID) is 1000 cells but for the YOPI group is probably lower. *L. monocytogenes* can occur not just in the environment, soils, but in the food and in the food processing, as well.

Listeriosis occurs as two different forms, a non-invasive form recognized as listerial gastroenteritis, or a severe invasive form of disease that frequently is accompanied by severe clinical manifestation. The non-invasive form of listeriosis results in a wide variety of symptoms ranging from muscle ache, fever and gastrointestinal symptoms such as diarrhea or nausea. *Listeria* infection can spread to the nervous system, symptoms such as headache, confusion, loss of balance stiff neck, or convulsions can occur. With brain involvement, listeriosis may mimic a stroke, and cause meningitis or encephalitis (Crum, 2002). The majority of listeriosis cases spontaneously recover without treatment in about seven days. However infected individuals at increased risk, (especially pregnant women), usually require antibiotic treatment to prevent, halt, or slow the development of more severe disease. Similarly to other pathogenic bacteria, antibiotic resistance was also observed in *L. monocytogenes*. One mechanism of this resistance could be efflux pump.

Two efflux pumps have been described in *L. monocytogenes* (Romanova et al., 2006). The efflux pump designated as MdrL, can extrude antibiotics (macrolides and cefotaxime), heavy metals, and EtBr (Mata et al., 2000). Another efflux pump, termed as Lde, is associated with fluoroquinolone resistance and, in part, with resistance to acridine orange and EtBr (Godreuil et al., 2003).

2.2.3. *Bacillus cereus*

Bacillus was chosen as a model organism of foodborne endospore forming pathogen. *B. cereus* is a facultative anaerobic Gram-positive bacterium. It is mostly detected in soil, grains, vegetables, raw and cooked rice, egg white, milk and meat (Magnusson et al., 2007; Arnesen et al, 2008). *B. cereus* is a potent pathogen associated with systemic and local infections such as severe ocular infections (Chan et al., 2003). Moreover, *B. cereus* causes food poisoning, which makes it a big concern in the food industry and especially in dairy products. Two types of food poisoning are associated to *B. cereus*: the emetic type and the diarrheal type (Granum and Lund, 1997; Schoeni and Wong, 2005). The emetic type is related to farinaceous foods, in particular fried or cooked rice and pasta (Jenson and Moir,

1997). However, the diarrheal type is associated in most cases with proteinaceous food like meat and is caused by enterotoxins (Granum and Lund, 1997; Schoeni and Wong, 2005).

The bacterial spore is a metabolically inactive or dormant cell type and is highly resistant to various environmental agents allowing it to survive in hostile conditions such as starvation, high temperatures, ionizing radiations, desiccation, hydrolytic enzymes and toxic chemicals (Nicholson et al., 2000). Dormant spores are able to survive for several years until nutrients are available.

Spore forming bacteria are implicated in food spoilage and foodborne disease. *Bacillus* spores can be found in a variety of food products such as rice, pasta, eggs and milk (Shi and Zu, 2009; Desai and Varadaraj, 2010; Parihar, 2014). The high resistance of the spores enables their survival to processing and food treatments in the food industry. However, the germination can be triggered once nutrients are available (Setlow, 2014). The spore will then change to the vegetative state. Food spoilage occurs when spore germination and vegetative cells growth take place in the final food product leading to foodborne illnesses.

Many methods were developed to treat and eliminate spores in the food industry. Beyond traditional heat treatments some of the methods to control *Bacillus* spores in food include UV irradiation (Koutchma, 2008). While others are non-thermal such as microfiltration and ultrasound to eliminate *Bacillus* spores (Guerra et al., 1997; Raso et al., 1998 and Gésan-Guiziou, 2010), high-pressure processing (HPP) (Zhang and Mittal, 2008), and pulsed electric field (PEF) (Soni et al., 2016).

2.2.4. *Chlamydia trachomatis*

Chlamydiae represents bacteria with medical importance and are obligate intracellulare pathogens that cause serious diseases in a wide range of hosts. They can either be symbiontic *Chlamydiae* in protozoa or pathogenic *Chlamydiae* in animals. The infections in mammals differ from bacterial strain to another as well as the site of infection (ocular, genital or respiratory tract). In humans, four different species of *Chlamydiae* have been isolated: *C. trachomatis, C. pneumoniae*, *C. psittaci* and *C. abortus*. *C. trachomatis* includes 15 different serovars responsible for different diseases in humans (Bush and Everett, 2001).

Trachoma is an eye infection which spreads through personal contact or by flies that had contact with an infected person. Bacteria develop in the conjunctiva of the eye causing conjunctival inflammation. According to the World Health Organization (WHO), trachoma is the leading cause of infectious and irreversible blindness. The causative serovars of trachoma are A, B, Ba and C while D-K cause different urogenital tract infections and L1-L3 provoke lymphogranuloma venereum (Pascolini and Mariotti, 2012).

C. trachomatis is considered the first cause of sexually transmitted infection (STI) of bacterial origin. Both men and women can be affected with infections in the lower parts of the genital tract or in the upper parts causing cervicitis, salpingitis or endometritis in women, and epididymitis and urethritis in men (Paavonen and Eggert-Kruse, 1999). Untreated infections may lead to ectopic pregnancies and infertility in women.

Lymphogranuloma venereum (LGV) is also sexually transmitted and they spread in the subepithelial tissues, infect monocytes and distribute to the regional lymph nodes, causing lymphadenitis or in some cases necrosis with pus formation (Mabey and Peeling, 2002).

For trachoma, the treatments may require surgical reorientation of the infected eye. In some cases, trachoma is treated with antibiotics such as tetracycline or azithromycin which are locally administered for 6 weeks (Hu et al., 2010). As for LGV, the typical treatment is 3 weeks of doxycycline administration, twice a week. In order to avoid an infection with *C. trachomatis*, a suitable strategy is the use sexual protection and focus on facial hygiene.

2.3. Multidrug resistant bacteria

When antibiotics were invented, deadly infectious diseases such as tuberculosis to pneumonia suddenly became easily curable. However, Alexander Fleming, who discovered penicillin, in his 1945 Nobel Lecture already predicted that bacteria could become resistant to antibiotics: "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them" (WHO, 2014).

Today, the number of bacteria that are antibiotic resistant are increasing, due to the overuse and misuse of antibiotics. In 2013, Center for Disease Control and prevention (CDC) published a list of the top 18 drug-resistant threats to the United States and classified them

as presenting urgent, serious and concerning threats (Table 3). These multidrug resistance (MDR) bacteria pose the greatest threat to human health, because of their frequency in causing infections, the increasing frequency of antibiotic resistance amongst them, and the lack of alternative antibiotics (CDC, 2013).

Table 3: Top 18 drug-resistant threats to the United States (CDC, 2013)

Urgent	**Serious**	**Concerning**
Clostridium difficile	Multidrug-resistant *Acinetobacter*	Vancomycin-resistant *Staphylococcus aureus*
Carbapenem-resistant Enterobacteriaceae (CRE)	Drug-resistant *Campylobacter*	Erythromycin-resistant Group A *Streptococcus*
Neisseria gonorrhoeae	Fluconazole-resistant *Candida*	Clindamycin-resistant Group B *Streptococcus*
	Extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae*	
	Vancomycin-resistant *Enterococcus* (VRE)	
	Multidrug-resistant *Pseudomonas aeruginosa*	
	Drug-resistant non-typhoidal *Salmonella*	
	Drug-resistant *Salmonella* Typhi	
	Drug-resistant *Shigella*	
	Methicillin-resistant *Staphylococcus aureus* (MRSA)	
	Drug-resistant *Streptococcus pneumoniae*	
	Drug-resistant *Mycobacterium tuberculosis*	

The CDC came together with the European Center for Disease Prevention and Control (ECDC) to standardize the terminology to describe the acquired resistance profiles. The term “multidrug resistant bacteria” refers to the resistance of the isolate "to at least one agent in three or more antimicrobial categories". The term “extensively drug-resistant (XDR) bacteria” is the resistance of the isolate "to at least one agent in all but two or fewer antimicrobial categories". Pandrug-resistant (PDR) bacterium have resistance to all the agents in all the antimicrobial categories (Magiorakos et al., 2012).

2.3.1. Efflux pump

Bacterial MDR efflux pumps (EPs) are cytoplasmic membrane proteins found in Gram-negative and Gram-positive bacteria that are capable of extruding of toxic biocides and antimicrobial agents (including all classes of antibiotics) and lower the intracellular concentration of the substrate antimicrobial by one of two mechanisms: either 1) overexpression of the efflux pump protein or 2) substitution of the amino acid of the protein that makes the protein more efficient at export (Borges-Walmsley et al., 2003; Piddock, 2006).

These EPs either use ATP hydrolysis or ion gradient to expel the antibiotics, they can be subdivided into different families of transport proteins (Fig. 4): the multidrug and toxic compound extrusion (MATE) superfamily, the resistance-nodulation-cell division (RND) superfamily, the major facilitator superfamily (MFS) and the small multidrug resistance (SMR) superfamily the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (Putman et al., 2000; Hemaiswarya et al., 2008).

Among these five super families, the MFS includes members that catalyze uptake, efflux, exchange transport or facilitated diffusion; the ABC superfamily includes members that catalyze both uptake or efflux; and functionally characterized members of the RND superfamily catalyze only efflux (Saier and Paulsen, 2001). Ethidium bromide (EtBr) and other fluorescent molecules, are used as substrates extruded by EPs to demonstrate efflux activity (Kern et al., 2006; Raherison et al., 2002).

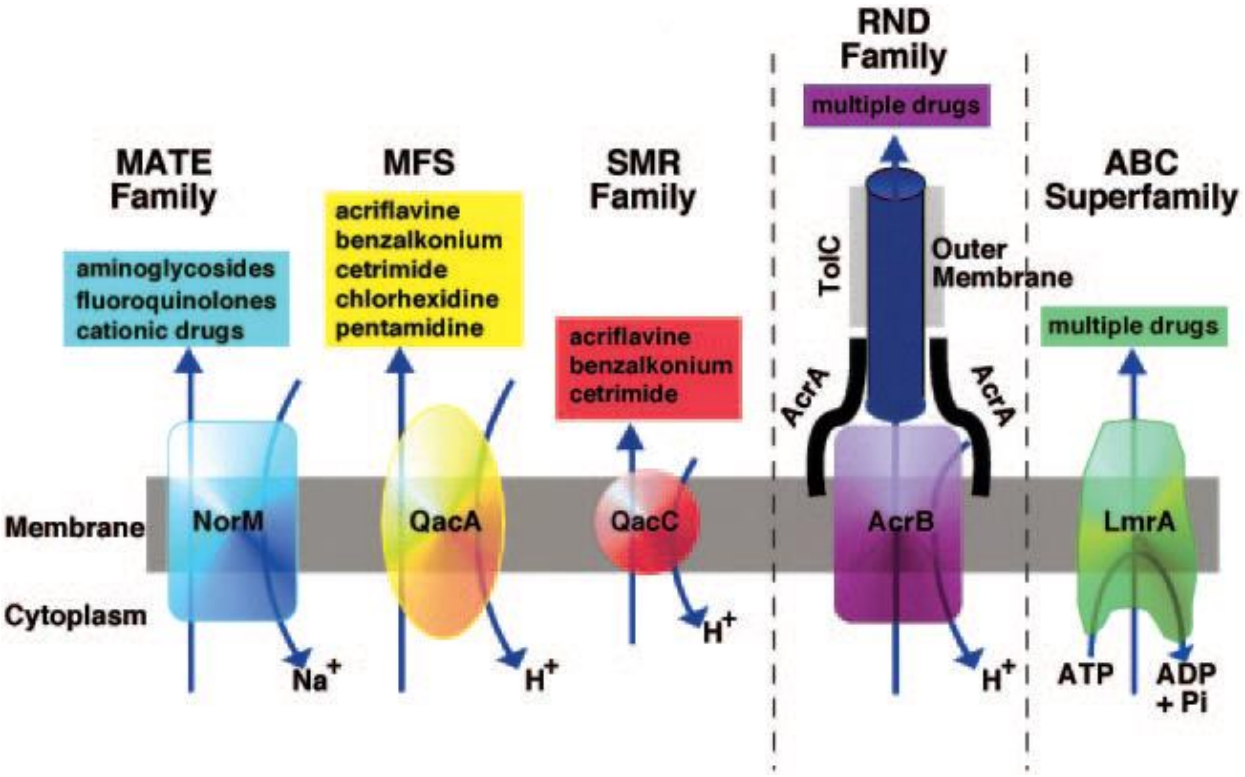


Figure 4: Diagrammatic comparison of the five main efflux pump families (Source: Piddock, 2006)

The appearance of the genomic era and the recent advances in DNA technology have led to the identification of numerous new members of these families. In the examination of bacterial genomes, it has been found that each contains various efflux pumps; this indicates that they are of ancient origin. It is estimated that ~ 5-10% of all bacterial genes are involved in transport and most of them encode efflux pumps (Saier and Paulsen., 2001; Lomovskaya et al., 2001).

In several cases, efflux pump genes are part of an operon, with a regulatory gene controlling expression. Increased expression is related to substrate resistance, for example, due to certain antibiotics, the overgrowth of the *acrAB* gene carried by *E. coli* can be observed (Thanassi et al., 1997). Although genes encoding efflux pumps are found on the plasmids, the mutant bacteria that over-express efflux pump genes can be selected without the need of new genetic material (Webber and Piddock., 2003).

Many efflux systems contributing to the development of antibiotic resistance have been described for clinically important bacteria, *Campylobacter jejuni* (CmeABC), *Salmonella typhimurium* (AcrAB-TolC), *Pseudomonas aeruginosa* (MexAB- OprM,

MexCD-OprJ, MexEF-OprN and MexXY-OprM), *Streptococcus pneumoniae* (PmrA), *E. coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD) and *S. aureus* (NorA, NorB and NorC). These systems extrude fluoroquinolones (e.g. ciprofloxacin) and RND pumps (CmeB, AcrB and Mex) pump complex antibiotics. Expression of efflux pumps may result from the mutation of local repressor genes or from the activation of a regulon (Webber and Piddock, 2003).

The wide substrate range of efflux systems is a cause of fear, because often overproduction of a pump results in resistance to antibiotics and other biocides including dyes, disinfectants and detergents. Another major problem is cross-resistance. Exposure to any substance that belongs to the substrate profile of the pump trigger the overproduction of the pump which creates cross-resistance to any other substrate of the pump. These substrates may contain clinically relevant antibiotics (Chuanchuen et al., 2001).

Nevertheless, excessive expression of multidrug resistant efflux pumps often does not alone provide a high level of clinically significant resistance to antibiotics. However, such bacteria are better equipped, to survive antibiotic treatment, and develop resistance to target sites of antibiotics by further mutation of the encoding genes (Kern et al., 2000). In addition, genes encoding the *E. coli* topoisomerase enzyme of very high fluoroquinolone resistance mutated and showed increased efflux mechanism. Additionally, increasing in MICs values of antibiotics and dyes have been observed with overproduction of multiple pumps, resulting in highly resistant *E. coli* (Webber and Piddock, 2001).

2.3.2. Efflux pump inhibitors

The importance of the inhibition of the efflux pump is to:

1) increase the intracellular concentration of the drug;
2) restore the activity of the drug against the resistant strains;
3) reduces additional development of resistant strains.

Inhibition of efflux pumps can be achieved by various mechanisms shown in Fig. 5.

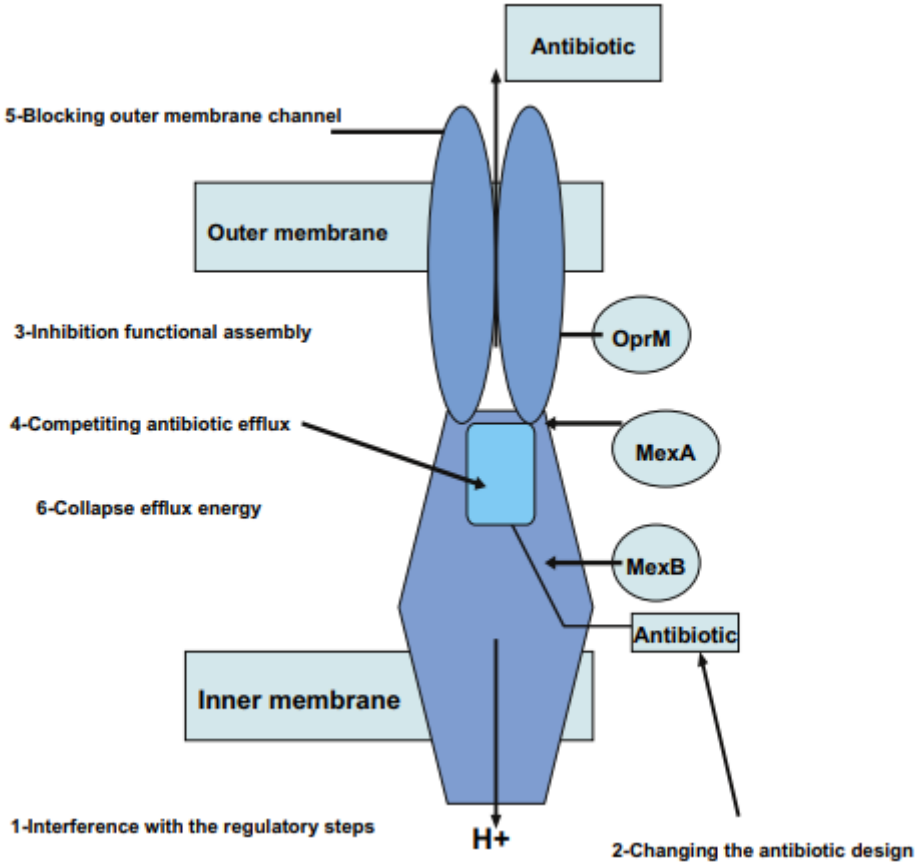


Figure 5. The general mechanisms of efflux pump inhibition factors influencing efflux pump activity (Askoura et al., 2011)

Description of efflux pumps inhibitors (EPIs) mechanism according to the Fig. 5:

1- interfering with the regulatory steps for the expression of the efflux pump;
2- chemical changes in the structure of the antibiotic, therefore, hinder the binding of this specific substrate;
3- disruption of the assembly of the efflux pump-components;
4- inhibition of binding of substrate (antibiotics) binding by either competitive or non-competitive binding using other compounds,
5- blocking the outer pores in charge of the antibiotics outflow;
6- collapse the energy required for the efflux activity (Pagès and Amaral, 2009).

The general methodology used to test the efficiency of these efflux inhibitors is simply performed by comparing the intracellular concentration of the added antibiotic to the bacterial cell culture before and after the addition of the EPIs such as reserpine. If the

compound under testing showed higher intracellular concentration of the antibiotic, it is considered a good efflux pump inhibitor and vice versa (Mahamoud et al., 2006).

2.3.3. The chemical EPIs

PAβN (phenylalanine arginyl β-naphthylamide)

The mechanism of action of the PA*β*N inhibitor (Table 4) is based on a competitive inhibitory mechanism where efflux pumps recognize them as a substrate instead of targeted antibiotics (quinolones, mainly ciprofloxacin) and as long as the pumps extrude these inhibitors from the intracellular, the antibiotics remain within the cell and increase their concentration. PA*β*N can compete with certain antibiotics (while for others there is no such competition) depending on the large substrate-binding sites and the nature of the efflux (Lomovskaya et al., 2001).

PA*β*N is also capable to restore the activity of other antibiotics such as macrolides and chloramphenicol. Therefore, it is considered a wide-spectrum efflux pump inhibitor. The main advantage of PA*β*N-derived (EPIs) inhibitors is that it is difficult to develop resistance to them. The disadvantage of these EPI components is their toxicity, which delays their clinical application. However, it is used to evaluate the efflux mechanism of various pathogenic bacteria with antibiotics (Askoura et al., 2011).

Carbonyle cyanide m-chlorophenylhydrazone

CCCP strongly affects the level of energy of the bacterial membrane. This compound decreases the bacterial viability and cause cell death via the dissipation of the proton-motive force of the membrane. On efflux activity, CCCP is regularly used as the benchmark to which the activities of novel efflux pump inhibitors are compared. CCCP functions as an inhibitor of many MDR efflux pumps, through the disruption of proton motive force (Kaatz et al., 1993).

In addition, CCCP is extremely harmful, strongly cytotoxic (Mahamoud et al., 2007).

Reserpine and verapamil

Reserpine and verapamil are common efflux inhibitors, which were first identified as inhibitors of vesicular monoamine transporters and blockers of transmembrane calcium channel blockers (Li and Nikaido, 2004).

Reserpine inhibits the activity of NorA and Bmr, two Gram-positive efflux pumps by altering the generation of the membrane proton-motive force required for the function of MDR efflux pumps. Verapamil is an inhibitor of MDR pumps for cancer cells and parasites and improves tobramycin activity as well, which is an aminoglycoside type antibiotic. Although, these molecules are capable to inhibit the ABC transporters involved in the extrusion of antibiotics, the concentrations necessary to block bacterial efflux are neurotoxic. Reserpine has been used as an anti-hypertensive drug; its concentration for this purpose is far lower than that employed for the inhibition of efflux. Reserpine and verapamil are used regularly to evaluate the activity of efflux pumps in Gram-positive bacteria (Li and Nikaido, 2004).

Table 4: Most common chemical efflux pump inhibitors and the type of efflux pump superfamily they block

Inhibitors	Efflux pump superfamily	Sources
PAβN	Resistance-nodulation-cell division	Cortez-Cordova and Kumar, 2011
CCCP	Major facilitator	Kumar et al., 2013
Reserpine	Major facilitator, ATP binding cassette	Schmitz et al., 1998; Kumar et al., 2013
Verapamil	Major facilitator	Singh et al., 2011

2.3.4. Antibiotics

General description of antibiotics

Antibiotics are chemical substances that can inhibit the growth of, and even destroy, harmful microorganisms. They are derived from special microorganisms or other living systems and are produced on an industrial scale using a fermentation process and can have a semi-synthetic production process, where chemical modifications are just as important. There are many types of antibiotics—antibacterial, antivirals, antifungals, and antiparasitic. Some drugs are effective against many organisms; these are called broad-spectrum antibiotics. Others are effective against just a few organisms and are called narrow spectrum antibiotics.

Antibiotics can be classified based on the cellular component or system they affect, in addition to whether they induce cell death (bactericidal drugs) or merely inhibit cell growth (bacteriostatic drugs).

The bactericidal antimicrobials inhibits the:

- DNA synthesis,
- RNA synthesis
- protein synthesis,
- cell wall synthesis (Kohanski et al., 2010).

Some microorganisms are naturally resistant to certain types of antibiotics. However, bacteria may become resistant to the antibiotics. The three essential mechanisms of antimicrobial resistance are:

1- enzymatic degradations of antibacterial drugs;
2- modification of bacterial proteins that are antimicrobial targets,
3- changes in membrane permeability (outer membrane protein) to antibiotics.

Antibiotic resistance can be either plasmid mediated or maintained on the bacterial chromosome (Dever et al., 1991).

There are well over 100 antibiotics, the majority come from only a few type of drugs. These are the main classes of antibiotics:

- Penicillins such as penicillin and amoxicillin,
- Cephalosporins such as cephalexin,
- Macrolides such as erythromycin and azithromycin,
- Fluoroquinolones such as ciprofloxacin and ofloxacin,
- Sulfonamides such as bactrim and trimethoprim,
- Tetracycline such as tetracycline and doxycycline,
- Aminoglycosides such as gentamicin and tobramycin.

Three types of antibiotics, a macrolide, a ciprofloxacin and a tetracycline, were chosen in this study based on the type of efflux pump resistance in the tested strains.

Erythromycin

Erythromycin ($C_{37}H_{67}NO_{13}$) is a broad-spectrum antibiotic which belongs to the group of macrolide antibiotics and produced by the Gram-positive bacteria *Streptomyces erythreus* (Fig. 6). It inhibits bacterial protein biosynthesis, especially of Gram-positive bacteria. Erythromycin diffuses through the bacterial membrane and reversibly bind to 50S ribosomal subunits. Binding inhibits peptidyl transferase activity and interferes with translocation of amino acids during translation and assembly of proteins. Erythromycin is mostly bacteriostatic, and bactericidal only at high concentrations (Alighardashi et al., 2009).

Macrolide antibiotics constitute an important treatment alternative in patients allergic to penicillin. Typically, these antibiotics are effective against Gram-positive strains such as *Staphylococcus spp., Streptococcus spp*. It only acts against some Gram-negative cocci (*Neisseria spp., Haemophilus spp., Legionella spp.* and *Chlamydia spp*.).

Against macrolide antibiotics there are two mechanisms of resistance: active efflux (particularly in *Streptococcus*) or target site modification. They are known as M-resistance respectively MLSB-resistance. Active efflux is encoded by *mef*-class genes (Varaldo et al., 2009).

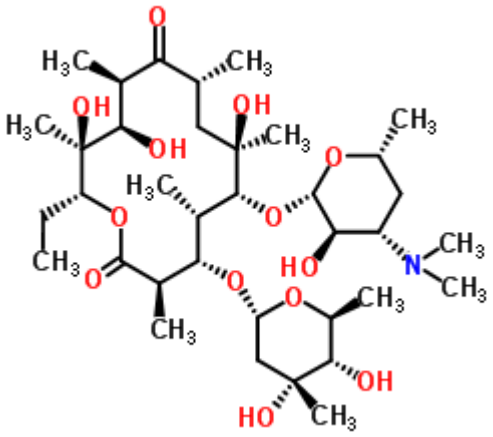


Figure 6: Structure of Erythromycin (Internet 4)

Tetracycline

Tetracycline ($C_{22}H_{24}N_2O_8$) is a broad-spectrum antibiotic which belong to the group of tetracycline antibiotics and produced by the Gram-positive bacterium *Streptomyces aureofaciens* (Backus et al., 1954) (Fig. 7). It inhibits protein synthesis by preventing the

attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Thus, it is inhibitor of growth (bacteriostatic) rather than killer of bacteria (Chopra and Roberts, 2001).

Tetracycline, according to Gu and Karthikeyan (2005) are the second most produced and used antibiotics worldwide. Tetracyclines are used against a variety of infectious diseases, including cholera, malaria, trachoma (a chronic infection of the eye), brucellosis, psittacosis (a disease transmitted by certain birds), and tularemia. Tetracyclines have also been used for the treatment of acne.

Against tetracycline antibiotics, there was widespread emergence of ribosome- and efflux-based resistance to first- and second- generation tetracyclines. Active efflux is encoded by *tet* and *otr* genes (Chopra and Roberts, 2001). The Gram-positive *tet* efflux genes are associated with small plasmids, while in Gram-negative they are found on transposons inserted into a diverse group of plasmids from a variety of incompatibility group (Chopra and Roberts, 2001).

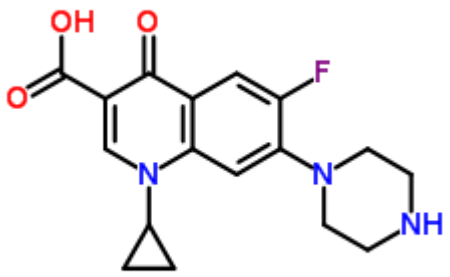


Figure 7: Structure of tetracycline (Internet 5)

Ciprofloxacin

Ciprofloxacin ($C_{17}H_{18}FN_3O_3$) is a broad-spectrum antibiotic which belongs to the fluoroquinolone group (Fig. 8). The primary functional route of this class of antibiotic is the inhibition of the enzyme, DNA gyrase (bacterial topoisomerase II), in bacterial cells. This enzyme is responsible for negative supercoiling of DNA during the replication process. The inhibition of this enzyme activity will, naturally, lead to a number of adverse and bactericidal effects as a result of inhibition of DNA replication. These effects include interfering with the

DNA replication, transcription and the separation of the bacterial chromosomes, and other cellular processes and damaging DNA (Oates et al., 1991).

Quinolones have bactericidal activity against most species of bacteria, with minimal bactericidal concentrations typically equal to or twofold higher than the minimal inhibitory concentrations. Ciprofloxacin is recognized as one of the most effective antibiotics of the quinolone drug class (Oates et al., 1991) and has been used for the treatment of urinary tract infections (Hickerson and Carson, 2006), bacterial prostatitis (Bundrick et al., 2003), infections of the respiratory (Fass, 1987) and digestive tract (Enzensberger et al., 1985).

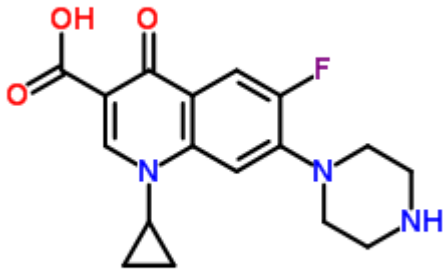


Figure 8: Structure of ciprofloxacin (Internet 6)

2.4. Biofilms

Biofilm is a very important problem both in food industy and in medical field. Biofilm formation can contaminate product through the introduction of pathogenic microorganisms (e.g. *L. monocytogenes* and *S. aureus*) or spoilage bacteria (*Pseudomonas* and *Enterococcus*).

Microorganisms aggregate to form matrix-enclosed clusters called biofilms, which adhere to biological and non-biological surfaces where bacterial cells stick to each other ("*Biofilms are matrix-enclosed clusters of microorganisms, which adhere to biological and non-biological surfaces where bacterial cells stick to each other"*. Jamal et al., 2015). Bacterial cells exist in two forms: single cell form known as planktonic and sessile aggregates referred to as biofilms. Biofilms communities can be attached to many surfaces such as rocks in a stream, wastewater channels, plants or animals. Even though, in the human

body, many surfaces are easily accessible for bacterial cells and hence for biofilms to colonize, these surfaces are continuously taken off and renewed, limiting the biofilms formation. Nevertheless, some non-shedding surfaces have biofilms on them, such as the teeth and dental plaques (Wilson, 2001).

2.4.1. Biofilm formation

Studies describe biofilm formation by these three main steps: attachment, growth and maturation, and dispersal. Once planktonic bacterial cells are inside the body, they search for the best adequate surface for adherence. They, then, slow down and attach to the surface. It occurs similarly in inorganic environments like food contact surfaces. Firstly, a conditional layer is formed as a foundation perturbing the charge of the surface as well as its tension and potential (Garrett et al., 2008). Attachment can be divided into two parts. The initial, reversible attachment is based on week forces such as electrostatic attraction and Van der Waals forces. During irreversible attachment biofilm bacteria excrete extracellular polymeric substances (also called EPS, sticky polymers), which hold the biofilm cells together and stick them to the surface. When the bacterial cells form a stable bond with the surface, they start to multiply and thus to colonize the surface. Several microcolonies start communicating with each other ensuring an exchange of nutrients and gases. Finally, in the dispersal phase, bacterial cells are released, and they return to their planktonic state. These planktonic cells are able to adhere to and colonize new surfaces.

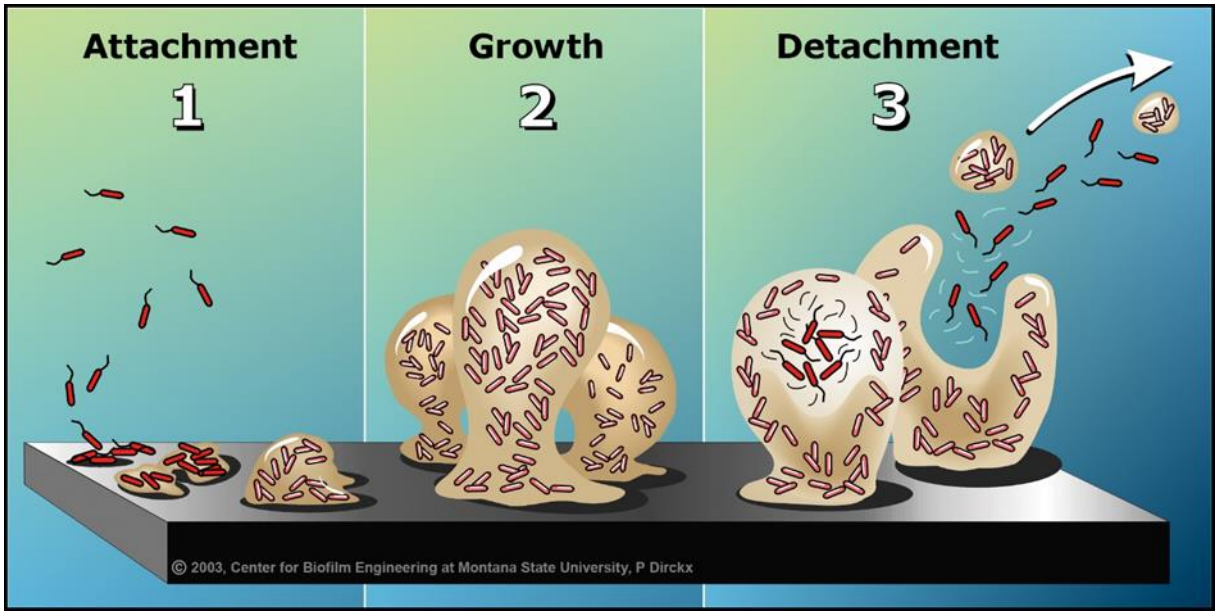


Figure 6: Steps of biofilm formation (Dirckx, 2003)

Environmental factors such as pH and temperature are important elements in the biofilm development. Some processes being very sensitive to pH changes, bacterial cells should be able to regulate their internal pH. Substances that affect pH are used to slow down bacterial growth and hence inhibit biofilm formation (Garrett et al., 2008). Temperature also influences the optimal efficiency of bacterial cells by inactivating or demolishing enzymes. Moreover, the nature of the surface plays also an important role in colony formation (Donlan, 2002). Rough surfaces enhance biofilm formation offering the bacterial cells extra space to grow.

2.4.2. Biofilm composition

Biofilms are composed of microorganisms producing extracellular polymeric substances (EPS). EPS contain mostly polysaccharides along with extracellular DNA and macromolecular components such as proteins and lipids, granting the structure and stability to the biofilms. Noting that EPS is highly hydrated, biofilms consist of approximatively 97% of water. The EPS matrix protects the biofilms from desiccation, predation, oxidizing molecules, radiation, and other damaging agents (Kostakioti et al., 2013).

2.4.3. Antimicrobial tolerance of biofilms

Biofilms have many mechanisms by which they resist to host immune systems and antimicrobial agents (Singh et al., 2017):

- glycocalyx or capsules, enclose antibiotics and antimicrobial agents delaying their diffusion into the matrix;
- enzyme neutralization, inactivating the antibiotics and high resistance occurs;
- heterogeneity in metabolism and growth rate, when the bacterial cells have decreased metabolic activity due to nutrients shortage, they become less susceptible to antibiotic activity;
- persistent cells, these cells survive antimicrobial treatments and may establish biofilm reformation;
- phenotypic alteration or adaptation reach the most resistant phenotype;
- structure of outer membrane, through repulsion of hydrophilic compounds;

2.4.4. Biofilms and quorum sensing

Quorum sensing (QS) is the process of communication between bacterial cells permitting them to share information about population density and therefore regulate their gene expression (Rutherford and Bassler, 2012) (Fig. 9). This cell-to-cell interaction is enabled due to robust structure of the biofilms. Yet, in some spaces with low levels of oxygen, water and nutrients, a community of division of labor is present. In this community, metabolically inactive cells are tolerant to many antibiotics and able to recolonize the biomass after treatment. Moreover, QS can promote antibiotic tolerance and drug resistance within the biofilm. Hence, infections-causing pathogens resist and induce the following cases: periodontitis (Kuramitsu and Wang, 2011), upper respiratory infections (Koch and Hoiby, 1993; Govan and Deretic, 1996), urinary tract infections (Foxman, 2010), catheter-induced and other device-associated infections (Venditti et al.,1993; Ferrieres et al., 2007; Jacobsen et al., 2008; Fey, 2010).

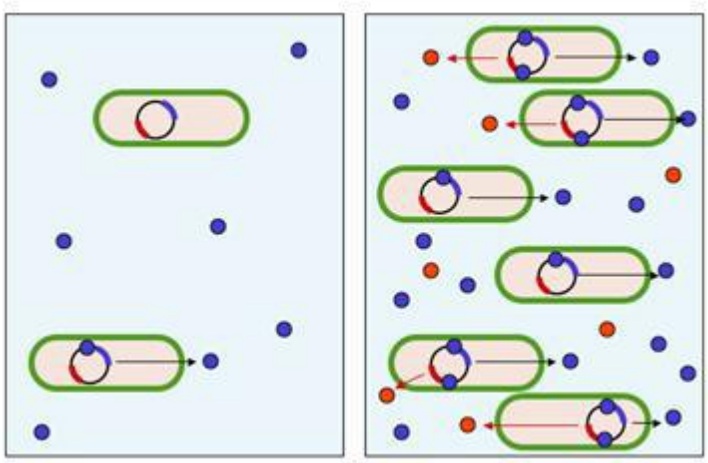


Figure 9: Schematic outline of QS (Margaret et al., 2008)

Left side: Low concentrations of bacteria and signal molecules (blue circles).

Right side: When the concentration of bacteria increases, the amount of signal molecules increases too.

2.4.5. Biofilms and chronic infections

Planktonic cells are usually responsible for mostly treatable acute infections, whereas biofilms generally cause chronic infections. The latter do not resolve easily and have a much slower progression rate than acute ones. Even though the immune system cannot fight efficiently against chronic infections, they cause gradual tissue damage with resistance to

antimicrobial agents. The National Health Institute (NIH) states that biofilm-associated infections present 80% of chronic infections cases (Høiby et al., 2015). Chronic infections caused by biofilms can be distributed into two categories foreign device-related infections (medical tools such as endotracheal tube, prostheses and urinary catheters) and tissue infections.

Cystic fibrosis: Patients with CF usually have endobronchial bacterial infections. The most common bacteria in CF is *P. aeruginosa*. Their biofilm goes through different adaptive changes with various variants.

Chronic wounds: Many different bacterial species can be found in dermal tissues such as the skin microflora, *S. aureus* and *P. aeruginosa* (Archer et al., 2011).

Chronic otitis media (COM): Patients suffering from COM have biofilms made up by a mixture of aerobic and anaerobic bacterial cells such as *P. aeruginosa*, *S. aureus* and *E. coli* (Akyildiz et al., 2012).

Treatment of biofilms of chronic infections

Antimicrobial agents are not sufficient to treat biofilms causing chronic infections. Therefore, new treatment strategies were established against biofilms. Dispersal agents leading to the dispersal of biofilms are emerging as a therapy method. As mentioned earlier, the dispersal phase allows free, planktonic bacterial cells to adhere to new surfaces and form biofilms. Two dispersal mechanisms might occur: passive and active dispersal. External shear forces or mechanically destroy the biofilm in the passive dispersal. Whereas, active dispersal is triggered by the biofilm microbes, bacteriophages or quorum sensing inhibition (Fleming and Rumbaugh, 2017).

Prevention of biofilms of chronic infections

Noting that medical devices in clinical practices are a common location for biofilm formations, approaches are being studied to prevent bacterial growth. Medical devices and materials are coated and impregnated by antibiotics. Moreover, another approach is to modify the surface by altering its hydrophobicity, charge and roughness which disables the attachment of bacterial cells to the surface (Bhattacharya et al., 2015).

2.4.6. Biofilms in food industry

2.4.7. Methods of studying biofilms

Many methods have been developed to test biofilms such as chemical, molecular or microscopy techniques measuring their structure or the viability (Azeredo et al., 2017). Applying the chemical or staining methods, the biofilms are cultivated and detected by dyes or fluorochromes. For the viability assays, XTT or resazurin are used. Biofilms are also detected by microscopic methods including light microscopy, Confocal Laser Scanning Microscopy (CLSM) and Scanning electron microscopy (SEM) (Azeredo et al., 2017).

3. MATERIALS AND METHODS

3.1. Reagents

Carvacrol (Car), thymoquinone (Thq), reserpine, carbonyl cyanide-m-chlorophenyl hydrazone (CCCP), ethidium bromide (EtBr), resazurin sodium salt, menadione, erythromycin and tetracycline were from Sigma-Aldrich (Germany), while ciprofloxacin (Cip) was obtained from MP Biomedicals, LLC (France) and p-cymene (p-cy) was purchased from Alfa Aesar (Germany). Iso-octane HPLC grade (Merck) was used as solvent, all the others were analytical grade chemicals.

3.1.1. Reserpine and CCCP

To prepare the stock solutions, 20 mg of reserpine and 20 mg of CCCP were dissolved in 1 mL of dimethyl sulfoxide (DMSO) and stored at 4 ºC. Further on, the stock solutions were dissolved in Phosphate Buffered Saline (PBS) or Tryptic Soy Broth (TSB) to get the appropriate concentration.

3.1.2. EtBr

To prepare the stock solution, 1.6 mg of EtBr was dissolved in 1 mL sterile double distilled water and stored at 4 ºC in aluminum folia in dark. Further on, the stock solution was dissolved with PBS to get the appropriate concentration.

3.1.3. Resazurin

2.8 mg of resazurin sodium salt was diluted in 1 mL sterile distilled water and added to 9 mL TSB (solution 1) and distributed in Eppendorf tubes (in each tube 900 µL). 1.4 mg of menadione was diluted in 1 mL of DMSO (solution 2). 100 µL of solution 2 was added to 900 µL of solution 1 to prepare the stock solution. The stock solutions were stored in freezer at -20 ºC.

3.1.4. Antibiotics

Erythromycin, ciprofloxacin and tetracycline were dissolved in ethanol (EtOH) in order to prepare the stock solutions, which were diluted with TSB to get the appropriate concentration.

3.1.5. *N. sativa* oil and its active compounds preparation

N. sativa seeds were purchased from local Turkish market and were cold pressed to produce the crude oil. The crude oil then was hydro-distilled at 100 °C in a Clevenger apparatus to extract the essential oil (EO). The essential oil was collected, dried over anhydrous sodium sulfate and stored finally at 4 °C for further analysis.

The crude oil, essential oil and thymoquinone were diluted using DMSO, while ethanol (EtOH) was used as diluent for carvacrol and p-cymene to prepare the stock solutions and further on diluted with TSB to get the appropriate concentration.

3.2. Fatty acids methylation and GLC analysis

The fatty acids methylation and GLC analysis was carried out by Toma and co-workers (2013) with some modifications according to the following: A quantity of 0.1 g of CO was dissolved in 2 mL iso-octane and 0.2 mL methanolic KOH (2 M) was added. The mixture was shaken for two minutes and allowed to stand for 10 minutes. The upper layer was removed and washed with water. The organic phase containing methyl esters of the fatty acids was diluted with iso-octane, then analyzed by Gas-Liquid chromatography (GLC) using a Thermo Finnigan Trace GC, with split/splitless injector and flame ionization detector, a capillary column: Supelco, SP 2340 (30 m × 0.32 mm × 0.25 μm), the carrier gas was nitrogen (N_2) with a flow rate 0,5 mL/min. The column temperature was programmed from 70 °C- 180 °C at a rate of 4 °C/min.

3.3. GC-MS analysis of the essential oil

The composition of the EO was determined by GC-MS. The GC analysis was carried out using an Agilent Technologies 6890 N instrument equipped with HP-5MS capillary column (5% phenyl, 95% dimethyl polysiloxane, length: 30 m, film thickness: 0.25 μm, id. 0.25 mm), programmed as follows: initial temperature 60 °C, heating at a rate of 3°C/min up to 240 °C; the final temperature was kept for 5 min; injector and detector temperatures: 250 °C; carrier gas: helium (constant flow rate: 1 mL/min); split ratio: 30:1.

The GC-MS analyses were carried out using an Agilent Technologies 6890 N GC equipped with an Agilent Technologies MS 5975 detector and ionization energy of 70 eV. The MS were recorded in full scan mode that revealed the total ion current (TIC) chromatograms. The linear retention indices (LRI) were calculated using the generalized equation of Van den

Dool and Kratz (1963). The MS and LRI were compared with those of commercial (NIST, Wiley) and home-made libraries. The proportions of the individual compounds were expressed as total fatty acids (TFA) area percentages.

3.4. Test organisms

Table 5 list all the strains that were used for the study. Bacterial isolates were subcultured at least twice from the stock solution on Tryptic Soy Broth (Sigma Aldrich, Germany) to prepare a fresh culture before analysis.

3.5. Evaluation of antimicrobial activity of *N. sativa* crude oil by agar well method

Antibacterial activity of *N. sativa* crude oil carried out against 27 Gram-positive strains and 2 Gram-negative strains (clinical isolates) (Chapter 3.4). Tryptic Soy Agar (TSA) (Sigma-Aldrich) was prepared, sterilized in autoclave at 121 °C for 15 min and poured into sterile Petri dishes. The strains were cultured at 37 °C on TSA medium for 18-24 h, diluted to approximately 10^6 CFU/mL, and the suspension aliquot (100 µL) was spread onto the surface of TSA medium. Wells of 5 mm diameter were made aseptically in the inoculated plates and 50 µL of the diluted CO (diethyl ether was used as diluent) was added to each well. The inoculated plates were incubated at 35 °C for 24 h. The antibacterial activity was evaluated by measuring the diameter of inhibition zone (expressed in millimeters) for the growth of test bacteria. The positive controls were obtained by preparing culture medium with the bacterial suspension and diethyl ether with the bacterial suspension corresponding to the highest concentration present in the preparation. The experiments were performed in triplicate.

Table 5: List of strains used in the study

Code	Strain	Source	Chapters
L1	*Listeria monocytogenes* E12/10.12.1	SZIE[a]	3.5; 3.6; 3.12
L2	*Listeria monocytogenes* 11/4.12 t03		3.5; 3.6; 3.12
L4	*Listeria monocytogenes* E ST/10.12.1		3.5; 3.6; 3.12
L6	*Listeria monocytogenes* E ST/10.12.2		3.5; 3.6; 3.12
L7	*Listeria monocytogenes* E ST/10.12.3		3.5; 3.6; 3.12
L9	*Listeria monocytogenes* 8/4.12. t0		3.5; 3.6; 3.12
LA	*Listeria monocytogenes* CCM 4699		3.5; 3.6; 3.12
LI	*Listeria monocytogenes* NCTC, serotype 4b		3.6; 3.12
L14	*Listeria monocytogenes* 10/10.12.2		3.6; 3.11; 3.12; 3.13; 3.14
	Listeria monocytogenes B01966		3.5
	Listeria monocytogenes NCAIM 1454		3.5
	Listeria monocytogenes 4b		3.5
	Listeria monocytogenes 1/2 c		3.5
	Listeria monocytogenes CCM 7202		3.5
	Listeria monocytogenes 3b		3.5
	Listeria monocytogenes #46		3.5
	Listeria grayi CCM 5990		3.5
	Listeria grayi CCM 4029		3.5
	Listeria innocua CCM 4030		3.5
	Listeria welshimeri CCM 3971		3.5
	Listeria ivanovii CCM 5884 T		3.5
	Micrococcus luteus		3.5
	Enterococcus faecalis		3.5
	Bacillus megaterium		3.5
	Bacillus licheniformis		3.5
	Rhodococcus eqvii		3.5
	Escherichia coli B 01909		3.5
	Proteus sp.		3.5
	Bacillus cereus T1		3.5; 3,7 ; 3.8
	Bacillus subtilis T1		3.5; 3,7 ; 3.8
	Staphylococcus aureus ATCC 6538	NCAIM[b]	3.5; 3.6
	Pseudomonas aeruginosa ATCC 9027		3.6
	Salmonella Hartford B 1310		3.6
	Escherichia coli B 01909		3.6
	Staphylococcus aureus ATCC 25923	IHTM[c]	3.6; 3.12; 3.13; 3.14; 3.15; 3.16
	Staphylococcus aureus MRSA 272123		3.6; 3.12; 3.13; 3.14; 3.15; 3.16
	Chlamydia trachomatis D (ATCC VR-885)	SZTE[d]	3.10

[a] Department of Microbiology and Biotechnology, Faculty of Food Science, Szent István University, Budapest, Hungary

[b] Institute of Hygiene and Tropical Medicine, Lisbon, Portugal

[c] NCAIM (National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary

[d] Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary

3.6. Determination of minimum inhibitory concentration (MIC)

The antimicrobial activity of *N. sativa* CO, EO, Thq, Car and p-cy were determined using broth microdilution method (Chapter 3.4) (Kovač et al., 2015). Briefly, the stock solution of the EO, Thq, Car or p-cy were serially half diluted in TSB in microtiter plates, and then each overnight culture was added at a concentration of 10^6 CFU/mL, to the final volume of 0.1 mL/well. After 24 h of incubation at 37ºC, 10 µL of resazurin reagent, which consisted of 10 M resazurin sodium salt and 0.8 mM menadione, was added to each well. Following 2-h incubation at 37ºC, the fluorescence intensity was measured at 550 nm and 959 nm, using a microplate reader (Victor x3, PerkinElmer/Waltham, USA). The minimum inhibitory concentrations (MICs) were defined as the minimal concentration at which the fluorescence signal declined to the level of the blank. All the MICs measurements were carried out in triplicates. The diluent controls were obtained by preparing culture medium with the bacterial suspension and DMSO or EtOH with the bacterial suspension corresponding to the highest concentration present in the preparation. The negative control was obtained by preparing the culture medium only or the culture medium with the given antimicrobial. The same assay was used for the determination of the MICs for the antibiotics and EtBr.

3.7. Spores preparation

B. cereus T1 and *subtilis* T1 cultures prepared on agar slants were inoculated into Tryptic Glucose extract (TGE) broth and incubated for 24 hours at 30 °C.

The morphology of the cells was checked by microscope, and 1 mL of the overnight culture was spread onto 200 mL TGE agar in large Petri dishes (measure diameter). Petri dishes were then incubated for 72 hours at 30 °C and then for 24 hours at 5 °C. The spores and vegetative cells were harvested by means of a sterile metal spatula and then transported into 25 ml of distilled sterile water. After that, the formation of spores was checked by spore staining and microscopic examination was carried out.

The spore suspension was treated with 2 mg/mL of lysozyme solution and allowed to stand for 24 hours at 5 °C. To separate vegetative cells and spores during treatment, the suspension was centrifuged at 5 °C at 4000 rpm for 20 minutes, then the supernatant and water were removed, and the spores were washed for 5 more times. At the end the spores were resuspended in sterile distilled water and kept at 4 °C until use. Spore concentration was determined by pour plating.

3.8. Combination of *N. sativa* crude oil and nisin against spores

The antimicrobial activity of *N. sativa* CO and nisin were tested against *B. cereus* and *B. subtilis* spores measuring the optical density using Multiskan machine. Briefly, the stock solution of the crude oil, or nisin were serially half diluted in TSB in microtiter plates, and then *B. cereus* or *B. subtilis* spores were added at a concentration of 10^6 CFU/mL, to the final volume of 300 µL/well.

Following 24-h incubation at 30 ºC, the optical density was measured at 595 nm, using a microplate reader (Multiskan plate reader, MTX Lab Systems, USA). The minimum inhibitory concentrations (MICs) were defined as the minimal concentration at which there was no growth detected after 24 h of incubation. All the MICs measurements were carried out in triplicates.

The combined effect of the crude oil and nisin was evaluated using the same method described above except that the medium was supplemented with sub-inhibitory concentration of the crude oil and nisin (Table 6).

Table 6: The composition of the crude oil and nisin combinations

Combinations	Concentration
1	1/2 MIC CO + 1/2 MIC nisin
2	1/4 MIC CO + 1/4 MIC nisin

3.9. Cytotoxicity assay

The effects of increasing concentrations of the *N. sativa* EO, Thq, Car and p-cy on HeLa 229 cell growth were tested in 96-well microtiter plates. 2×10^4 cells in 100 µl of Modified Eagle Medium (Sigma-Aldrich), supplemented with 10% fetal calf serum, 0.5%

glucose, 0.3 mg of L-glutamine/ml, 4 mM HEPES, and 25 µg of gentamycin/ml, were added to each well, with the exception of the medium control wells. After an overnight incubation period the compounds were diluted in 100 µl of medium and added to the cells. The plates were further incubated at 37 °C for 48 h. At the end of the incubation period, 20 µl of thiazolyl blue tetrazolium bromide (MTT; Merck KGaA) solution (from a 5 mg/ml stock) was added to each well. After incubation at 37 °C for 4 h, 100 µl of sodium dodecyl sulfate (SDS; Merck KGaA) solution (10% in 0.01 M HCI) was added to each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA).

Inhibition of the cell growth was determined according to the following formula:

$$\text{IC50} = 100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control} \right] x100$$

Where the inhibitory concentration (IC50) is defined as the inhibitory dose that reduces the growth of the compound-exposed cells by 50%.

This test was performed at the Department of Medical Microbiology and Immunobiology, University of Szeged, Hungary.

3.10. Anti-chlamydial assay

Elementary bodies of *Chlamydia trachomatis* D (Chapter 1.5) ($1x10^3$ IFU/ml) were incubated with *N. sativa* EO, Thq, Car and p-cy at various concentrations (50, 25 µM; 0,005%, 0,0025% EO in DMSO) in sucrose-phosphate-glutamic acid buffer (SPG) for 1 h at 37 °C. As a control, *C. trachomatis* D was also incubated in SPG alone. To quantify the anti-chlamydial effects of compounds, HeLa cells were seeded in 24-well tissue culture plates with 13-mm cover glasses. After 24 h, the confluent cells were infected with compounds-treated *C. trachomatis* D or the non-treated controls. After 48 h, the cells were fixed with acetone at -20°C for 10 min and stained with murine monoclonal anti-Chlamydia LPS antibody (AbD Serotec, Oxford, UK) and FITC-labelled secondary anti-mouse IgG (Merck KGaA, Darmstadt, Germany). The number of *C. trachomatis* D inclusions was counted under a UV microscope, and the titer was expressed in inclusion forming units/ml (IFU/ml).

This test was performed at the Department of Medical Microbiology and Immunobiology, University of Szeged, Hungary.

3.11. Application of *N. sativa* crude oil in food matrix

Minced pork chop (1000 g) was used for the experiment, which was purchased from a local shop in Budapest on the first day of the experiment.

Different samples were prepared for the experiment (from each type of samples 3-3 parallel for each sampling day):

- MP – control minced pork,
- MP-Lm – minced pork inoculated with *L. monocytogenes*,
- MP-CO+Lm – minced pork with 2% Nigella sative CO and inoculated with *L. monocytogenes*,

Samples that were inoculated with the pathogen had 10^5 CFU/g initial cell count of *L. monocytogenes*. Samples (10 g) were measured into sterile stomacher bags, sealed and stored in a refrigerator during the experiment. Microbiological examinations were performed on the day of sample preparation and after 6 day refrigerated storage.

40 ml maximum recovery diluent was added to each sample and homogenised for 2 minutes in stomacher homogenizer and decimal serial dilutions were prepared. *L. monocytogenes* counts were determined by spreadplating the appropriate dilutions on Palcam agar (BIOKAR BK145 with Palcam Selective Supplement BIOKAR BS004). Plates were incubated at 37 °C for 24-48h and evaluated.

3.12. Resistance-modulation assay

Modulation of antimicrobial resistance for the antibiotics, erythromycin, ciprofloxacin, and EtBr was evaluated using the same microdilution method (Chapter 1.5), except that the medium was supplemented with sub-inhibitory concentration of the EO or Thq (1/2 MIC). The modulation factor was defined as the ratio of the MICs for the antimicrobials (Ery, Cip or EtBr) alone and for the antimicrobial agent in the presence of the EO, Thy, Car and p-cy. All the MICs measurements were carried out in triplicates. The chemical EPI, Reserpine (20 μg/mL) was used as positive control reference. The positive control wells were prepared with the bacterial suspension only, the bacterial suspension and ½ MIC of the EO, Thq, Car or p-cy and with DMSO or EtOH with the bacterial suspension

corresponding to the highest concentration present in the preparation. A modulation factor >2 was set as the cut-off for biologically significant resistance modulation (Kovač et al., 2015).

3.13. EtBr accumulation assay

The influence of *N. sativa* EO, Thq, Car and p-cy on EtBr accumulation in *L. monocytogenes* L14 and *S. aureus* (ATCC 25923 and MRSA 272123) were determined (Kovač et al., 2014). Briefly, 150 μL of overnight culture (4 mL inoculum, 24 h) was added to 9.9 mL TSB, centrifuged at 6000 g for 5 minutes, then the cells were two times washed and resuspended in phosphate-buffered saline (PBS) (OD_{600}, 0.2). *N. sativa* EO, Thq, Car or p-cy were added to the culture to the appropriate concentration (1/2 MIC). After 15 min incubation at 37 ºC, 96.74 μL of the untreated culture, the treated culture containing EO, Thq, Car or p-cy, were pipetted in black microtiter plates followed by adding 3.26 μL EtBr to a final concentration of 0.5 μg/mL. The kinetics of intracellular EtBr accumulation was measured at 490 nm and 579 nm using a Victor x3 plate reader (PerkinElmer/USA), at 45 s intervals for 1 h. Additionally reserpine (100 μg/mL) and CCCP (10 μg/mL)was used as a positive control reference in the assay. Measurements were carried out in triplicate and the means of the last 10 times points of the measurements were used in the statistical analysis.

This test was performed at the Institute of Enzymology, Hungarian Academy of Science, Hungary.

3.14. Membrane integrity assay

The influence of *N. sativa* EO, Thq, Car and p-cy on membrane integrity of *L. monocytogenes* L14 and *S. aureus* (ATCC 25923 and MRSA 272123) were assessed using LIVE/DEAD Baclight^{TM} Bacterial Viability Kits (L-7012, Molecular Probes, Eugene, Oregon, USA). The BacLight^{TM} kit is composed of two nucleic acid-binding stains: SYTO 9TM and propidium iodide (PI). SYTO 9TM penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells (Simoes et al., 2008). Briefly, exponential phase culture was washed two times and resuspended in phosphate-buffered saline ($OD_{600,}$ 0.2) 1 mL of the culture was heat treated at 80 ºC for 15 min to obtain the dead culture, while the treated cultures with the EO, Thq, Car or p-cy were incubated at 37 ºC for 15 min. A mixture of SYTO 9TM (6 μL) and propidium iodide (6 μL) was added to 2 mL filtered (0.2

*µ*m pore size filter) distilled water. This dye mixture was added to 100 *µ*L *L. monocytogenes* culture (1:1, *v/v)* that was untreated or treated with *N. sativa* EO, Thq, Car or p-cy or heat treated. The kinetics of propidium iodide and SYTO 9 intracellular penetration were followed by measuring the relative fluorescence units (RFU) in 60-s intervals over 1 h, at 490 nm and 535 nm using a microplate reader (Tecan, Männedorf, Switzerland). The experiments were carried out in triplicate in black microtiter plates. The membrane disruption (%) was calculated from the kinetic measurements of the treated relatively to the untreated cultures over the last 10 min of the assay.

This test was performed at the Institute of Enzymology, Hungarian Academy of Science, Hungary.

3.15. Expression analysis of genes by real-time reverse transcriptase quantitative polymerase chain reaction

S. aureus ATCC 25923 and *S. aureus* 272123 strains were cultured in TSB broth and were incubated overnight at 37°C with shaking at 140 rpm. On the day of RNA isolation, the bacterial suspensions (OD of 0.6 at 600 nm) were transferred to 10 mL tubes in 3 mL aliquots, and 5 µM of *N. sativa* EO, or 0.5 µM of compound**s** (Thq, Car, p-cy) were added to the tubes, which were incubated at 37ºC. After 4 hours of culturing, the tubes were centrifuged at 12,000 × *g* for 2 min. Pellets were suspended in 100 µL TE (Tris-EDTA) buffer containing 1 mg/mL lysozyme by vigorous vortexing, and they were incubated at 37°C for 10 min. The total RNA was isolated in an RNase-free environment using the NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes and was maintained at −20ºC until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry at 260 nm. Expression of the efflux pump genes *mepA* was studied by reverse transcription of the total RNA. The data obtained for gene targets were normalized against the *S. aureus* 16S ribosomal RNA measured in the same sample.

The primers used in the assay were the following (Couto et al., 2008):

1. Sequence (5'-3') of *mepA* (198 bp) TGCTGCTGCTCTGTTCTTTA (Fw)
   GCGAAGTTTCCATAATGTGC (Rv)
2. Sequence (5'-3') of *16S rRNA* (492 bp) AGAGTTTGATCMTGGCTCAG (Fw)
   GWATTACCGCGGCKGCTG (Rv)

Real-time quantification of the RNA templates by real-time one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA), following the manufacturer's recommendations of the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany). Briefly, each well of the 96-well microtiter plates in a final volume of 20 µL contained 10 µL of the 2x SensiFAST™ SYBR No-ROX One-Step Mix, 0.2 µL Reverse Transcriptase, 0.4 µL RiboSafe RNase Inhibitor, 5.4 µL Diethylpyrocarbonate (DEPC)-treated water, 500 nM of each primer and approximately 20 ng of the total RNA in RNAase-free water. The RT step involved incubation at 45°C for 10 min, followed by DNA Polymerase activation for 2 min at 95°C. Thermal cycling was initiated with a denaturation step of 5 min at 95°C, followed by 40 cycles, each of 10 s at 95°C, 30 s at 57°C and 20 s at 72°C. The ΔΔCt method was used for the quantification of the gene expression.

This test was performed at the Department of Medical Microbiology and Immunobiology, University of Szeged, Hungary.

3.16. Biofilm formation assay

Biofilm formation of *S. aureus* (ATCC 25923 and MRSA 272123) was studied under defined growth conditions that experimentally approximate different stress conditions. M9 minimal media (containing NH_4Cl [1.9 mM], Na_2HPO_4 [42.3 mM], KH_2PO_4 [22 mM], NaCl [8.56 mM], $MgSO_4$ [2 mM], $CaCl_2$ [0.1 mM], and TSB broth were used for this purpose to enable an assessment of the inhibition of biofilm formation capacity using *N. sativa* EO, Thq, Car and p-cy at different concentration. Initially, overnight cultures were adjusted to 1 McFarland with M9 minimal media, and 200µL of the bacterial cell suspension was dispensed across a 96-well microtiter plate. Plates were incubated for 72 h at 37 ºC.

Crystal violet was used to detect total biofilm biomass formed. Briefly the 96-well plate containing culture medium was discarded after growth and the plate was washed with

PBS. Crystal violet (0.4% [v/v]) was added and incubated at room temperature for 15 min. The plate was then washed again with PBS and dried in a sterile air flow hood for 15 min. Once dried, 200 μL 33% [v/v] acetic acid was added to each well and incubated at room temperature for 15 min, then moved to another clean microplate and the absorbance was recorded in a Multiskan^{TM} FC Microplate Photometer (Thermo Scientific, Dublin, Ireland) at 650 nm.

3.17. Statistical analysis

The results were statistically analyzed using Microsoft Excel program (2016). Comparisons of the group mean values and the significance of the differences between the groups were verified by one-way ANOVA. The results were considered significant when $p \leq 0.05$.

4. RESULTS AND DISCUSSION

4.1. Chemical composition of *N. sativa* oils

4.1.1. Fatty acids methylation and GLC analysis of the crude oil

The results of fatty acids analysis of *N. sativa* crude oil (Table 7) showed ten saturated and unsaturated fatty acids. The major fatty acid of CO was linoleic acid (18:2n-6) with a level of 55.03g/100g total fatty acids (TFA), followed by oleic (18:1n-9) with a level of 22.53 g/100g TFA and palmitic (16:0) with a level of 14.39 g/100g TFA, respectively. The ratio of linoleic acid to oleic was about 2:1. This ratio agreed with previous published data (Atta, 2003; Nickavar et al., 2003; Piras et al., 2013).

Chemical composition of the oils depends on many factors, such as the origin of the samples and climatic conditions. Therefore, different variety of chemotypes have been described in the literature. Our results (55.0% linoleic acid and 22.5% oleic acid) are in accordance with previously published data (Atta, 2003), while in a study from Marocco a chemotype with 33.0% p-cymene and 26.8% thymol was reported and a chemotype with 60.2% p-cymene and 12.9% γ-terpinene was reported in another publication for *N. sativa* oil from Poland (Piras et al., 2013).

N. sativa oil, similarly to wheat germ oil (Piras et al., 2013), corn oil, cottonseed oil, and soybean oil (Ong and Goh, 2002) represents a source of the essential fatty acid, linoleic acid which cannot be synthesized by humans. Linoleic acid is essential for human and it has an important role e.g. in the prevention and treatment of cardiovascular diseases (Bhatnagar & Durrington, 2003). This suggest that the cold-pressed black cumin oil may serve as a potential dietary source of polyunsaturated fatty acid (PUFA).

Table 7: Fatty acids composition of *N. sativa* crude oil

Fatty acid	Retention time (min)	Percentage (w/w%)
Myristic acid (C14:0)	17.132	0.35
Palmitic acid (C16:0)	20.835	14.39
Stearic acid (C18:0)	24.283	4.77
Arachidic acid (C20:0)	27.507	0.24
Behenic acid (C22:0)	30.523	1.44
Lignoceric acid (C24:0)	32.668	0.3
Palmitoleic acid (C16:1)	20.835	0.45
Oleic acid (C18:1)	27.738	22.53
Linoleic acid (C18:2)	25.823	55.03
Linolenic acid (C18:3)	27.177	0.49
TSFA	….	21.49
TUFA	….	78.05

4.1.2. GC-MS analysis of the essential oil

The identified active components, their percentages, their retention indices and their class can be seen in Table 8.

The results showed that 10 components were identified in the EO (seven monoterpenes hydrocarbon (MH), two oxygenated monoterpenes (MO) and one sesquiterpene hydrocarbon (SH), representing 94.03% of the total amount. The oil consisted mainly of MH with 71.69 % followed by lower contents of MO with 20.51 % and very low amounts of SH (1.83 %). Therefore, among of the monoterpenes hydrocarbons, p-cymene represent the major constituent (52.24%) while thymoquinone was dominating the oxygenated monoterpenes (18.76%).

The amount of p-cymene, which was found as a major compound in our oil, was higher than those reported in the literature (Nickavar et al., 2003; Piras et al., 2013) and quite

similar to those reported by Toma and co-workers (2010) in the case of the hydrodistillation of Tunisian *Nigella sativa* essential oil extracts. However, thymoquinone that was found as a second major compound of the essential oil, was similar to those reported by (Benkaci-Ali et al., 2006) in the case of the hydrodistillation of Algerian *Nigella sativa* essential oil extracts and much higher than those reported for the Tunisian and Iranian *N. sativa* essential oils (Nickavar et al., 2003; Toma et al., 2010). These differences could be attributed to several factors such as origin of the samples, environmental, climatic conditions and harvest year.

Table 8: Chemical composition of *N. sativa* essential oil

Component	**Retention time (min)**	**Percentage (%) ***	**LRI**	**Class**
α-thujene	5.31	6.08±0.30	928	MH[1]
α-pinene	5.56	1.46±0.04	938	MH
Sabinene	6.52	0.96±0.02	976	MH
ß-pinene	6.64	2.07±0.06	981	MH
p-cymene	8.09	52.24±1.04	1026	MH
Limonen	8.19	2.21±0.11	1029	MH
γ-terpinene	9.78	6.67±0.33	1033	MH
Thymoquinone	17.93	18.76±0.38	10,6	MO[2]
Carvacrol	19.71	1.75±0.11	1251	MO
Longifolene	24.92	1.83±0.07	1290	SH[3]

*Minor components (<1%) were not evaluated

[1] Monoterpenes hydrocarbon

[2] Oxygenated monoterpenes

[3] Sesquiterpene hydrocarbon

4.2. Evaluation of antimicrobial activity of *N. sativa* oils

4.2.1. Agar well method

The crude oil of *Nigella sativa* seeds was evaluated for its antimicrobial activity (Table 9). Antibacterial activity was evaluated against different microbial strains, including Gram-positive and Gram-negative bacteria, using agar well method and the diameters of inhibition zones was measured (Table 9). The activity of different concentrations of *N. Sativa* crude oil are presented in appendix (A2).

Table 9: Diameters of inhibition zones (mm) of *N. sativa* crude oil (%) against food spoilage bacteria and foodborne pathogens.

Strains	**crude oil (w/w %)**	**Zone of inhibition (mm)**
		Mean ± SD
Listeria monocytogenes E12/10.12.1	10	23±1.1
Listeria monocytogenes 11/4.12 t03	10	16±1.5
Listeria monocytogenes E ST/10.12.1	10	16±1.5
Listeria monocytogenes E ST/10.12.2	10	27±2
Listeria monocytogenes E ST/10.12.3	10	24±2.5
Listeria monocytogenes 8/4.12. t0	10	22±1
Listeria grayi CCM 5990	10	12±1
Listeria innocua CCM 4030	10	21±0.5
Listeria grayi CCM 4029	10	15±2
Listeria monocytogenes B01966	5	18±2
Listeria monocytogenes CMM 4699	5	14±4.5
Listeria monocytogenes NCAIM 1454	2,5	15±4.2
Listeria monocytogenes 4b	10	24±1
Listeria monocytogenes 1/2 c	10	26±1
Listeria welshimeri CCM 3971	5	14±1
Listeria monocytogenes CCM 7202	10	19±3.2
Listeria monocytogenes 3b	10	25±0.5
Listeria monocytogenes #46	1,25	17±2.1
Listeria ivanovii CCM 5884 T	10	23±1.7
Micrococcus Luteus	0,33	15±0
Staphylococcus aureus	0,63	12±0
Enterococcus faecalis	20	21±2.6
Bacillus subtilis	2,5	29±5
Bacillus megaterium	2,5	12±1
Bacillus licheniformis	2,5	28±2.1
Bacillus cereus	5	33±2.1
Rhodococcus eqvii	5	19±0.5
E. coli	100	No effect
Proteus sp.	100	No effect

Antimicrobial activity of *N. sativa* crude oil toward of standard and isolated strains is reported in Table 9. Previous study on the antimitotic and the antibacterial effect of *N. sativa* L. seed (Özmen et al., 2007) showed that the oil extracted by diethyl ether was determined as ineffective against *E. coli* and *Proteus spp.* which is in accordance with our results. For gram-positive bacteria, CO showed the best activity against *Micrococcus* at 0,33 % and the lowest activity was for *Enterococcus* at 20 %. The oil was less active against *Listeria* strains.

It is generally expected that when antimicrobial activity is measured most of the materials tested would be active against Gram-positive than Gram-negative bacteria (McCutcheon et al., 1992). In this study CO inhibited especially Gram-positive bacteria.

4.2.2. Minimal inhibitory concentration

The results of minimal inhibitory concentration (MIC) of the *N. sativa* crude and essential oils evaluated by microdilution method against 7 bacteria (spoilage and/or pathogenic) are shown in Table 10. The results of the antimicrobial activity showed that both oils of *N. sativa* had a good activity on the tested strains. DMSO, as a diluent of oils, did not show any inhibitory effect on the growth of the strains.

Table 10: Minimal inhibitory concentration (MIC) of *N. sativa* crude oil and essential oil

Strains	Crude Oil (w/w %)	Essential Oil (w/w %)
Bacillus cereus	0.06	0.006
Bacillus subtilis	0.06	0.003
Staphylococcus aureus	0.25	0.025
Listeria monocytogenes	0.125	0.025
Pseudomonas aeruginosa	0.25	0.025
Salmonella Hartford	no effect	0.46
Escherichia coli	no effect	0.23

The activity of essential oil was 10 times more than the crude oil. In addition, the MIC of essential and crude oils was varied between 0.003-0.46% and 0.06-0.25% for all bacteria tested respectively.

The mode of action of essential oils depends on the properties of the essential oil and on the type of microorganisms, which is mainly related to their cell wall structure. Generally Gram-positive bacteria are more sensitive to essential oils than Gram-negative bacteria. Resistance of Gram-negative bacteria to a wide variety of essential oils is associated with the hydrophilic surface of their lipopolysaccharide rich outer membrane that serves as a permeability barrier. Small hydrophilic molecules can pass through the outer membrane but hydrophobic macromolecules (e.g. essential oil constituents) are not able to penetrate this barrier (Kalemba & Kunicka, 2003). Our results were in accordance with these statements, as the order of sensitivity of microorganisms was Gram-positive (in decreasing order: *Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, Bacillus subtilis*) followed by Gram-negative (in decreasing order: *Salmonella* Hartford, *Escherichia coli, Pseudomonas aeruginosa*) for both oils. However, no inhibitory activity was observed against *Salmonella* and *E. coli* with the crude oil, at the maximum concentration tested, 100% (it was checked with disc diffusion method, (10 µL of the undiluted crude oil/disk) results are not shown).

Previous study has shown that long chain of fatty acid has an antimicrobial effect against a few strains of *Staphylococcus aureus* (Kabara et al., 1972). Kabara and co-workers compared the antimicrobial effect of saturated and unsaturated fatty acids and found that as compared with their saturated counterparts, the C14:1, C16:1, and C18:2 derivatives were more active. They also stated that the second double bond further increased the bacteriostatic effect of the C18 derivatives. Therefore, the antibacterial activity of our crude oil may be attributed to the notable amount of oleic (C18:1 - 22.53%) and linoleic acids (C18:2 - 55.03%) as the main components in the oil composition of *N. sativa*.

Characterization of *N. sativa* essential oil composition by gas chromatography-mass spectrometry analysis has revealed the presence of a variety of compounds possessing antimicrobial properties, including p-cymene and γ-terpinene (Güllüce et al., 2003), thymoquinone (Halawani, 2009), carvacrol (Suntres et al., 2015) and limonene (Oumzil et al., 2002). Oxygenated monoterpenes (e.g. thymoquinone and carvacrol) exhibit stronger antimicrobial activity, than hydrocarbon derivatives (e.g. p-cymene, limonene, γ-terpinene).

The relative low inactivity of hydrocarbons can be attributed to their limited hydrogen bound capacity and water solubility (Knobloch et al., 1988) that limits their penetration through the cell wall (Oumzil et al., 2002). Although p-cymene (52.24%) formed a significant amount of our essential oil, its antimicrobial effect was more likely caused by Thq (22.53%), which has greater antimicrobial potential as an oxygenated monoterpene. Crude oil also contained these compounds (as the essential oil was extracted from it), but the concentration of these compounds was much higher in the essential oil due to the chemical extraction step resulting much stronger antimicrobial activity.

4.2.3. Combination of crude oil and nisin against spores

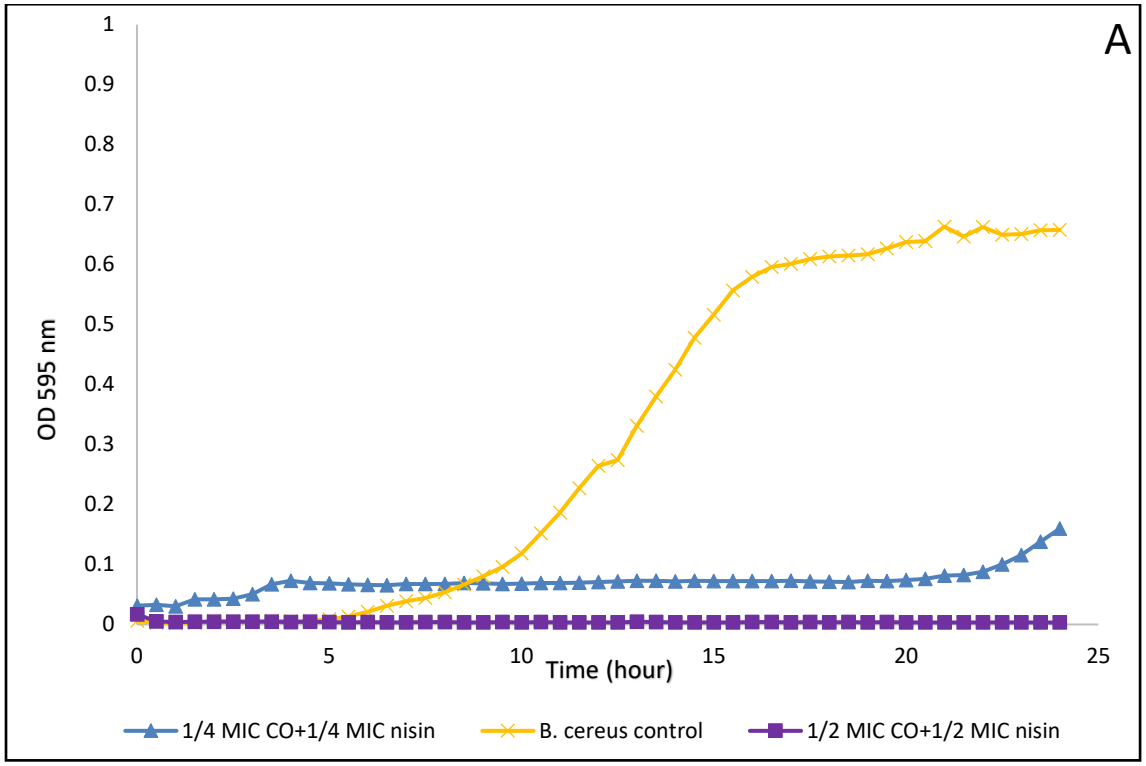
The MIC of *N. sativa* crude oil and nisin were determined by measuring the optical density using Multiskan machine (Table 11).

Table 11: Minimal inhibitory concentrations of CO and nisin

Spores	Crude oil (%)	Nisin (mg/ml)
B. cereus	0.125	0.3
B. subtilis	0.25	0.3

Both compounds were found to inhibit *Bacillus* spores. From these MICs values, two combinations between *N. sativa* crude oil and nisin (1/2 MIC + 1/2 MIC or 1/4 MIC + 1/4 MIC) were used to check if *N. sativa* crude oil is able to enhance the activity of nisin against *B. cereus* and *B. subtilis* spores.

Fig. 10, shows the effect of the combination between *N. sativa* crude oil and nisin on *B. cereus* spores (A) and *B. subtilis* spores (B).



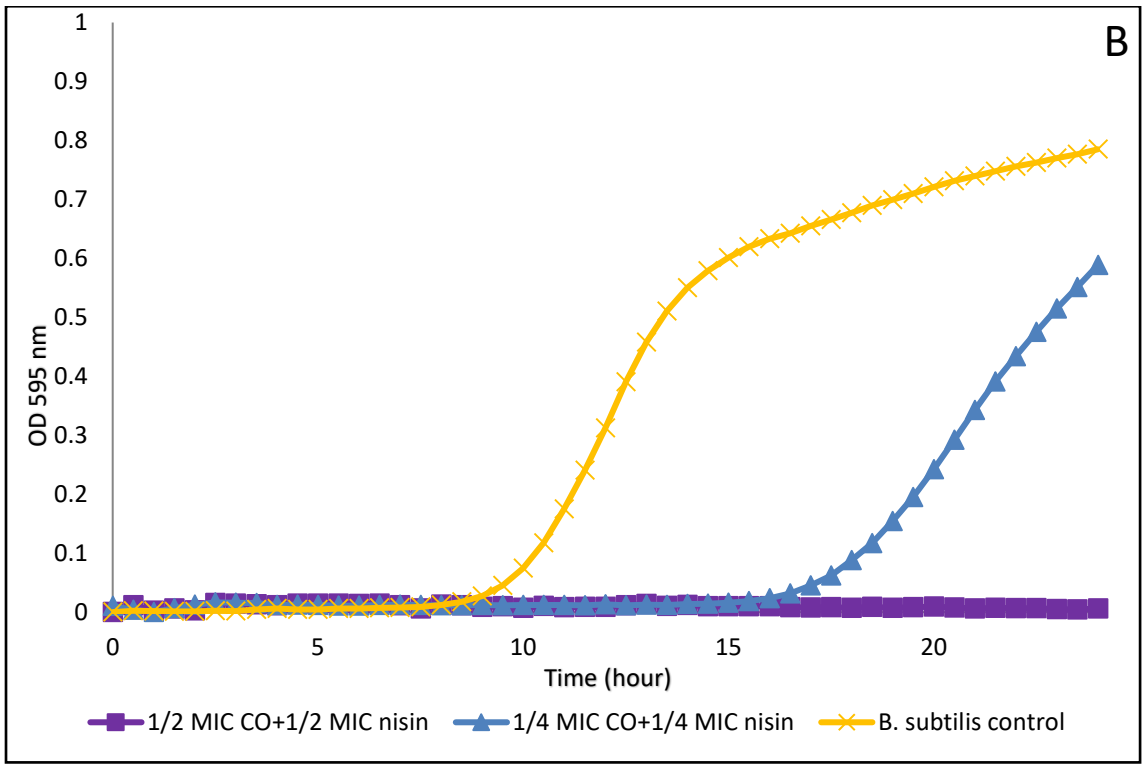


Figure 10: The effect of *N. sativa* crude oil (CO) in combination with nisin against *B. cereus* (A) and *B. subtilis* (B) spores

In case of *B. cereus* spores the first combination (1/2 MIC + 1/2 MIC) resulted in full inhibition of the strain. As for the second combination (1/4 MIC + 1/4 MIC) it was less effective than the first one, yet the optical density was much lower and the time needed to

detect growth was much longer than the positive control (Fig. 10A). In case of *B. subtilis* spores the first combination resulted in full inhibition of the strain, while as for the second combination after 15 hour the optical density increased and reached optical density values close to the positive control (Fig. 10B). Interestingly, combining nisin with *N. sativa* crude oil resulted in extra reduction of the OD. This clearly indicates that *N.sativa* crude oil enhance the activity of nisin.

In the food industry, the pathogenic microorganisms and the defense against them are a major challenge. Foodborne diseases affect millions of people each year, so minimizing the risks of microbial food safety is of fundamental importance. Because of conscious consumer behavior, foods with naturally occurring materials that are free from artificial preservatives are becoming more common.

Antimicrobial substances are generally used in combination with other substances or physical methods (Pol and Smid, 1999), as it often avoids undesirable organoleptic changes in food. However, the combined effect of *N. sativa* crude oil and nisin has not yet been examined.

In our approach, we focused on extending previous research, by applying *N. sativa* and examining its combined effect. For all combinations used, the synergistic effect was detectable, that is, they worked together more efficiently than separately.

Combination of nisin and *N. sativa* crude oil enables us to use lower amounts of both compounds for effective food preservation. The actual mechanism of this effect is not known. *N. sativa* crude oil might enhance nisin's action by increasing the life-time of pores created by nisin or by increasing the number or size of the pores formed, both leading to an increased reduction of viable numbers. This needs to be elucidated.

4.2.4. Anti-chlamydia

The IC50 of *N. sativa* EO, Thq, Car and p-cy on HeLa cells were determined (Table 12), where the inhibitory concentration (IC50) is defined as the inhibitory dose that reduces the growth of the compound-exposed cells by 50%. The IC50 of *N. sativa* EO was indicated to be 0.009%, while the IC50 of Thq, Car and p-cy was higher than 100 μM (Table 12)

Table 12: Half maximal inhibitory concentration (IC50) in μM of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene on HeLa cells

Bioactive compounds	**IC50 (µM)**
N. sativa essential oil	0.009 (%)
Thymoquinone	>100
Carvacrol	>100
p-cymene	>100

To determine whether they possess anti-chlamydial activity, *N. sativa* EO, and its active compounds, Thq, Car and p-cy were co-incubated individually with *C. trachomatis* D at 1/2 and 1/4 IC50 of each compound (Fig. 12).

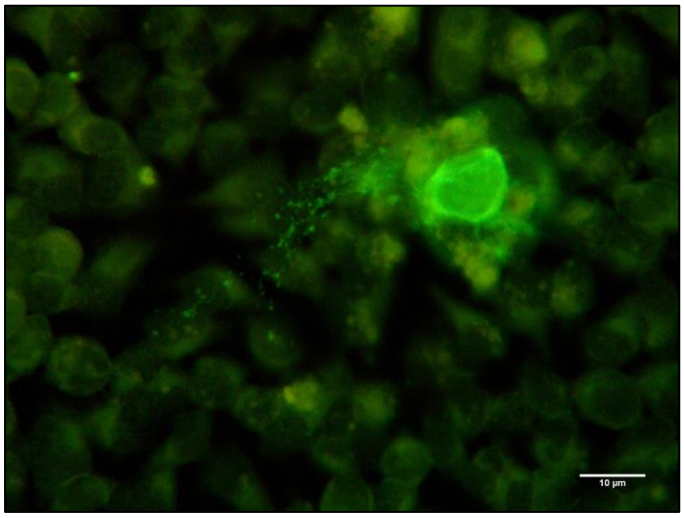


Figure 11: *C. trachomatis* D immunostained with murine monoclonal anti-Chlamydia LPS antibody and FITC-labelled secondary anti-mouse IgG (Green staining)

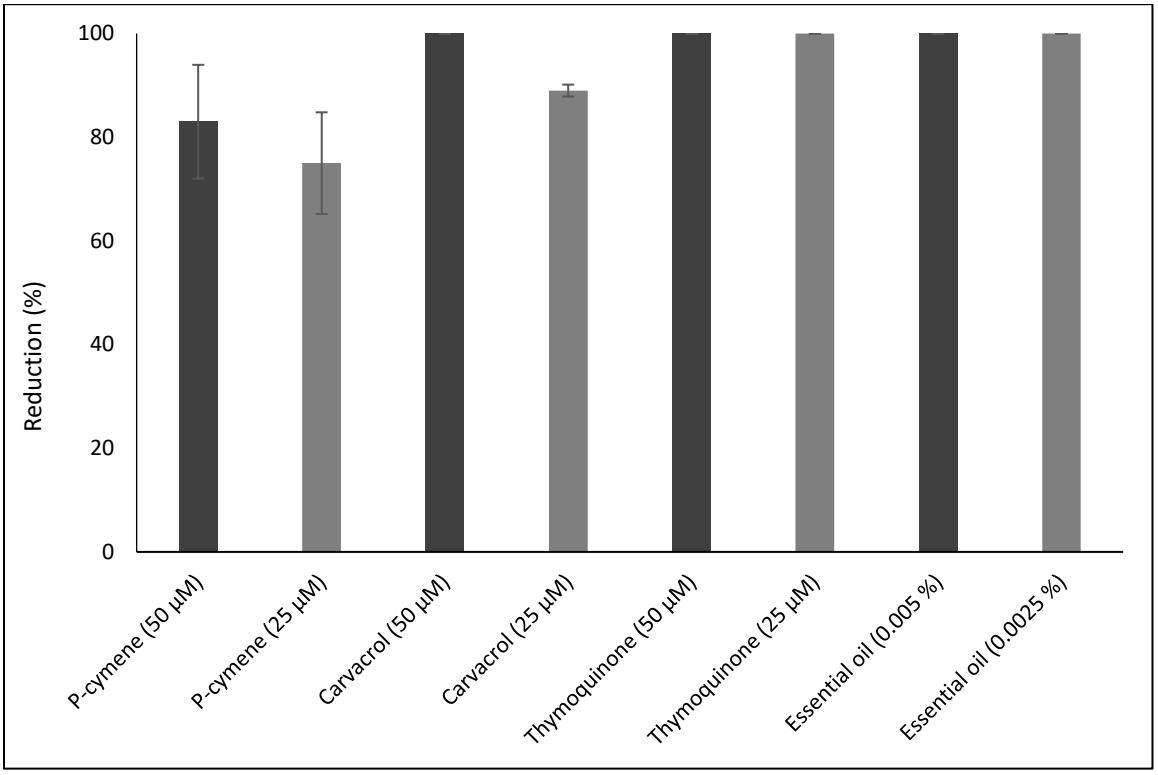


Figure 12: Reduction percentage of *C. trachomatis* D inclusions by *N. sativa* essential oil, thymoquinone, carvacrol and p-cymene.

Counting of the number of viable *C. trachomatis* inclusions demonstrated that all the compounds were highly effective killers of *C. trachomatis*. *N. sativa* EO, Thq and Car (at ½ IC50) exhibited 100% reduction in the viability of *C. trachomatis*, while p-cymene showed lower reduction by 83 % (Fig. 12).

C. trachomatis is the most important cause of sexually transmitted diseases in both developed and developing countries. There are an estimated 90 million new cases of genital infections occurring annually among adults. The development of effective new antimicrobial compounds is necessary if the late severe sequelae of the infections, such as infertility and ectopic, are to be avoided (WHO, 1996). Although the antimicrobial activity of *N. sativa* EO against bacteria, fungi has been tested (Forouzanfar et al., 2014), the anti-chlamydial activity of *N. sativa* EO has not yet been tested. Hughes and co-workers (2009) suggested that quinones have powerful inhibitory effect against *Chlamydia muridarum*. *N. sativa* EO and its active compounds, Thq, Car and p-cy appears to be potentially promising candidates against *C. trachomatis* D.

4.2.5. Application of *N. sativa* crude oil in food matrix

The control, oily and bacterial inoculated food samples were subjected to a storage experiment to investigate the ability of the crude oil to inhibit *L. monocytogenes* proliferation and deterioration of the meat product. For each type of sample, the results were derived from 3 parallel measurements.

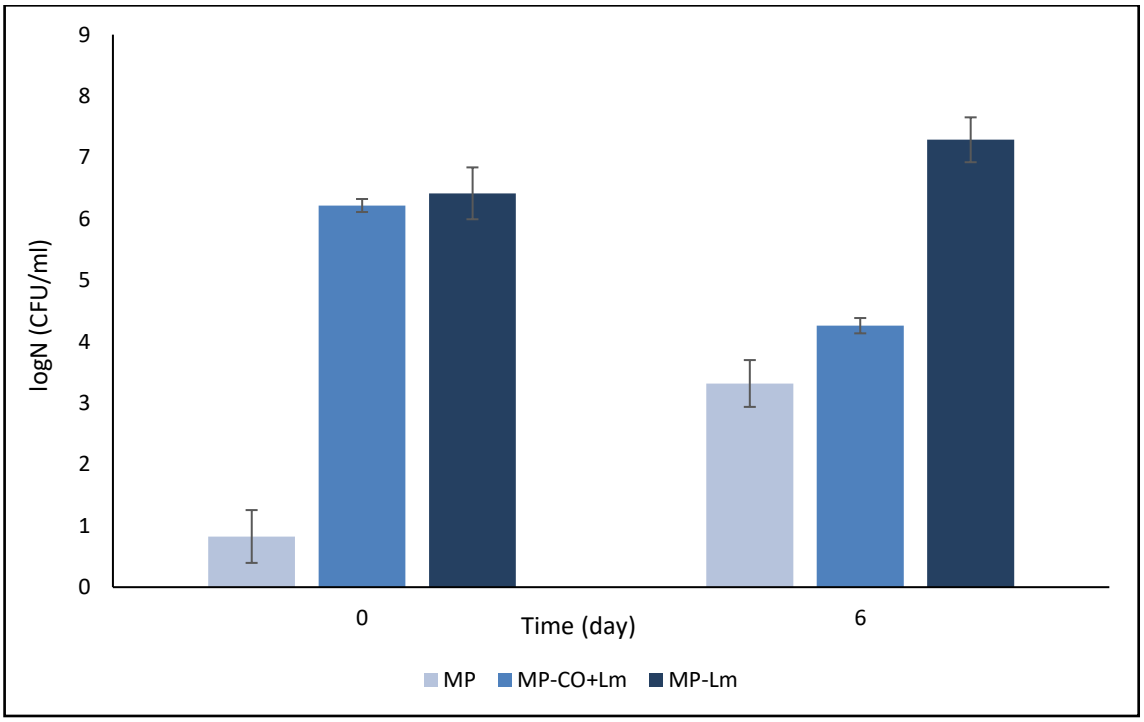


Figure 13: effect of *N. sativa* crude oil on *L. monocytogenes* count in meat samples stored at 10 ºC

Fig. 13 shows that the starting minced meat sample also contained *L. monocytogenes* as a natural contamination, but its number was negligible compared to the inoculation (presumptive *Listeria monocytogenes* colonies were not confirmed with biochemical tests). In Fig. 13, it can be observed that on the 6th day of storage, the maximum number of *L. monocytogenes* bacteria was found at the absolute control (MP). While for the oily sample, the oil inhibited the growth of *Listeria monocytogenes* by 2 log.

It is clear that *N. sativa* crude oil has an antimicrobial effect on the *L. monocytogenes* pathogenic bacterium and is therefore an alternative natural preservative. In further experiments, it would be beneficial to investigate the inactivation potential of *Nigella sativa* oil in other foods.

4.3. Resistance modifying activity of *N. sativa* essential oil and its active compounds against *L. monocytogenes*

4.3.1. Determination of minimum inhibitory concentrations (MICs)

The antibacterial activity of *N. sativa* EO and its active compounds were determined by broth microdilution method.

As presented in Table 13, the essential oil of *N. sativa*, thymoquinone and carvacrol were active against all the tested strains of *L. monocytogenes*. The MIC values of *N. sativa* EO ranged from 116 to 466 μg/mL, thymoquinone exhibited a significant antibacterial activity with a MIC values of 40 μg/mL, and carvacrol exhibited the MIC of 150 μg/mL, while p-cymene showed no inhibitory activity at the tested maximum concentration (2144 μg/mL) against any strain.

Table 13. Minimal inhibitory concentration in μg/mL of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene on nine *L. monocytogenes* strains

***L. monocytogenes* strain**	***N. sativa* EO**	**Thymoquinone**	**Carvacrol**	**p-cymene**
L2	116	40	150	>2144
L6	116	40	150	>2144
L14	116	40	150	>2144
L7	233	40	150	>2144
L4	233	40	150	>2144
L1	233	40	150	>2144
L9	233	40	150	>2144
LI	466	40	150	>2144
LA	233	40	300	>2144

4.3.2. Resistance-modulation assay

As a potential modulator of antimicrobial resistance, *N. sativa* essential oil, thymoquinone, carvacrol and p-cymene were tested at 1/2 MIC (58 μg/mL, 20 μg/mL, 75

μg/mL and 1072 μg/mL, respectively) in combination with the antibiotics erythromycin, ciprofloxacin, and the antimicrobial efflux pump substrate EtBr, on nine *L. monocytogenes* test strains (Table 14). A modulation factor >2 was set as the cut-off for biologically significant resistance modulation (Kovač et al., 2015).

Table 14: Modulation of antimicrobial activity of antibiotics and EtBr in the presence of half MIC of *N. sativa* essential oil and its bioactive compounds and reserpine (20 μg/mL) for 9 *L. monocytogenes* strains

Antimicrobial	***Listeria monocytogenes* strain MIC (μg/ml)**								
	L2	**L6**	**L14**	**L7**	**L4**	**L1**	**L9**	**LI**	**LA**
Ery [a]	0.125	0.125	0.125	0.25	0.25	0.25	0.25	0.25	0.25
Fold-decrease in MIC of Ery									
+1/2 MIC EO	2	2	2	2	4	2	2	4	2
+1/2 MIC Thq	2	2	2	4	2	2	2	2	2
+1/2 MIC Car	2	2	2	4	2	2	2	2	2
+1/2 MIC p-cy	2	2	2	1	1	2	2	2	2
+20 μg/mL Res	1	1	1	1	1	1	1	1	1
Cip [a]	1	0.25	0.25	0.5	0.25	0.25	0.125	0.25	0.25
Fold-decrease in MIC of Cip									
+1/2 MIC EO	2	4	8	4	4	4	4	4	2
+1/2 MIC Thq	4	4	2	4	2	4	4	4	2
+1/2 MIC Car	4	4	4	4	4	4	4	2	4
+1/2 MIC p-cy	2	2	4	4	2	2	2	2	2
+20 μg/mL Res	4	4	4	2	2	4	4	2	2
EtBr [a]	128	64	128	128	128	128	128	128	64
Fold-decrease in MIC of EtBr									
+1/2 MIC EO	16	8	16	8	4	8	4	4	8
+1/2 MIC Thq	8	8	8	8	8	8	8	4	8
+1/2 MIC Car	16	16	8	8	16	16	8	4	2
+1/2 MIC p-cy	2	4	4	2	2	2	2	2	4
+20 μg/mL Res	8	8	8	8	8	8	8	8	8

[a]: Tet: tetracycline; Cip: ciprofloxacin; EtBr: ethidium bromide; EO = *N. sativa* essential oil; Thq = thymoquinone; Car = carvacrol, p-cy = p-cymene, Res = reserpine and MIC = minimal inhibitory concentration.

Data presented in Table 14 showed that the supplementation of *N. sativa* EO (at 1/2 MIC) decreased the MICs of erythromycin from two-fold to four-fold and decreased the MIC of ciprofloxacin from two-fold up tp eight-fold against all *L. monocytogenes* strains. Additionally, a four-fold up to sixteen-fold reduction of EtBr was observed against *L. monocytogenes* strains in the presence of the EO.

Whereas, the supplementation of thymoquinone (at 1/2 MIC) induced decrease in the MIC of ciprofloxacin four-fold against seven *L. monocytogenes* strains and two-fold decrease against the other three strains. Furthermore, eight-fold reduction of EtBr was noted against eight *L. monocytogenes* strains and four-fold against 1 strain (Table 14).

Carvacrol supplementation (at 1/2 MIC) decreased the MIC of ciprofloxacin: two-fold reduction was observed for all the tested strains of *L. monocytogenes.* Additionally, a two-fold up to sixty-four-fold reduction of EtBr MIC was detected against *L. monocytogenes* strains in the presence of carvacrol (Table 14).

p-cymene supplementation (at 1/2 MIC) showed two-fold reduction in the MIC of erythromycin against six *L. monocytogenes* strains and had no activity against the other three strains. P-cymene induced decrease in the MIC of EtBr from two-fold up to four-fold against all *L. monocytogenes* strains (Table 14).

Reserpine supplementation (20 μg/mL) showed two up to four-fold reduction in the MIC of ciprofloxacin, and decreased the MIC of EtBr eight-fold against all *L. monocytogenes* strains (Table 14).

4.3.3. EtBr accumulation assay

In order to elucidate the mechanism of modulatory activity of *N. sativa* essential oil and its active compounds, thymoquinone, carvacrol, and p-cymene in *L. monocytogenes* L14, we have evaluated the potency of the compounds to increase the accumulation of the common efflux pump substrate EtBr indicating efflux inhibition.

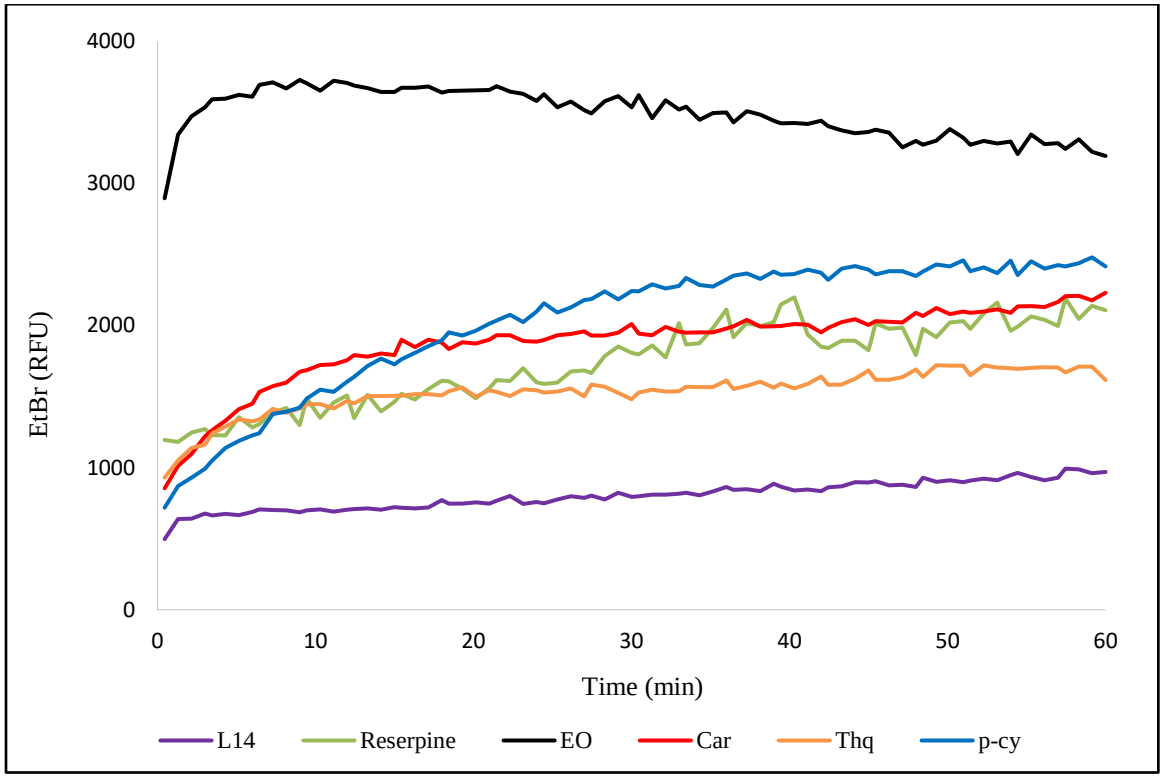


Figure 14. Ethidium bromide accumulation in *L. monocytogenes* L14 in the presence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene, and reserpine as positive control. RFU= relative fluorescence units; L14= *L. monocytogenes* L14; EtBr=ethidium bromide; EO= essential oil; Thq=thymoquinone; Car=carvacrol and p-cy=p-cymene.

The level of EtBr accumulation in cultures treated with half of the MIC values of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene were compared, relatively to the untreated culture, to evaluate whether it can potentiate intracellular EtBr accumulation. The known efflux pump inhibitor reserpine was included in the study as a positive control. The results showed significant ($p<0.0001$) increase in the EtBr accumulation in the presence of *N. sativa* essential oil, p-cymene, carvacrol, and thymoquinone, respectively, compared to the untreated culture of *L. monocytogenes* (Fig. 14). *N. sativa* essential oil increased the EtBr accumulation significantly compared to reserpine. P-cymene and carvacrol activity were comparable to reserpine, while thymoquinone activity was lower than reserpine.

4.3.4. Membrane integrity assay

Again, 1/2 MIC of *N. sativa* EO, Thq, Car, and p-cymene were tested for their influence on membrane integrity in *L. monocytogenes* L14, to determine whether membrane permeability is the main mechanism of its modulation of antimicrobial resistance (Fig. 15).

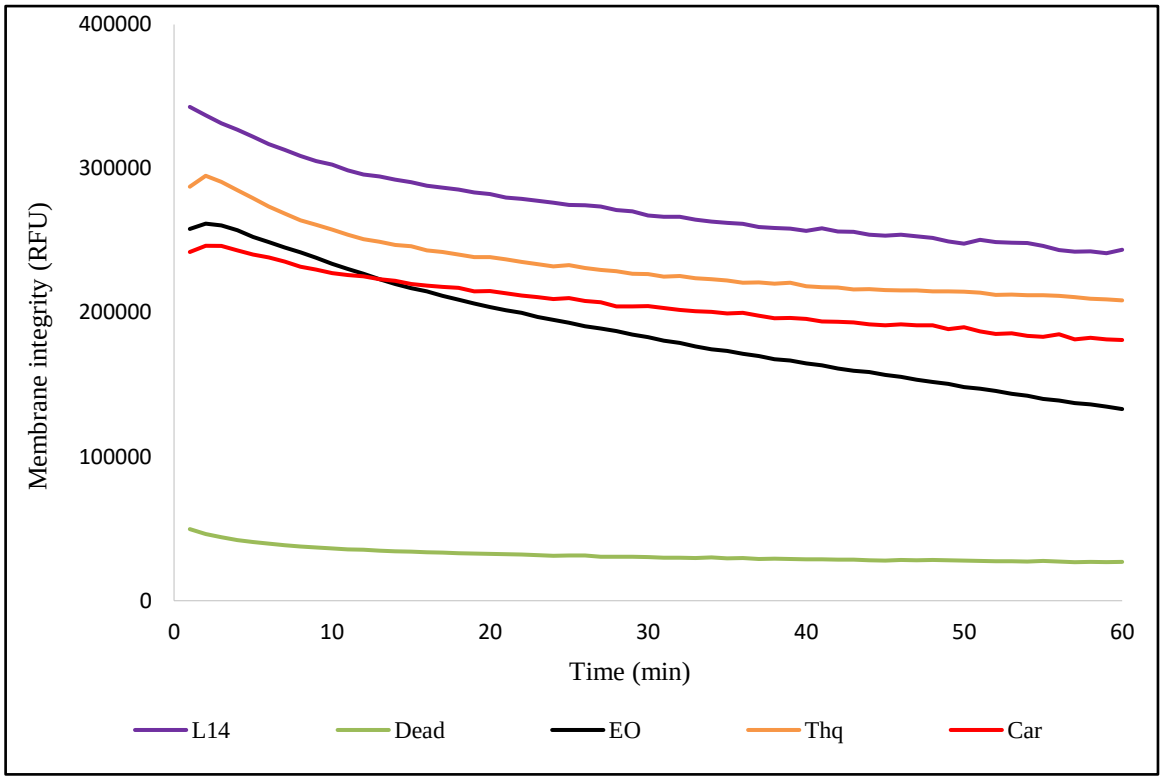


Figure 15: Influence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol and p-cymene on membrane integrity of *L. monocytogenes* L14. RFU= relative fluorescent unit; L14= *L. monocytogenes* L14; EO=essential oil; Thq=thymoquinone; Car=carvacrol and p-cy=p-cymene.

The membrane integrity of cultures treated with 1/2 MIC of *N. sativa* essential oil, thymoquinone or carvacrol decreased by 43%, 14% and 25% respectively. Hence, at this half MIC concentration of the essential oil and its active compounds, the disruption of the membranes is likely to have contributed to their antimicrobial resistance modifying effect. Cultures incubated at 80°C for 15 min were used as positive controls for disrupted membranes, and these showed 89% decreased membrane integrity, compared to the untreated control cultures. These differences were calculated based on the kinetics measurements over the last 10 min of the 1h assay, and they were statistically significant ($p<0.00001$). The preliminary results showed that the membrane integrity assay is not suitable to examine the activity of p-cymene on the membrane permeability, due to the

change in fluorescence when p-cymene was added to the wells without bacteria as negative control. One could explain this results by the high concentration used of p-cymene (1072 μg/mL).

In general, antimicrobial resistance alters the activity of antibiotics through one of the following mechanisms: antimicrobial target modification (decreasing drug affinity), a decrease in drug absorption, activation of efflux mechanisms to expel the toxic molecules (overexpression of efflux pumps) or global changes in important metabolic pathways through the modulation of regulation networks (Munita and Arias, 2016). The search of new efflux pump inhibitors is necessary to combat the emergence of antibacterial resistant strains (Stavri et al., 2007).

Data presented in Table 13 showed that all tested strains were sensitive to *N. sativa* EO, Thq and Car, while p-cy did not show any activity against the tested *L. monocytogenes* strains. These results support previous studies which reported an effective antibacterial activity of *N. sativa* EO (Piras et al., 2013), thymoquinone (Kouidhi et al., 2011) and carvacrol (Magi et al., 2015) against Gram-positive bacteria. 1/2 MIC of *N. sativa* EO, Thq, Car or p-cy as positive control did not show any inhibitory effect in the performed test against any of *L. monocytogenes* strains. DMSO and EtOH as diluents did not show any inhibitory effect on the growth of the strains.

Efflux is an important mechanism of resistance in *L. monocytogenes* (Mata et al., 2000). The efflux pumps are proteins of bacterial membranes which extrude and limit the intracellular accumulation of antibiotics and other antimicrobial agents (Piddock, 2006). It was identified previously that the increased resistance to ciprofloxacin (a fluoroquinolone) confers also increased resistance to EtBr, also increased expression of the Lde transporter (Godreuil et al., 2003). Morvan and co-workers (2010) on the basis of three-fold or greater decrease of ciprofloxacin MIC in the presence of reserpine, found twenty *L. monocytogenes* isolates to be resistant to fluoroquinolones. Based on this, our data shows that five *L. monocytogenes* are resistant to ciprofloxacin and all the nine strains are resistant to EtBr. Our data showed the potential of *N. sativa* EO, Thq, Car and p-cy to decrease the MIC of ciprofloxacin at least two-fold and up to eight-fold.

Additionally, in a previous study, increased susceptibility to macrolides resulted in the inactivation of the MdrL in *L. monocytogenes* (Mata et al., 2000). The MIC of

erythromycin (a macrolide) decreased in the presence of *N. sativa* EO, Thq, Car and p-cy at least two-fold up to four-fold, while reserpine had no activity with erythromycin. Determination of the erythromycin MIC against these strains in the absence and presence of reserpine did not lead to a decrease in the MIC, ruling out an efflux mechanism. Resistance in this case could be due to a chromosomal mutation.

EtBr is a substrate of many MDR pumps and causes fluorescence when bounded to the DNA, multidrug efflux pumps are known to cause resistance to this agent, thus the effects of inhibition of efflux can be assessed fluorometrically (Kouidhi et al., 2011; Magi et al., 2015). As found previously, the MICs of EtBr were also lower in combination with *N. sativa* EO, Thq, Car and p-cy (Table 14).

Nigella sativa EO, Thq, Car and p-cy induced the increase of EtBr accumulation in the treated strain and was comparable to the chemical EPI, reserpine. The inhibition of EtBr *via* a number of efflux pumps has been already reported for *L. monocytogenes* (Romanova et al., 2006; Mata et al., 2000).

Since plant essential oils and their components are hydrophobic in nature, their primarily target is the bacterial membrane to make it more permeable, which could have permitted an increase uptake of the antimicrobial agent by the bacterial cell (Carson et al., 2002).

These observations presume the modulating activity of *N. sativa* EO and Thq through efflux pumps inhibition leading to increased accumulation of antibiotics in the cells enhancing their effects at lower doses. So, the inhibition of EtBr efflux supports the hypothesis of antibacterial activity of *N. sativa* EO and its active components thymoquinone, carvacrol, and p-cymene through pump efflux inhibition.

Therefore, the results presented in this study suggest that the essential oil of *N. sativa* and its major components thymoquinone and carvacrol act as putative efflux pump inhibitors in *Listeria*, modulating the bacterial resistance to antibiotics. To the best of our knowledge, this is the first report on resistance modifying activity of *N. sativa* EO against *L. monocytogenes*.

4.4. Resistance modifying activity of *N. sativa* essential oil and its active compounds against *S. aureus*

4.4.1. Determination of minimum inhibitory concentrations

As presented in Table 15, the antimicrobial activity of *N. sativa* EO, Thq, Car and p-cy against the tested strains were as follows: 30 μg/mL for *N. sativa* EO, 10 μg/mL for Thq and 75 to 150 μg/mL for Car, while p-cy showed no inhibitory activity at the maximum tested concentration (2144 μg/mL) against both strains.

Table 15: Minimal inhibitory concentration in μg/mL of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene on *S. aureus* (ATCC 25923 and MRSA 272123)

Bioactive compounds	***S. aureus* ATCC**	***S. aureus* MRSA**
N. sativa essential oil	30	30
Thymoquinone	10	10
Carvacrol	150	75
p-cymene	>2144	>2144

4.4.2. Resistance-modulation assay

Data presented in Table 16 showed that the presence of Thq at half MIC, in combination with Tet resulted in decrease of MIC up to 16-fold in the MRSA strain. We observed also an 8-fold reduction in combination with Cip in the MRSA strain (Table 16). Whereas, the supplementation of *N. sativa* EO at half MIC induced a 2-fold decrease in the MIC of EtBr in both strains. A modulation factor >2 was set as the cut-off for biologically significant resistance modulation (Kovač et al., 2015).

Table 16: Modulation of antimicrobial activity of antibiotics and EtBr in the presence of half MIC of *N. sativa* essential oil and its bioactive compounds of *S. aureus* (ATCC 25923 and MRSA 272123)

Antimicrobial	**MIC (**μg/mL**)**	
	***S. aureus* ATCC**	***S. aureus* MRSA**
Tet[a]	0.78	25
	Fold-decrease in MIC of Tet	
+1/2 MIC EO	1	1
+1/2 MIC Thq	2	16
+1/2 MIC Car	1	1
+1/2 MIC p-Cy	1	1
Cip[a]	0.625	12.5
	Fold-decrease in MIC of Cip	
+1/2 MIC EO	1	2
+1/2 MIC Thq	4	8
+1/2 MIC Car	2	4
+1/2 MIC p-cy	1	2
EtBr[a]	7.81	7.81
	Fold-decrease in MIC of EtBr	
+1/2 MIC EO	2	2
+1/2 MIC Thq	2	2
+1/2 MIC Car	4	2
+1/2 MIC p-cy	1	4

[a]: Tet: tetracycline; Cip: ciprofloxacin; EtBr: ethidium bromide; EO = *N. sativa* essential oil; Thq = thymoquinone; Car = carvacrol, p-cy = p-cymene and MIC = minimal inhibitory concentration.

4.4.3. EtBr accumulation assay

In order to elucidate the mechanism of modulatory activity of *N. sativa* EO and its active compounds, Thq, Car, and p-cy on *S. aureus (*ATCC 25923 *and* MRSA 272123), the

potency of the compounds was evaluated regarding the increased accumulation of the common efflux pump substrate EtBr indicating efflux pump inhibition (Fig. 16 and 17).

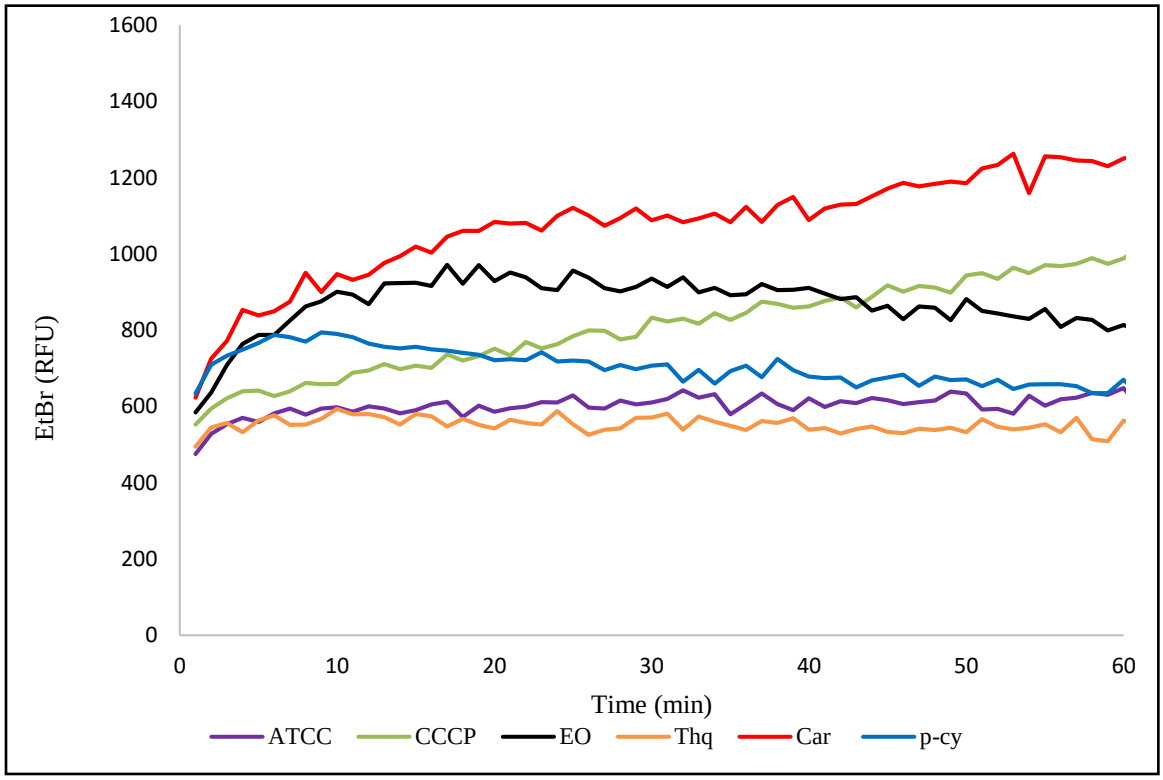


Figure 16: Ethidium bromide accumulation by *S. aureus* ATCC 25923 in the presence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene, and carbonyl cyanide m-chlorophenylhydrazone as positive control. RFU = relative fluorescence units; EtBr = ethidium bromide; ATCC= untreated control; EO = *N. sativa* essential oil; Thq = thymoquinone; Car = carvacrol; p-cy = p-cymene and CCCP=carbonyl cyanide m-chlorophenylhydrazone.

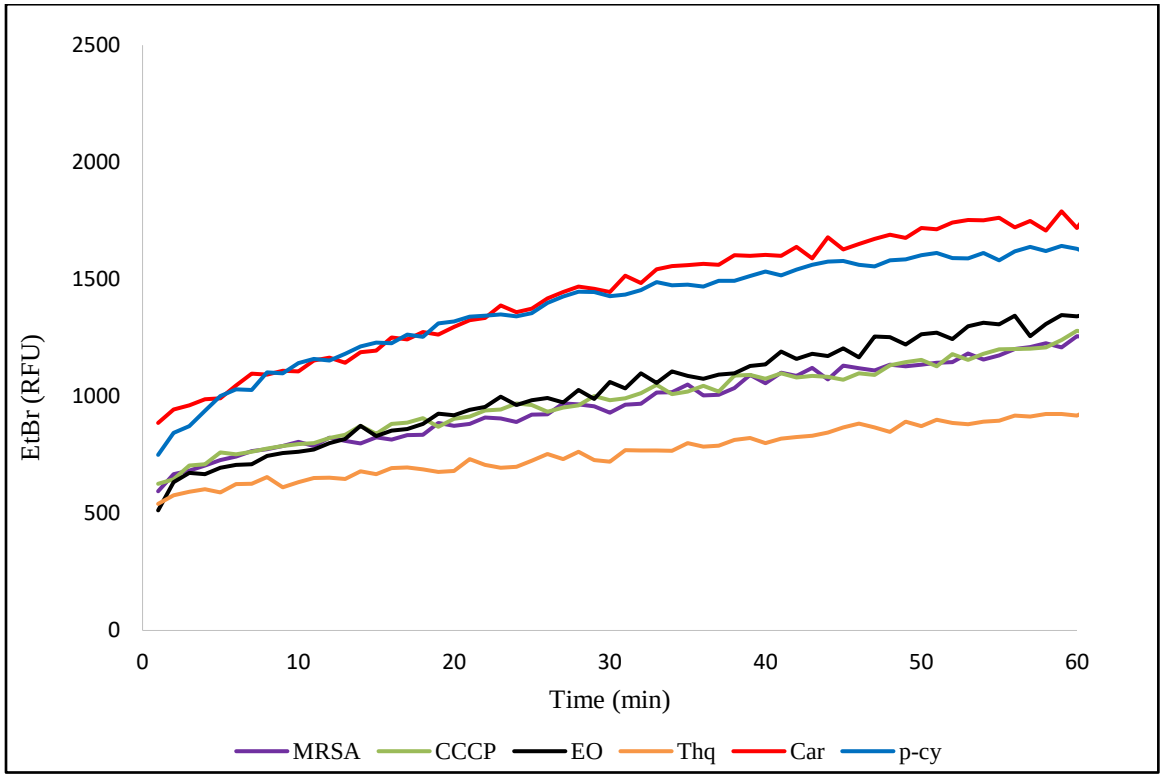


Figure 17: Ethidium bromide accumulation by *S. aureus* MRSA 272123 in the presence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene, and carbonyl cyanide m-chlorophenylhydrazone (10 µL) as positive control. RFU = relative fluorescence units; EtBr = ethidium bromide; MRSA= untreated control; EO = *N. sativa* essential oil; Thq = thymoquinone; Car = carvacrol; p-cy = p-cymene and CCCP = carbonyl cyanide m-chlorophenylhydrazone.

The level of EtBr accumulation in cultures treated with half of the MIC of *N. sativa* EO, Thq, Car, and p-cy (at 1072 μg/mL) was compared, relatively to the untreated culture, in order to evaluate whether they can potentiate the intracellular accumulation of EtBr. The known efflux pump inhibitor CCCP (10 μg/mL) was included in the study as a positive control. The results showed significant ($p<0.0001$) increase in the EtBr accumulation in the presence of *N. sativa* EO and Car in the ATCC strain and elevated intracellular EtBr concentration was observed in the presence of *N. sativa* EO in the MRSA strain (Fig. 16 and 17). Compound p-cy had no effect on the EtBr accumulation in the ATCC strain, while the activity of Thq was lower than the EtBr accumulated by the untreated ATCC and MRSA strains. CCCP showed activity on the ATCC strain while marginal effect was detected in case of the MRSA strain. Car was the most active compound on both strains.

4.4.4. Membrane integrity assay

With the aim of detecting the influence of these bioactive compounds on membrane integrity, the membrane damaging effect was evaluated in the presence of half of the MIC of *N. sativa* EO, Thq, and Car in *S. aureus* (ATCC 25923 and MRSA 272123) (Fig. 18 and 19) to determine whether the compounds affect the membrane permeability, thus contributing to the modulation of antimicrobial resistance.

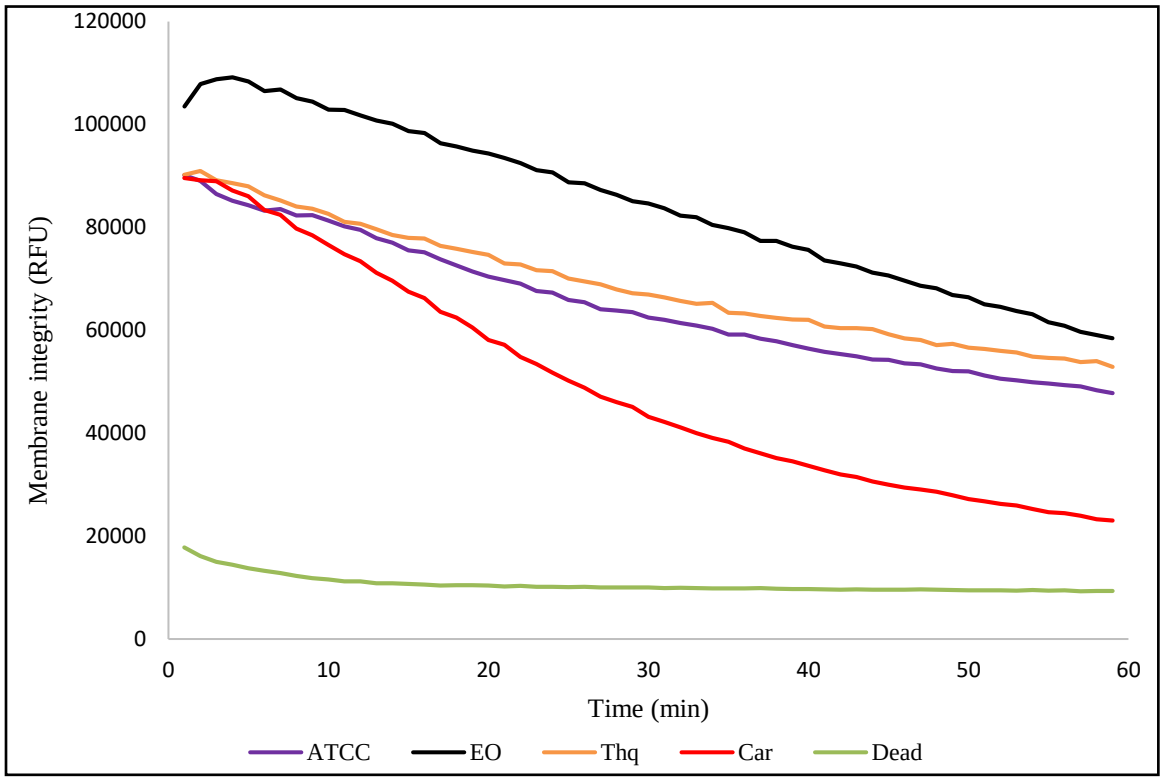


Figure 18: Influence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol and p-cymene on membrane integrity of *S. aureus* ATCC 25923. RFU = relative fluorescent unit; ATCC= untreated control; EO = *N. sativa* essential oil; Thq = thymoquinone; and Car = carvacrol.

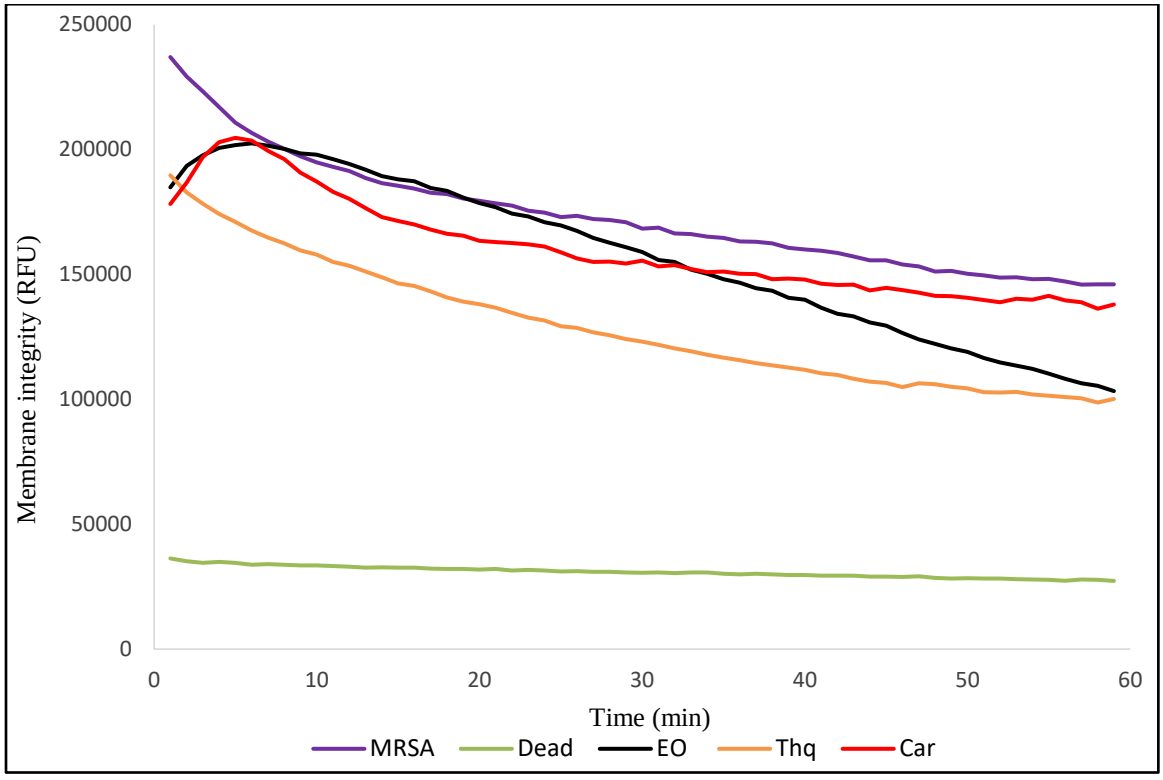


Figure 19: Influence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol and p-cymene on membrane integrity of *S. aureus* MRSA 272123. RFU = relative fluorescent unit; MRSA= untreated control; EO = *N. sativa* essential oil; Thq = thymoquinone; and Car = carvacrol.

Cultures incubated at 80°C for 15 min were used as positive controls for disrupted membranes, and these controls showed decreased membrane integrity by 81% in both strains, compared to the untreated control cultures. These differences were calculated based on the kinetics measurements over the last 10 min of the 1h assay, and they were statistically significant ($p<0.00001$).

The membrane integrity of *S. aureus* ATCC 25923 treated with Car decreased by 50%, while *N. sativa* EO and Thq increased the membrane integrity by 24% and 11%, respectively.

The membrane integrity of *S. aureus* MRSA 272123 treated with 1/2 MIC values of *N. sativa* EO, Thq or Car decreased by 25%, 31% and 6%, respectively for *S. aureus* MRSA 272123. Hence, the disruption of the membranes at half of MIC could have contributed to their antimicrobial resistance modifying effect of the essential oil and its bioactive constituents.

The preliminary results showed that the membrane integrity assay is not suitable to examine the activity of p-cymene on the membrane permeability, due to the change in fluorescence when p-cymene was added to the wells without bacteria as negative control. One could explain this results by the high concentration used of p-cymene (1072 μg/mL).

4.4.5. Expression analysis of genes by real-time reverse transcriptase quantitative polymerase chain reaction

In real-time quantitative RT-PCR assay, the gene of MepA transporter was investigated to evaluate the effect of compounds on the relative expression of this efflux pump gene in both *S. aureus* strains (Fig. 20).

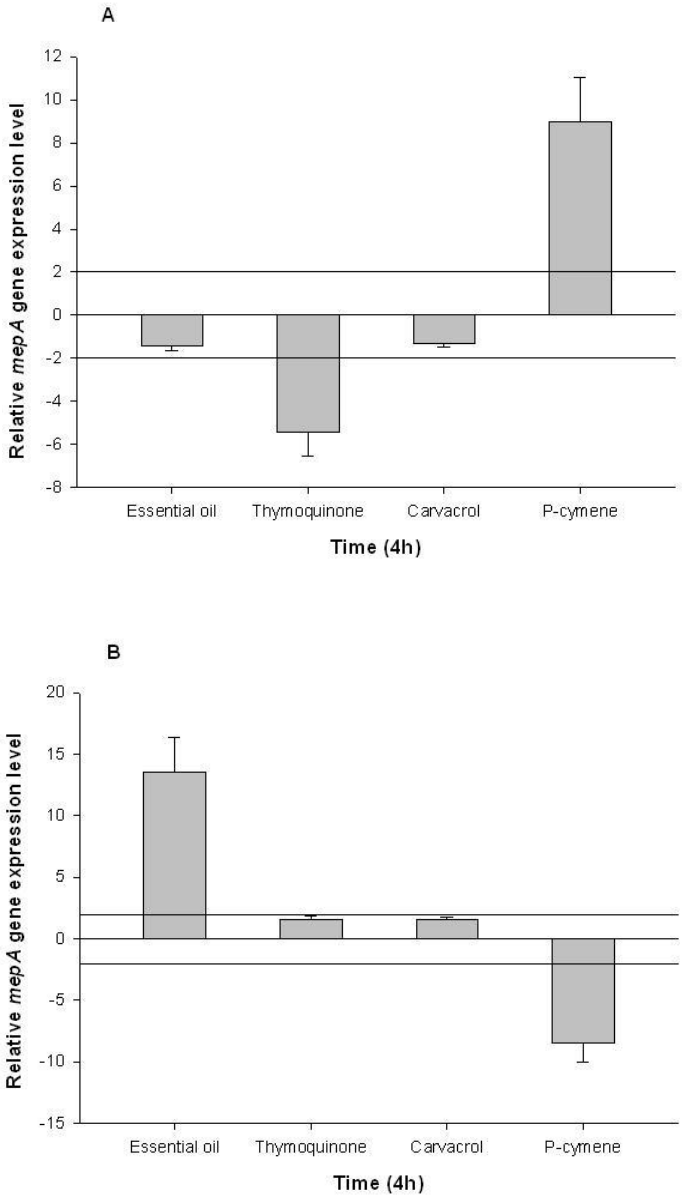


Figure 20**:** Relative gene expression level of the *mepA* efflux pump gene in the presence of *N. sativa* essential oil and its bioactive constituents on *Staphylococcus aureus* ATCC 25923 (A) and MRSA *Staphylococcus aureus* 272123 (B) strains after 4 h exposure (treatment: 5 µM of *N. sativa* EO or 0.5 µM of compound**s** Thq, Car, and p-cy). The line denotes the threshold value, which was set at a two-fold increase in transcripts.

As shown in Fig. 20A, Thq significantly down-regulated the expression of *mepA* after 4 hours of exposure, while p-cy up-regulated the expression of *mepA* in the ATCC strain. In the case of MRSA strain (Fig. 20B), *N. sativa* EO significantly up-regulated the expression of *mepA* after 4 hours of exposure, and p-cy down-regulated the expression of *mepA* after 4 hours of exposure.

4.4.6. Activity of *N. sativa* EO and its compounds against biofilm

In this study, *N. sativa* EO, Thq and Car could reduce effectively the development of bacterial performed biofilm of *S. aureus* (ATCC and MRSA) (Table 17). The reduction ranged from 30 to 40 % after EO treatment, furthermore 18 to 22 % and 11 to 35 % when the medium was supplemented with Thq and Car, respectively (Table 17).

Table 17: Anti-biofilm effect of *N. sativa* essential oil, thymoquinone and carvacrol expressed in the percentage (%) of decrease in biofilm formation of *S. aureus* (ATCC 25923 and MRSA 272123)

Bioactive compounds	**Concentration**	***S. aureus* ATCC**	***S. aureus* MRSA**
EO[a]	0.1%	35	28
	0.2%	36	30
	0.4%	40	33
Thq[a]	2.5 mM	20	18
	5 mM	21	18
	10 mM	22	23
Car[a]	2.5 mM	11	31
	5 mM	20	32
	10 mM	30	35

[a]: EO = *N. sativa* essential oil; Thq = thymoquinone and Car = carvacrol

Essential oils are mixtures of secondary metabolites produced by aromatic plants, which are responsible for the fragrance of the plants and numerous essential oils possess antimicrobial activity and they can enhance the effect of antibiotics *in vitro* (Bouhdid et al., 2010).

The activity of essential oils against staphylococci and their adjuvant role in the combination with antibiotics, namely the reduction in MICs of antibiotics have been investigated by several studies. *Cinnamomum verum* essential oil exhibited a strong

antibacterial activity against *S. aureus* and induced initially the leakage of intracellular K^+ from cells of *S. aureus* cells affecting the membrane permeability followed by reduction in metabolic activity and replication (Bouhdid et al., 2010).

Tea tree oil (TTO) extracted from *Melaleuca alternifolia* has been shown to have activity against *S. aureus* because TTO influenced the regulation of genes related to heat shock response and cell wall metabolism, furthermore TTO induced the expression of a gene encoding a putative resistance, nodulation and cell division efflux pump being involved in the bacterial stress response (Cuaron et al., 2013).

It has been demonstrated that essential oil extracted from *Origanum vulgare* L., as well as its individual constituents carvacrol and thymol have anti-staphylococcal effect and they could reduce the MIC of tetracycline in *S. aureus*. In addition, it has been confirmed that the essential oil and its bioactive ingredients carvacrol and thymol could be possible efflux pump inhibitors (Cirino et al., 2015).

Recently, five essential oils such as eugenol, carvacrol, thymol, p-cymene, and γ-terpinene were tested alone and in combination with tetracycline on bacteria present in the oral flora for example oral strains of *S. aureus*. All of the above mentioned essential oils could inhibit the efflux activity of *S. aureus* ATCC 25923, moreover they exhibited synergistic interaction with tetracycline on biofilm inhibition on polystyrene and tooth surfaces (Miladi et al., 2017).

In order to reverse resistance in *S. epidermidis*, essential oils from *Salvia fruticosa*, *Salvia officinalis* and *Salvia sclarea* reduced the minimal inhibition concentration of tetracycline and showed synergism, in addition they decreased the efflux of this antibiotic and reduced the expression of *tet(K)* gene in tetracycline resistant clinical isolates of *S. epidermidis* (Chovanová et al., 2015).

Based on a study published lately the essential oil of *Chenopodium ambrosioides* L. leaves alone did not present relevant antibacterial activity, but when combined in a sub-inhibitory concentration (¼ MIC) with tetracycline, the interaction resulted in synergism (Limaverde et al., 2017).

In our study we have evaluated *N. sativa* EO and its bioactive compounds, Thq, Car, and p-cy, regarding their antimicrobial, efflux pump inhibiting, anti-biofim and membrane

disrupting effects and their adjuvant role in combined antibiotic therapy against methicillin-susceptible and methicillin-resistant *S. aureus* strains.

Data presented in Table 15 show that both strains were sensitive to *N. sativa* EO, Thq and Car, while p-cy had no inhibitory activity against *S. aureus* strains. Compound p-cy lacks a hydroxyl group, which is thought to play an important role in antimicrobial activity (Chovanová et al., 2015). These results are in accordance with previous studies reporting the effective antibacterial activity of *N. sativa* EO (Forouzanfar et al., 2014), Thq (Limaverde et al., 2017) and Car (Ultee et al., 2002) against *S. aureus*. 1/2 MIC of *N. sativa* EO, Thq, Car or p-cy as positive control did not show any inhibitory effect in the performed test against any of *S. aureus* strains. The diluents EtOH and DMSO did not show any inhibitory effect on the growth of the strains.

Based on our results, Thq decreased the MIC of ciprofloxacin, tetracycline, and EtBr by at least two-fold up to 16-fold in both *S. aureus* strains. *N. sativa* EO and Car induced the increase of EtBr accumulated by both *S. aureus* strains.

Since plant essential oils and their components are hydrophobic in nature, their primary target is the bacterial membrane to make it more permeable, which could permit an increased uptake of the antimicrobial agent by the bacterial cell (Chaieb et al., 2011). P-cy has a high affinity for membranes and causes membrane expansions and affects the membrane potential of intact cells (Chovanová et al., 2015).

Regarding the relative gene expression studies *mepA* is overexpressed in the MRSA strain and it has low expression level in the ATCC strain. For this reason, *N. sativa* EO had more potent efflux pump inhibitory activity causing higher intracellular EtBr accumulation by the MRSA strain compared to the lower EtBr accumulation in the ATCC strain suggesting that EO has an influence on overexpressed efflux pump systems. This fact can be supported by the MIC reduction assay as well, since EO was more potent on the MRSA strain applied in combination with Cip.

It can be concluded that there is an elevated *mepA* expression in the MRSA strain due to the stress response against EO. P-cy downregulated the expression level of *mepA* in the MRSA strain that is why it could be applied as an EPI in the MRSA strain. Compound p-cy probably influences the transporter protein MepA and downregulates the expression of the *mepA* gene.

In the ATCC strain *mepA* was upregulated after treatment with p-cy showing that the sensitive ATCC strain is under stress and a stress response was induced against p-cy in order to survive the effect of this noxious agent because normally MepA is not expressed in sensitive isolates.

5. CONCLUSIONS AND SUGGESTIONS

Multidrug resistant bacteria are a growing threat to human health and welfare. The aim of this study was to investigate antimicrobial properties and resistance modifying activities of *N. sativa* oils. In particular their antibacterial effect against food spoilage organisms, foodborne and non-foodborne pathogens, their anti-spore and anti-biofilm activity.

In our study the major fatty acids of *N. sativa* crude oil were linoleic (18:2n-6), oleic (18:1n-9) and palmitic (16:0) acids. While, the major compounds of its essential oil were p-cymene and thymoquinone. The essential oil of *N. sativa* showed 10 times higher antimicrobial activity than the crude oil, however, the crude oil still showed good inhibitory effect against Gram-positive bacteria.

The present study showed that *N. sativa* essential oil was a source of biological active compounds, which contributed to its antimicrobial properties. *N. sativa* and its examined active compouds were highly effective killers of *C. trachomatis*. The crude oil was also effective against endspores of *Bacillus* strains and showed good activity against *Listeria monocytogenes* not only in laboratory media but in food matrix, as well. Our findings enhance further study to use this oil as a potential food preservative and as a medical commodity.

Nigella sativa essential oil and its active compounds, thymoquinone and carvacrol are confirmed as efficient modulators of antimicrobial resistance in *L. monocytogenes*, with at least two different mechanisms that contribute synergistically to their activity. Half MIC of *N. sativa* essential oil, thymoquinone, and carvacrol modulates antibiotic resistance in *L. monocytogenes* against various antimicrobial, showing increased EtBr accumulation. Additionally, targeting the membrane, they caused increased permeability, thereby promoting the influx of antimicrobials. P-cymene had no antimicrobial activity; however, it increased the membrane permeability. Due to the modulation of the antimicrobial resistance in *L. monocytogenes*, *N. sativa* essential oil, thymoquinone, and carvacrol has the potential to be promising modifiers of antimicrobial resistance in *L. monocytogenes*.

According to the results obtained in our study, *N. sativa* essential oil and its bioactive compounds such as carvacrol and p-cymene could be applied as potent efflux pump inhibitors in MRSA strains, furthermore p-cymene downregulates the expression level of

efflux pump gene *mepA* in MRSA. Influencing efflux pumps and their genes, the resistant isolates can lose their virulence and we can provide better perspectives to treat MRSA related infections. In addition, essential oil, thymoquinone, and carvacrol might be used as adjuvants in combination with antibiotics enabling a better therapeutic efficacy in *S. aureus* related infections.

These findings prove that *Nigella sativa* oils represent a potential source of bioactive compounds to use in food preservation and different fields of medicine.

Further studies are needed to apply the oil as food preservative, and to check the mechanism of action between the crude oil and nisin whether it's synergistic or additive effect.

Additional research is required to determine precisely which efflux pump in *L. monocytogenes* is inhibited by *N. sativa* essential oil

Due to the promising effect of p-cymene on *mepA* gene expression, more genes included in the resistance to antibiotics should be tested.

Further examination are needed to assess the effect of *N. sativa* essential oil and its bioactive compounds against *S. aureus* quorum sensing in biofilm.

6. NEW SCIENTIFIC RESULTS

1- The MIC of *N. sativa* crude oil (0.125 % for *B. cereus* spores and 0.25 % for *B. subtilis*) and MIC of nisin (0.3 mg/ml) were determined against *B. cereus* and *B. subtilis* spores. I proved that the combination of crude oil with nisin enhanced the activity of nisin against *Bacillus* spores.

2- I demonstrated that *N. sativa* essential oil and its active compounds carvacrol and p-cymene possess anti-chlamydial activity against *Chlamydia trachomatis.*

3- I proved that *N. sativa* essential oil modulate antimicrobial resistance of *L. monocytogenes* and methicillin resistant *S. aureus* by targeting the efflux pump and the membrane integrity. Furthermore, I confirmed p-cymene downregulates the expression level of efflux pump gene *mepA* in MRSA.

4- The formation of *S. aureus* biofilm was proved to be partially inhibited by *N. sativa* essential oil, thymoquinone and carvacrol.

7. SUMMARY

The search for alternatives to available antibiotics is becoming more and more needed in the developed countries due to the misuse of antibiotics. Alexander Fleming, in his 1945 Nobel lecture speech, warned against the resistance that develops in bacteria when under-exposed to antibiotics. The deeper knowledge of essential oils and their bioactive compounds would help to overcome an increasingly upsetting crisis of human illness from antibiotic resistance that is an international one, because the antimicrobial mechanisms of these bioactive compounds often differ from the current classes of conventional antibiotics, greater knowledge about them could help guide development of new classes of antibiotics.

Nigella sativa L. (Black cumin) is well known for its benefits in the field of traditional medicine. The aim of this study was to determine the chemical composition and investigate the antimicrobial activity of crude oil and essential oil of *Nigella sativa* L. on spoilage bacteria, foodborne and non-foodborne pathogenic bacteria, and the resistance modifying activity of *N. sativa* EO, thymoquinone, carvacrol, and p-cymene against methicillin susceptible and methicillin resistant *S. aureus* and *L. monocytogenes* strains.

N. sativa essential oil, thymoquinone, carvacrol, and p-cymene was assessed for antimicrobial activity, modulation of antimicrobial resistance, inhibition of antimicrobial efflux, membrane disrupting effect and anti-biofilm activity by broth microdilution, ethidium bromide accumulation, LIVE/DEAD BacLightTM assays and real-time reverse transcriptase quantitative polymerase chain reaction.

N. sativa essential oil was a source of biological active compounds, which contributed to its antimicrobial properties. Our findings enhance further study to use this oil as a potential food preservative and as a medical commodity. *N. sativa* EO and its bioactive compounds such as carvacrol and p-cymene could be applied as potent efflux pump inhibitors in MRSA strains. *N. sativa* EO might have a potential for controlling the antibiotic resistance in *Listeria*.

APPENDIX

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A2 Diameters of inhibition zones (mm) of *N. sativa* crude oil (%)

Strains	**crude oil (w/w %)**						
	20%	10%	5%	2.50%	1.25%	0.63%	0.33%
	Zone of inhibition (mm) mean ± SD						
Listeria monocytogenes E12/10.12.1	36±1.7	23±1.1	X**	X	X	X	X
Listeria monocytogenes 11/4.12 t03	36±1.1	16±1.5	X	X	X	X	X
Listeria monocytogenes E ST/10.12.1	36±4.1	16±1.5	X	X	X	X	X
Listeria monocytogenes E ST/10.12.2	39±1.1	27±2	X	X	X	X	X
Listeria monocytogenes E ST/10.12.3	39±7.2	24±2.5	X	X	X	X	X
Listeria monocytogenes 8/4.12. t0	34±1.1	22±1	X	X	X	X	X
Listeria grayi CCM 5990	15±0.5	12±1	X	X	X	X	X
Listeria innocua CCM 4030	26±0.5	21±0.5	X	X	X	X	X
Listeria grayi CCM 4029	19±3.4	15±2	X	X	X	X	X
Listeria monocytogenes B01966	26±2.8	25±4.3	18±2	X	X	X	X
Listeria monocytogenes CMM 4699	23±1	18±0.5	14±4.5	X	X	X	X
Listeria monocytogenes NCAIM 1454	44±5.3	36±2.6	23±1.5	15±4.2	X	X	X
Listeria monocytogenes 4b	total*	24±1	X	X	X	X	X
Listeria monocytogenes 1/2 c	total	26±1	X	X	X	X	X
Listeria welshimeri CCM 3971	total	22±0.5	14±1	X	X	X	X
Listeria monocytogenes CCM 7202	total	19±3.2	X	X	X	X	X
Listeria monocytogenes 3b	total	25±0.5	X	X	X	X	X
Listeria monocytogenes #46	total	37±0.7	24±1.4	20±1.4	17±2.1	X	X
Listeria ivanovii CCM 5884 T	total	23±1.7	X	X	X	X	X
Micrococcus luteus	total	total	total	31±1.5	19±1	14±2.8	15±0
Staphylococcus aureus	total	total	23±5.5	14±0.5	13±1	12±0	X
Enterococcus faecalis	21±2.6	X	X	X	X	X	X
Bacillus subtilis	total	total	32±2.8	29±5	X	X	X
Bacillus megaterium	total	total	23±3.5	12±1	X	X	X
Bacillus licheniformis	total	37±3.5	30±1.4	28±2.1	X	X	X
Bacillus cereus	total	total	33±2.1	X	X	X	X
Rhodococcus eqvii	total	total	total	36±4	19±0.5	X	X
E. coli	X	X	X	X	X	X	X
Proteus sp.	X	X	X	X	X	X	X

*total = full inhibition

**X = no inhibition

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