

Nanodispersion Enhances Antimicrobial Efficacy of Lipophilic Essential Oil Compounds in
Laboratory Medium and Food Models

Tarik Bor

North Carolina A&T State University

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Major Professor: Dr. Salam A. Ibrahim

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The Graduate School
North Carolina Agricultural and Technical State University
This is to certify that the Doctoral Dissertation of

Tarik Bor

has met the dissertation requirements of
North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2015

Approved by:

Dr. Salam A. Ibrahim
Major Professor

Dr. Jane Walker
Committee Member

Dr. Mulumebet Worku
Committee Member

Dr. Abolghasem Shahbazi
Committee Member

Dr. Keith Schimmel
Department Chair

Dr. Godfrey Ejimakor
Committee Member

Dr. Sanjiv Sarin
Dean, The Graduate School



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Biographical Sketch

Tarik Bor was born in 1977 in Heilbronn, Germany. He received a BS degree in Biology Department, Basic and Industrial Microbiology section in Ege University, Izmir, Turkey. He also received a Master of Science Degrees in Biology from Ege University, Izmir, Turkey in 2001 and in Food Science and Nutrition from North Carolina Agricultural and Technical State University at Greensboro, NC in 2009. Tarik is a candidate for the Doctor of Philosophy degree in Energy and Environmental Systems.



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List of Symbols and Abbreviations

ABTS	: 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid
A/C assay	: Aldehyde/Carboxylic Assay
A/E	: Attaching-And-Effacing
ANOVA	: Analysis of Variance
ATP	: Adenosine Tri Phosphate
BHI	: Brain Heart Infusion
BHT	: Butylated Hydroxytoluene
CD essay	: Conjugate Diene Essay
CDC	: Centers for Disease Control and Prevention
CFU	: Colony Forming Unit
CFU/g	: Colony Forming Unit / Gram
CFU/ml	: Colony Forming Unit / Milliliter
Cu	: Copper
CVD	: Cardiovascular Disease
DAEC	: Diffusely adherent <i>E. coli</i>
DMF	: Dimethylformamide
DNA	: Deoxyribonucleic Acid
DPPH	: 2,2'-diphenyl-1-picrylhydrazyl
<i>E. coli</i>	: <i>Escherichia coli</i>
EAEC	: Enteroaggregative <i>E. coli</i>
EDTA	: Ethylene Diamine Tetraacetic Acid
EHEC	: <i>Escherichia coli</i> O157:H7, Enterohemorrhagic <i>E. coli</i>
EIEC	: Enteroinvasive <i>E. coli</i>

EO	: Essential Oil
EOC	: Essential Oil Constituents
EOs	: Essential Oils
EPEC	: Enteropathogenic <i>E. coli</i>
ETEC	: Enterotoxigenic <i>E. coli</i>
FDA	: Food and Drug Administration
FE	: Free Eugenol
Fig(s)	: Figure(s)
FRAP	: Ferric Reducing Antioxidant Power
g	: Gram
GC	: Gas Chromatography
GC/FID	: Gas Chromatography/Flame Ionization Detector
GC/MS	: Gas Chromatography/Mass Spectrometer
GRAS	: Generally Recognized as Safe
h	: Hour(s)
HC	: Hemorrhagic Colitis
HIPEF	: High-Intensity Pulsed Electric Fields
HIV	: Human Immunodeficiency Virus
HSP	: Heat Shock Protein
HSV	: Herpes Simplex Virus
HUS	: Hemolytic Uremic Syndrome
L	: Liter
LA	: Lactic Acid

log	: Logarithm (10)
(n) log	: 10 ⁿ times
LP	: Lipid Peroxidation
LT	: Heat-Labile Toxin
MA/GC	: Malonaldehyde/Gas Chromatography
ml	: Milliliter
mm	: Millimeter
mM	: Millimolar
μL	: Microliter
MAP	: Modified Atmosphere Packaging, Passive MAP
MBC	: Minimum Bactericidal Concentration
MD	: Maltodextrin
MIC	: Minimum Inhibitory Concentration
MID	: Minimum Inhibitory Dose
MLC	: Minimum Lethal Concentration
MPN	: Most Probable Number
ND	: Nanodispersed
NE	: Nanodispersed Eugenol
NT	: Nanodispersed Thymol
PEF	: Pulsed Electric Fields
PGSS	: Particles from Gas Saturated Solutions
pI	: Isoelectric point
PLA	: Polyactide

Ppm	: Parts per million
RAPD	: Random Amplified Polymorphic DNA
ROS	: Reactive Oxygen Species
RPSO	: Red Pepper Seed Oil
RSC	: Radical Scavenging Activity
RSV	: Respiratory Syncytial Virus
RTE	: Ready to Eat
sp.	: Species
SDS	: Sodium Dodecil Sulfate
SE	: <i>Staphylococcus aureus</i> Enterotoxin
SMART	: Somatic Mutations and Recombination Test
SMP	: Skim Milk Powder
SPEF	: Soy Protein Edible Films
ST	: Heat-Stable Toxin
STEC	: Shiga Toxin Producing <i>E. coli</i>
stx	: Shiga Toxin
TBARS	: Thiobarbituric Acid Reactive Species
TC	: <i>trans</i> -cinnamaldehyde
TSA	: Tryptic Soy Agar
TSB	: Tryptic Soy Broth
USDA	: United States Department of Agriculture
UV	: Ultra Violet
v/v	: Volume by Volume

WPI : Whey Protein Isolate

WPC : Whey Protein Concentrate



Abstract

Foodborne disease is an important environmental health issue that affects tens of millions of people and kills thousands. To control foodborne pathogens, the food industry is interested in expanding the use of natural ingredients which do not have the side effects of currently used chemical preservatives.

The objectives of this study were: (a) to evaluate the effectiveness of certain essential oils (EOs) and the combination of low concentrations of copper (Cu) and lactic acid (LA) to control *E. coli* O157:H7 in brain heart infusion medium (BHI), (b) to investigate the antimicrobial efficacy of nanodispersed (ND) eugenol against *E. coli* O157:H7 in BHI, and (c) to investigate the antibacterial activity of nanodispersed (ND) eugenol against *E. coli* O157:H7 in ground beef and carrot juice during refrigerated storage.

Antibacterial activities of Cu (25 and 50ppm) - LA (0.1, 0.15, and 0.2%v/v), armoise, and clove EOs (0.006, 0.0125, 0.025, 0.05, 0.1, and 0.2%) eugenol in free, emulsion, and ND forms were tested against *E. coli* O157:H7 in BHI. Antibacterial efficacy of ND eugenol (0.1, 0.2, 0.4, and 0.5%v/w) against *E. coli* cocktail was tested in ground beef and carrot juice. When Cu (50ppm) and LA (0.2%) were added together into BHI, the bacterial growth was significantly inhibited ($p < 0.05$). Armoise (0.15% v/v) and clove bud (0.1% v/v) EOs caused ~6 log reduction on growth of *E. coli* O157:H7 in BHI. Eugenol addition into BHI at $\leq 0.05\%v/v$ concentration in emulsion or ND forms caused more reduction than free eugenol at the same concentration. ND eugenol significantly inhibited the growth of *E. coli* O157:H7 in carrot juice ($\geq 0.1\%v/v$) and ground beef (0.5%v/w) during refrigerated storage. Due to its strong antimicrobial activity, ND eugenol can cause at least 5-log reduction in carrot juice at low concentrations.

CHAPTER 1

Literature Review

1.1 Foodborne Illnesses and Major Foodborne Pathogens

1.1.1 *Escherichia coli* O157:H7. *Escherichia coli* (abbreviated as *E. coli*) was first isolated by Theodor Escherich in 1885 from newborn infant stools. It is a rod-shaped, gram (-) facultative anaerobic bacterium. First he called it *Bacterium coli commune* (Kaper, 2005). *E. coli* O157:H7 (somatic O, and flagellar H antigens) was first categorized as a human pathogen after two hemorrhagic colitis outbreaks in 1982 (Buchanan & Doyle, 1997).

1.1.1.1 Pathogenic *E. coli* types and related diseases. Pathogenic *E. coli* strains are categorized into pathotypes. Six pathotypes, as listed below, are associated with diarrhea and collectively referred to as diarrheagenic *E. coli*.

- **Shiga toxin-producing *E. coli* (STEC) or Enterohemorrhagic *E. coli* (EHEC).**
These bacteria attach and efface into intestinal epithelial cells and secrete shiga toxin (stx), and cause hemorrhagic colitis (HC) and bloody diarrhea.
- **Enterotoxigenic *E. coli* (ETEC).** These bacteria cause diarrhea by secreting at least one of two enterotoxin groups: heat-stable toxin (ST) and heat-labile toxin (LT).
- **Enteropathogenic *E. coli* (EPEC).** Their infections are characterized by attaching-and-effacing (A/E) histopathology. They recognize, attach and disrupt intestinal cell ultrastructure and intimately adhere to epithelial cells in the intestine with signal transduction. They cause infant diarrhea.
- **Enteraggregative *E. coli* (EAEC).** This group of bacteria form biofilm and aggregates and bind to epithelial cells. This aggregation increases toxicity and inflammation.

- **Enteroinvasive *E. coli* (EIEC).** These bacteria are usually non-motile. They bind to the intestinal cell surface by secreting their own antigens.
- **Diffusely adherent *E. coli* (DAEC).** These bacteria have surface fimbria and can be completely internalized by means of these fimbria and protect themselves from host attacks (Nataro & Kaper, 1998).

1.1.2 *Salmonella enterica*. *Salmonella* is the most frequently reported cause of foodborne diseases. These bacteria are gram (-) and rod shaped enterobacteria that cause diarrhea in humans. *Salmonella* species are commonly associated with food poisoning all over the world. There are two species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. The most commonly known one is the *Salmonella enterica*. *S. enterica* have over 2,300 serovars and the most common cause for food poisoning is related to Serovar Enteritidis (USDA, 2011). Salmonellosis caused by *Salmonella spp.* creates an estimated 1.4 million cases of foodborne illness and more than 400 deaths annually in the United States. The symptoms of salmonellosis are diarrhea, abdominal cramps, fever, chills, headache, nausea, and vomiting. Salmonellosis can be life-threatening, especially for people who have weakened immune system such as infants, elderly people, pregnant women, and their unborn babies (USDA, 2011).

1.1.3 *Listeria monocytogenes*. *Listeria monocytogenes*, named after Joseph Lister, is a gram (+), facultative anaerobic bacteria that cause listeriosis. These bacteria are motile with flagella at 30°C and below. An estimated 20-30% of listeriosis infections result in death. *Listeria* is found in soil and vegetation and therefore it can be easily transmitted by herd animals. The symptoms of invasive listeriosis are abortion, sepsis, febrile gastroenteritis syndrome, and meningoencephalitis (Ramaswamy et al., 2007).

The recent outbreaks associated with the foodborne pathogens *E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, and *Campylobacter* spp. are shown in Figure 1. The frequency of diseases is expressed as incidence per 100,000 populations. Table 1 shows the pathogenic bacteria causing outbreaks associated with fresh produce and the type of produce on which these foodborne pathogens grow.

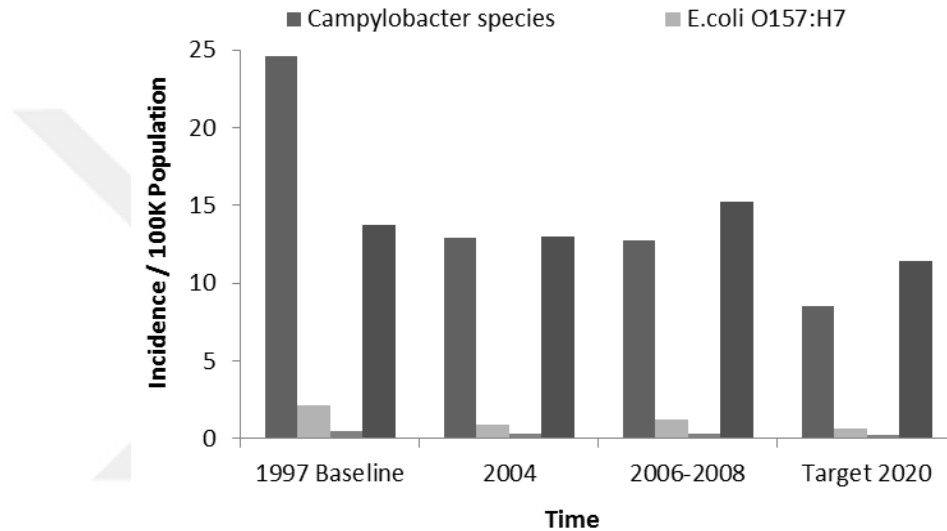


Figure 1. Foodborne outbreaks and association of these three foodborne pathogens. (CDC, 2005, 2009; “Healthy People 2020”).

Table 1

List of Shiga Toxin Producing *E. coli* Related Outbreaks and Their Sources of Infection

Date	STEC <i>E. coli</i> type	Sources of Infection	# of States	# of Hospitalizations	# of Deaths	Case Count
11/2013	O157:H7	Ready-to-Eat salads	3	6	0	26
05/2013	O121	Farm Rich Brand Frozen Food Products	19	9	0	35
12/2012	O157:H7	Organic Spinach and Spring Mix Blend	5	13	0	33
07/2012	O145	Not identified	9	4	1	18
04/2012	O26	Raw Clover Sprouts at Jimmy John’s Restaurants	11	7	0	29

Table 1

(Cont.)

Date	STEC <i>E. coli</i> type	Sources of Infection	# of States	# of Hospitalizations	# of Deaths	Case Count
03/2012	O157:H7	Romaine Lettuce	9	33	0	58
07/2011	O104:H4	Travel to Germany	5		1	6
04/2011	O157:H7	Infections associated with Lebanon Bologna	5	3	0	14
04/2011	O157:H7	In-Shell Hazelnuts	3	4	0	8
10/2011	O157:H7	Bravo Farms Cheese	5	15	0	38
05/2010	O145	Shredded Romaine Lettuce from a Single Processing Facility	5	12	0	30
01/2010	O157:H7	Beef from National Steak and Poultry	16	9	0	21
11/2009	O157:H7	Beef from Fairbank Farms	8	19	2	26
06/2009	O157:H7	Beef from JBS Swift Beef Company	9	12	0	23
06/2009	O157:H7	Eating Raw Refrigerated, Prepackaged Cookie Dough	30	34	0	72
07/2008	O157:H7	Beef from Kroger / Nebraska Ltd	7	27	0	49
11/2007	O157:H7	Totino's / Jeno's Pizza	10	8	0	21
10/2007	O157:H7	Topp's Ground Beef Patties	8	21	0	40
10/2006	O157:H7	Fresh Spinach	26	102	3	199

1.2 Antimicrobials from Plants

Antimicrobials extracted from different plants have a strong capacity to be used as alternative preservatives (Wallace, 2004). There are different groups of antimicrobial compounds in plants such as phenolic compounds and phenolic acids (simple phenols, flavones, quinones, flavonoids, flavonols, tannins, coumarins), terpenoids, EOs, alkaloids, lectins and polypeptides, saponin, and sesquiterpenes (Cowan, 1999; Tajkarimi et al., 2010). A number of research studies have been published about antimicrobial activities of these plant extracts and their effects against

foodborne pathogens (Bajpai, Rahman, & Kang, 2008; Burt, 2004; Ceylan & Fung, 2004; Tajkarimi et al., 2010; Tiwari et al., 2009).

1.3 Herbs, Spices and Plant Extracts

Herbs and spices have been used as food additives all over the world. Not only do they improve organoleptic properties of food, they can also increase their shelf life by reducing the number or removing foodborne pathogens (Lai & Roy, 2004). The majority of herbs and spices exert antimicrobial activity against different bacteria, yeasts, and molds (Friedman, Henika, Levin, & Mandrell, 2004; Friedman, Henika, & Mandrell, 2002; Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2008; Tajkarimi et al., 2010). Phenolic compounds obtained from herbs and spices show biological activities and can be potentially used as food preservatives (Lai & Roy, 2004).

The extract of *Capsicum annuum* bell pepper prevented the growth of *Salmonella typhimurium* at 1.5 ml/100g concentration in minced beef. For *Pseudomonas aeruginosa*, bell pepper extract showed a bacteriostatic effect at 0.3 ml/100g and bactericidal effect at 3 ml/100g concentrations (Careaga et al., 2003). Tornuk et al. (2011) showed the inhibitory effects of the hydrosols of thyme, black cumin, sage, rosemary, and bay leaf against *Salmonella typhimurium* and *Escherichia coli* O157:H7 in carrots and apples, and thyme hydrosol had the highest antibacterial effect on both *S. typhimurium* and *E. coli* O157:H7 counts.

Different type of spices were grouped based on their antimicrobial activities as strong (cinnamon, clove, mustard), medium (allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage, thyme), and weak (black pepper, red pepper, ginger; Zaika, 1988). Ethanol and hexane extracts of oregano, clove, sage, rosemary, and celery exerted relatively strong antimicrobial activities against *Escherichia coli*, *Listeria innocua*, *Staphylococcus aureus* and

Pseudomonas fluorescens, but water extracts showed weak or no antimicrobial activity (Witkowska, Hickey, Alonso-Gomez, & Wilkinson, 2013). Aqueous extracts of *Mentha spp* have antimicrobial activities against *Bacillus fastidiosus*, *Proteus mirabilis*, *Proteus vulgaris*, *Salmonella choleraesuis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Serratia odorifera* at different concentrations (1:1, 1:5, 1:10, and 1:20, respectively; Al-Sum & Al-Arfaj, 2013).

Green tea was particularly effective against *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes*, and rosemary had a strong inhibitory effect against *B. cereus* and *S. aureus* in tryptic soy broth. When green tea or rosemary was added to rice cakes at 1-3% concentration, the growth of *B. cereus* and *S. aureus* were significantly reduced in rice cakes after 3 days of storage at 22°C (Lee, Gwon, Kim, & Moon, 2009). The mixed extracts of *Scutellaria*, *Forsythia*, honeysuckle, cinnamon, and rosemary and clove oil showed strong antimicrobial effects in vacuum-packaged fresh pork from 1.81 to 2.32 log reductions in *Escherichia coli*, *Pseudomonas fluorescens*, and *Lactobacillus plantarum* counts during 28 days of storage when compared with the control (Kong, Wang, & Xiong, 2007).

Rosemary extracts and dry powders of orange and lemon were effective in controlling bacterial spoilage during 12 days of storage at 8°C (Fernandez-Lopez, Zhi, Aleson-Carbonell, Perez-Alvarez, & Kuri, 2005). Ahn, Grun, and Mustapha (2007) showed that Activin™ (grape seed extract) and Pycnogenol® (pine bark extract) can be used as food additives to maintain the quality and safety of cooked beef. Green, jasmine, and black tea aqueous extracts suppresses the growth of *S. aureus* by 5.0 log CFU/ml and *L. monocytogenes* by 3.0 log CFU/ml in brain heart infusion broth (Kim, Ruengwilysup, & Fung, 2004).

The plants *Pelargonium purpureum* and *Sideritis scardica* exerted the strongest antimicrobial effects against *Escherichia coli* O157:H7 NCTC 12900, *Salmonella enteritidis* PT4, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ScottA, *Pseudomonas putida* AMF178, and *Bacillus cereus* FSS134, but especially on *E. coli* and *L. monocytogenes* (Proestos, Zoumpoulakis, & Sinanoglou, 2013). The methanol extract of *Peganum harmala*, the EO of *Satureja bachtiarica*, the ethanol extract of *Juglans regia* and *Trachyspermum copticum* with MIC of 105, 126, 510 and 453 µg/ml, respectively are the most active plant extracts against *Lactococcus garvieae* from diseased *Oncorhynchus mykiss*, whereas some of the extracts such as *Quercus branti* Lindley and *Glycyrrhiza glabra* L had lower activity against *L. garvieae* with MIC values of 978 and 920 µg/ml, respectively (Fereidouni, Akhlaghi, & Alhosseini, 2013).

The addition of 5% olive or apple skin extracts reduced *E. coli* O157:H7 populations to below the detection limit and by 1.6 log CFU/g, respectively, and 1% lemongrass oil reduced *E. coli* O157:H7 to below detection limits, whereas clove bud oil reduced the pathogen bacteria by 1.6 log CFU/g. The formation of carcinogenic heterocyclic amines (HCAs) was reduced with the addition of olive and apple extracts by 76-85% and from 35 to 53% with clove bud oil (Rounds, Havens, Feinstein, Friedman, & Ravishankar, 2012).

The aqueous extracts of *Psidium guajava*, *Citrus limonium*, *Allium sativum*, and *Zingiber officinale* were found active against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Salmonella* species. Guava leaves had stronger antibacterial effect against *B. subtilis*, whereas *S. aureus* was inhibited with garlic cloves in a more effective way. Lemon leaves extract and juice inhibited the growth of *P. aeruginosa* and *E. coli*, respectively, to a high level (Kumar, Marwaha, Singh, & Kumar, 2012).

Cinnamon, clove, star anise, picklyash peel, and common fennel water extracts were mixed with chitosan at 0.2 g/l, 3.0 g/l, 0.7 g/l, 2.2 g/l, and 0.6 g/l concentrations, respectively, to design a new food preservative formula. Chilled steak samples treated with this preservative formula remained fresh after 9 days of storage (Wang et al., 2012).

Rosemary, sage, peppermint, and spearmint inhibited the growth of *Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus*. Rosemary and sage showed a stronger antibacterial effect than green and black teas of *Camellia sinensis* (Chan, Kong, Yee, Chua, & Loo, 2012). *Laurus nobilis* (L.), *Rosmarinus officinalis* (L.), *Equisetum arvense* (L.), *Lavandula officinalis* (L.), and *Lavandula stoechas* (L.) leaves showed antibacterial activity. Only aqueous extracts of *L. nobilis* showed anticandidal activity. The most sensitive bacterium was *S. aureus* and the most resistant one was *B. cereus* (Ceyhan, Keskin, & Ugur, 2012).

Lai and Roy (2004) showed the antimicrobial activity of cinnamon, clove, and mustard against mycotoxigenic *Aspergillus*, *Aspergillus parasiticus*, *Salmonella enterica*, *L. monocytogenes*, *E. coli*, *Shigella sonnei*, and *Shigella flexneri*. The application of garlic-derived organosulfur compounds in meat could enhance microbial safety (Yin & Cheng, 2003).

In broth model systems 0.25-1.0% clove and garlic showed bacteriostatic and bactericidal activities against *E. coli* O157 and *Salmonella enterica* serovar Enteritidis. Garlic at 1% concentration reduced the viable cells *S. enteritidis* in mayonnaise (Leuschner & Zamparini, 2002). The addition of fresh garlic (30g/kg) or garlic powder (9g/kg) into raw chicken sausage significantly reduced the aerobic plate counts and extended the shelf life of the product to 21 days (Sallam, Ishioroshi, & Samejima, 2004). Gaekwad and Trivedi (2013) showed that fresh garlic extract, combined with ampicillin or ciprofloxacin, can synergistically increase the zone of inhibition (Ankri & Mirelman, 1999).

Allicin, an active compound extracted from fresh garlic, in its pure form exerts antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria, including drug resistant Enterotoxigenic *E. coli* strains, antifungal (*C. albicans*), antiparasitic (*Entamoeba histolytica* and *Giardia lamblia*), and antiviral activities (Ankri & Mirelman, 1999). Aqueous extract of garlic and garlic shoot juice in combination with nisin showed synergistic bactericidal activity against nisin resistant *Listeria monocytogenes* (Kim, Choi, Bajpai, & Kang, 2008; Singh, Falahee, & Adams, 2001).

Turmeric (*Curcuma longa* L.) is a tropical herb which belongs to the Zingiberaceae family and is indigenous to southern Asia. Turmeric is used in foods as a condiment and in medicine as a carminative, anthelmintic, laxative and as a cure for liver ailment (Negi, Jayaprakasha, Rao, & Sakariah, 1999). The ethanolic extract of turmeric showed high antimicrobial activity at 3.11 and 5.65 mg gallic acid/ml extract against *E. coli*, *Staphylococcus aureus*, *Saccharomyces sake*, and *Aspergillus oryzae* (Falco, Martínez, Rodríguez, Villavicencio, & Sevillano, 2011).

The total six extracts of black pepper (*Piper nigrum*) and turmeric (*Curcuma longa*) showed antibacterial activity against *Bacillus subtilis*, *B. megaterium*, *B. sphaericus*, *B. polymixa*, *S. aureus*, *E. coli*, and eleven molds, *Aspergillus luchuensis*, *A. flavus*, *Penicillium oxalicum*, *Rhizopus stolonifer*, *Scopulariopsis sp*, *Mucor sp* (Pundir & Jain, 2010).

The ethanolic extracts of unripe banana, lemongrass, and turmeric were effective against *Escherichia coli* ATCC 25922, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *S. aureus* ATCC 25921, and *Bacillus subtilis*. MIC ranged from 4 to 512 mg/ml while MBC ranged from 32 to 512 mg/ml depending on bacterial isolates and extracting solvent. *S. aureus* ATCC 25921 was killed in less

than 2 h with unripe banana extract, turmeric had less than 3 h killing time for *E. coli*, and lemongrass had more than 3 h killing time for *S. paratyphi* (Fagbemi, Ugoji, Adenipekun, & Adelowotan, 2009).

The hexane extract eluted with 5 % ethyl acetate was the most active fraction of curcumin, the yellow color pigment of turmeric, against *Bacillus cereus*, *B. coagulans*, *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Negi et al., 1999). Turmeric extract (1.5% v/v) alone or combined with shallot extract (1.5% v/v) could enhance quality characteristics and extend shelf life of vacuum-packaged rainbow trout (*Onchorhynchus mykiss*) during 20 days of storage at 4±1 °C (Pezeshk, Rezaei, & Hosseini, 2011).

Cinnamon is extracted from cinnamon bark, fruit, leaf, and their EOs, and many *Cinnamomum* species produce a volatile oil on distillation with different compositions and aroma characteristics (Jayaprakasha, Rao, & Sakariah, 1997; Kaul, Bhattacharya, Rao, Syamasundar, & Ramesh, 2003; Negi, 2012). Bioactive fraction obtained from fruits of *Cinnamomum zeylanicum* having moisture 4-6%, greenish white color and mild salty flavor, along with one or more pharmaceutically acceptable additives, use of this antibacterial agent is under patent (Jayaprakasha, Negi, Rao, & Sakariah, 2003). The oil of cinnamon (*Cinnamomum zeylanicum*) was highly effective against *Bacillus sp.*, *Listeria monocytogenes*, *E. coli*, *Klebsiella sp.*, and fungi *Rhizomucor sp* (Gupta, Garg, Uniyal, & Kumari, 2008).

The extracts of *Cinnamomum cassia* Blume (cassia bark, Chinese cinnamon) bark exerted antibacterial activity (7-29 mm/20 µl inhibition zone) against 13 bacterial species by in vitro agar diffusion method. The alcohol extracts showed 7 mm/20 µl inhibition zone against *Bacillus megaterium* and *Enterococcus faecalis*. The alcohol extracts of *Pimpinella anisum* (L.) (anise,

aniseed) seeds were effective against *Micrococcus luteus* and *Mycobacterium smegmatus* (8 mm/20 µl inhibition zone), however the extracts of *Glycyrrhiza glabra* roots showed various antibacterial activities (7-11 mm/20 µL inhibition zone) against tested microorganisms (Ates & Erdogrul, 2003).

The addition of 5 µl cinnamon EO per 100 ml of carrot broth in combination with refrigerated storage produced conditions required for inhibiting *Bacillus cereus* for 60 days (Valero & Salmeron, 2003). The mixture of Chinese chive (*Allium tuberosum*), cinnamon (*Cinnamomum cassia*), and corni fructus (*Cornus officinalis*) extracts exhibited better inhibitory effects on growth of *E. coli* than potassium sorbate at 2-5 mg/ml and inhibited growth of *Pichia membranaefaciens* at levels as low as 2 mg/ml concentration. With addition of food additives, its microbial activity was slightly enhanced except for citric acid. The mixed extract was effective in orange juice, pork and milk, dumplings, guava juice, and green and black tea as food models (Hsieh, Mau, & Huang, 2001; Mau, Chen, & Hsieh, 2000).

The antimicrobial activity of polyphenols, tannins, and flavonoids is well known. Pomegranate (*Punica granatum* L.) is rich in tannins which show strong antimicrobial activity. Fruits, peels, leaves, flowers, seeds, and roots of pomegranate have been widely used in herbal remedies and medicine in many countries (Al-Zoreky, 2009; Negi, 2012). The 80% methanolic extract of pomegranate peels had an inhibitory effect against *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Yersinia enterocolitica*, and showed more than 1 log reduction of *L. monocytogenes* in fish during refrigerated storage (Al-Zoreky, 2009).

Pomegranate fruit fractions showed antimicrobial activity (inhibition zone) against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Opara, Al-Ani, & Al-Shuaibi, 2009). Pomegranate aril extracts had antimicrobial effects on *Bacillus megaterium* DSM 32,

P. aeruginosa DSM 9027, *S. aureus* Cowan 1, *Corynebacterium xerosis* UC 9165, *E. coli* DM, *Enterococcus faecalis* A10, *Micrococcus luteus* LA 2971 and three fungi, *Kluyveromyces marxianus* A230, *Rhodotorula rubra* MC12, and *Candida albicans* ATCC 1023 with 30 to > 90 µg/ml MIC values (Duman, Ozgen, Dayisoğlu, Erbil, & Durgac, 2009). Braga et al. (2005) showed that pomegranate extract at 1% v/v eliminated the growth of *Staphylococcus aureus* FRI 722 and inhibited enterotoxin (SE) production at 0.05% v/v concentration (Braga et al., 2005). The addition of pomegranate peel extract to chicken meat products extended their shelf life by 2-3 weeks during chilled storage (Kanatt, Chander, & Sharma, 2010).

Garcinia kola, commonly known as Bitter Kola, is a medium sized tree. Different species of *Garcinia* possess antimicrobial, antioxidant, antitumorogenic, and cytotoxic activities through their secondary metabolites (Akerele, Obasuyi, Ebomoyi, & Oboh, 2010; Negi, 2012). The crude ethanol extract of *Garcinia kola* Heckel showed inhibitory activity against clinical bacterial isolates of *S. aureus*, *B. subtilis*, *Streptococcus viridians*, *E. coli*, *P. aeruginosa*, and *K. pneumoniae* and fungi like *Penicillium notatum*, *Aspergillus niger* and *Candida albicans*. The MIC values range between 2.5 and 7.5 mg/ml (Akerele et al., 2010).

The compounds extracted from *Garcinia brasiliensis*, which are active against *S. aureus* and *B. cereus* were pericarp hexane extract 4.0 µg/ml and 2.4 µg/ml, seed ethanol extract 10.00 µg/ml and 12.6 µg/ml, respectively (Naldoni et al., 2009). Methanolic crude extract of the stem bark of *Garcinia lucida* Vesque exerted a good inhibitory effect against *Candida albicans* (MIC value was 64 µg/ml). Only cycloartenol obtained from dichloromethane fraction exhibited antimicrobial activity against *E. coli* and *P. aeruginosa* (Momo, Kuete, Dufat, Michel, & Wandji, 2011).

Pericarp extract of *Garcinia mangostana* Linn. (mangosteen) was effective against *Staphylococcus aureus*, *Staphylococcus albus*, and *Micrococcus luteus* (Vishnu Priya, Jainu, Mohan, Saraswathi, & Chandra, 2010). The crude methanolic extracts of *Garcinia atroviridis* had inhibitory effect against *Bacillus subtilis* B28 mutant, *B. subtilis* B28 wild type, methicillin resistant *S. aureus*, *E. coli*, and *P. aeruginosa* UI 60690 at a minimum inhibitory dose (MID) of 15.6 µg/disc (Mackeen et al., 2000). The ethanol extracts of *Garcinia kola* produced inhibition zones ranging from 8 to 18 mm against *S. aureus*, *P. aeruginosa*, *E. coli*, and *Candida albicans* (Ezeifeke, Orji, Mbata, & Patrick, 2004). Chomnawang, Surassmo, Nukoolkarn, and Gritsanapan (2005) showed that *Garcinia mangostana* had a strong inhibitory effect against *Propionibacterium acnes* and *Staphylococcus epidermidis* and this plant can be used for an alternative treatment of acne. The natural xanthenes isolated from the fruit hulls of *Garcinia mangostana* showed good inhibitory activity against phytopathogenic fungi, *Fusarium oxysporum* *vasinfectum*, *Alternaria tenuis*, and *Dreschlera oryzae* (Gopalakrishnan, Banumathi, & Suresh, 1997). The MIC exerted by the seed extracts of *Garcinia kola* against *E. coli*, *S. aureus*, and *P. aeruginosa* ranged between 3.125 and 25 mg/ml and inhibition zones were between 4.0 and 10.5 mm. The crude ethanol extract was found to be most effective against all bacteria at different treatment regimens (Ghamba, Agbo, Umar, Bukbuk, & Goje, 2012).

The antimicrobial activities of seven Turkish spice (cumin, *Helichrysum compactum* Boiss (HC), laurel, myrtle, oregano, sage and thyme) methanolic extracts were investigated against *Escherichia coli* O157:H7. Thyme and oregano showed higher inhibitory effects and their antibacterial effects vary related to the concentration of extracts used (Sagdic, Kuscu, Ozcan, & Ozcelik, 2002). High concentration of rosmarinic acid in methanolic extract of *Orthosiphon stamineus* was closely related with antibacterial activity of plant extract against

Vibrio parahaemolyticus and a similar inhibitory effect was obtained with 5 % lactic acid (Ho, Noryati, Sulaiman, & Rosma, 2010).

Tea (*Camellia sinensis*) is a popular nonalcoholic beverage consumed by over two-thirds of the world's population due to its taste and medicinal properties. Among all tea polyphenols, epigallocatechin-3-gallate is responsible for anti-inflammatory, antimicrobial, antitumor, antioxidant activities and protection from cardiovascular disease (CVD), anti-obesity and anti-aging properties. Tea and its polyphenols possess antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori*, *Bacillus spp*, *Clostridium spp*, *Streptococcus spp* and some other bacteria, antiviral activity against influenza virus, HIV, Epstein-Barr virus, Hepatitis B virus, Herpes simplex virus (HSV) and some other viruses, and antifungal activity against *Candida albicans* and some other fungi (Bansal et al., 2013). Methanolic extracts of green tea (*Camellia assamica*) at 10, 20, 30 μ L concentrations had significant antibacterial activity against *Staphylococcus sp*, *Streptococcus sp*, and *Bacillus sp* and they were least active against *Pseudomonas sp* and *Proteus sp* (Kumar, Kumar, et al., 2012), ethanolic extracts of green tea had potent antimicrobial activity against *Streptococcus mutans*, *S. sobrinus*, *Listeria monocytogenes*, *Shigella flexneri*, and *Salmonella enterica* (Oh, Jo, Cho, Kim, & Han, 2013).

The green tea kombucha exhibited the highest antimicrobial activity against *Staphylococcus epidermidis* (22 mm), *Listeria monocytogenes* (22 mm) and *Micrococcus luteus* (21.5 mm). However, anticandidal activity of green tea kombucha was revealed by the reaction against *Candida parapsilosis* (Battikh, Chaieb, Bakhrouf, & Ammar, 2013). Aqueous green tea leaf extract was effective against *Staphylococcus epidermidis*, *Pseudomonas fluorescens*, *Micrococcus luteus*, *Brevibacterium linens*, and *Bacillus subtilis* by disc diffusion assay (zone of inhibition ≥ 7 mm). MIC was determined via nitro blue tetrazolium (NBT) assay (0.156-0.313

mg/ml). The extract was not toxic to Vero cell line up to a concentration of 500 µg/ml (Sharma, Gupta, Sarethy, Dang, & Gabrani, 2012).

Incorporation of green tea extract into chitosan film enhanced antimicrobial activities of the film and therefore maintained the sensory quality and extended shelf life of the pork sausages (Siripatrawan & Noipha, 2012). Aqueous and methanolic extracts of Lipton® black tea in Nigeria contain tannin and reduced sugar and have antimicrobial activity against *E. coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa* at 2%-10% concentrations (Funmilayo, Kamaldeen, & Buhari, 2012). Black tea and tea with milk beverages significantly reduced cariogenic oral *Streptococcus mutans* and *Lactobacillus sp.* counts (43.6%-83.3%) and they are recommended as natural anti-cariogenic beverages (Abd Allah, Ibrahim, Abd Allah, & Amin, 2012).

Murali, Kumar-Philips, Rath, Marcy, and Slavik (2012) found green and white tea extracts are most antibacterial against *C. jejuni*, *E. coli* O157:H7, *Salmonella enteritidis*, and *Staphylococcus aureus* among the different teas tested and white tea killed all tested bacteria except *C. jejuni* 81176 within 48 hours of incubation. Longjing Tea extract could reduce the *Vibrio parahaemolyticus* count by 0.8 log MPN/g in shucked Pacific oysters and that green tea treatment of oysters could enhance reducing *V. parahaemolyticus* while retarding the growth of total bacteria in oysters during 5±1°C storage (Xi, Liu, & Su, 2012).

Caffeine (1, 3, 7-trimethylexanthine) is a methylated xanthine alkaloid derivative present in plant species and it has shown significant inhibitory effect against *E. coli* O157:H7 at a concentration of 0.5 % (Gyawali & Ibrahim, 2012; Ibrahim, Salameh, Phetsomphou, Yang, & Seo, 2006), *Serratia marcescens*, *Enterobacter cloacae*, and *Salmonella enterica* (Almeida, Farah, Silva, Nunan, & Glória, 2006), *Staphylococcus aureus* and *Vibrio sp.* (MIC values of 192±91 µg/ml and 162±165 µg/ml, respectively; Taguri, Tanaka, & Kouno, 2004).

Processed and fresh forms of sloe berry purees reduced *Salmonella spp.* in range of 4.24-6.70 log units within 24 h at 25°C (Gunduz, 2013).

1.4 Antimicrobial Herbs and Spice Compounds

1.4.1 Introduction. Consumers are vigorously demanding good quality, safe food. Due to the increase in the number of foodborne outbreaks caused by pathogen microorganisms, new concerns have arisen about food safety. Considerable challenges arise with these concerns. Consumers are especially concerned about the use of chemical and artificial antimicrobial compounds and preservatives to inhibit the growth of pathogens (Tajkarimi et al., 2010).

Consumer demand for keeping nutrition and quality features the same while providing food safety has increased the desire for using alternative preservation methods to inactivate pathogen microorganisms and enzymes in food products. These attributes include flavor, odor, color, texture, and nutritional value. Foods preserved with natural additives have become popular. New aspects for using natural preservatives obtained from plants, animals, or microflora were considered with this increasing demand (Holley & Patel, 2005; Tiwari et al., 2009). As a result, natural antimicrobials are gaining attention for controlling microorganisms. They play an important role in food control through preventing microbial contamination by pathogens and extending shelf life by removing undesirable pathogens. Antibiotic resistance of pathogens can be decreased and the immune system can be boosted by using natural antimicrobials (Tajkarimi et al., 2010).

There are two major reasons for using antimicrobials in food models: to prevent natural spoilage (preservation) and to control the growth of microorganisms (safety). Animals, plants and microorganisms are the major sources of natural antimicrobials. Natural antimicrobials can be used in fruits and vegetables in particular with great potential. Nevertheless, methods and

mechanisms of action of natural antimicrobials are not fully understood. Great efforts have been made to find new methods for the use of antimicrobials in ensuring food safety (Tajkarimi et al., 2010).

Besides their seasoning effects, some herbs and spices have antimicrobial effects against human and plant pathogens (Brandi, Amagliani, Schiavano, De Santi, & Sisti, 2006). Food processing techniques such as chemical preservatives usage cannot totally kill foodborne pathogens or stop microbial spoilage (Gutierrez, Barry-Rian, & Bourke, 2009).

Cold storage of perishable food can help in preservation, but overall product quality and safety would not be guaranteed. Furthermore eating habit changes, food processing techniques, and increasing the trend for ready-to-eat foods are very effective on preservation. Reports for the presence of foodborne pathogens in fruits and vegetables have increased since the pathogens are present in such raw materials (Lanciotti et al., 2004; A. Li, Zhu, et al., 2008; H. Li, Tajkarimi, & Osburn, 2008).

Recent food preservation methods such as pulsed light, high pressure pulsed electric, and magnetic fields were used for controlling pathogen and spoilage microorganisms in food (Tajkarimi et al., 2010). Nevertheless, the evaluation of the bactericidal effect at pH 4.0 indicated that most compounds assayed were very efficient against *Escherichia coli* and *Listeria monocytogenes*. Excellent synergistic lethal effects were obtained with mild heat (54°C/10 min) or PEF (30 kV/cm/25 pulses) when they were combined with 0.2 µl/ml of some antimicrobials. Five log reductions were achieved through a combination of treatments. These combined treatments were more efficient in apple than in orange juice (Ait-Ouazzou et al., 2011).

Nevertheless, preservation technologies such as heat processing, modified atmosphere packaging (MAP), and refrigeration are not quite efficient for removing pathogens or slowing

microbial spoilage process down. Furthermore, some preservation methods, for example vacuum cooling, can increase the risk for food contamination. The attachment of natural antimicrobials to packaging materials to insulate the surface of the food instead of the food itself has been developed as well. For example, the use of pure essential oils (eugenol, thymol or menthol) in combination with MAP is a new and useful method as an alternative to using synthetic fungicides in fruits and vegetables, specifically for those which are highly perishable and have a reduced shelf-life. Some of the essential oils, such as eugenol, the bioactive compounds with antioxidant activity, were also enhanced during prolonged storage (Serrano et al., 2008; Tajkarimi et al., 2010).

The recently expanding data shows that there is remarkable potential for natural antimicrobial usage in food products, specifically application to fresh fruits and vegetables for their antioxidant effect against lipids and improvement of the overall quality of the food besides their considerable effects against fungi (Serrano et al., 2008; Tajkarimi et al., 2010). EOs extracted from plants and spices have an antimicrobial effect against *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Shigella dysenteriae*, *L. monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* at concentrations between 0.2 and 10 µl/ml (Burt, 2004). Two percent of citric acid or up to 0.1% of cinnamon bark oil added into tomato juice and then treated with high intensity pulsed electric fields caused at least 5-log reduction (Mosqueda-Melgar, Raybaudi-Massilia, & Martin-Belloso, 2008a, 2008b).

Plant-derived natural antimicrobials have been used a long time for preservation of foods. Some spices and EOs were used by the early Egyptians and have been used for centuries in China and India. Spices such as clove, cinnamon, mustard, garlic, ginger, and mint are still used as alternative health remedies in India. EO production can be traced back over 2,000 years to the

Far East with the modern technologies in Arabia in ninth century. Spices and plant extracts have antimicrobial effects against Gram-positive pathogen microorganisms such as *Listeria monocytogenes*. These antimicrobial compounds can increase storage time and stability with active ingredients such as phenols, alcohols, aldehydes, ketones, ethers, and hydrocarbons, specifically in cinnamon, clove, garlic, mustard, and onion. The first scientific study on preservative effects of spices was in the 1880s showing the antimicrobial effect of cinnamon oil against *Bacillus anthracis* spores (Tajkarimi et al., 2010).

Clove was used to prevent spoilage in meat, syrups, sauces, and sweet meats. Cinnamon and mustard were identified as preservatives in applesauce in the 1910s. It has been reported that allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage, and thyme have strong bacteriostatic effects (Tajkarimi et al., 2010). The majority of spices have originated from the eastern world; nevertheless, the New World discovery also caused the introduction of some spices such as chili and sweet peppers, allspice, annatto, chocolate, epazote, saffron, and vanilla (Ceylan & Fung, 2004).

1.4.2 Major antimicrobial compounds of herbs and spices. Plants have a nearly endless potential to synthesize mostly phenolic aromatic compounds or their oxygen substituted analogs (Geissman, 1963). The majority of them are secondary metabolites and approximately 12,000 of them have already been isolated but it does not even correspond to 10% of the total number of metabolites (Schultes, 1978). Most of the time, these materials allow plants to defend themselves against microorganisms, insects, and herbivores. Some of them, for example terpenoids give the plants their characteristic scent whereas quinones and tannins are related to the plant pigment. Most of these metabolites give the plants their flavor such as the terpenoid from chili peppers and some herbs and spices used by people to marinate food produce useful

medicinal compounds. Several different groups of useful antimicrobials are summarized in Table 2 (Cowan, 1999).

Table 2

Major Classes of Antimicrobial Compounds from Plants (Cowan, 1999)

Class	Subclass	Example(s)	Mechanism
Phenolics	Simple phenols	Catechol Epicatechin	Substrate deprivation Membrane disruption
	Phenolic acids Quinones	Cinnamic acid Hypericin	Bind to adhesions, complex with cell wall, inactivate enzymes
	Flavonoids Flavones	Chrysin	Bind to adhesions Complex with cell wall
		Abyssinone	Inactivate enzymes Inhibit HIV reverse transcriptase
	Flavonols	Totarol	Unknown
	Tannins	Ellagitannin	Bind to proteins Bind to adhesions Enzyme inhibition Substrate deprivation Complex with cell wall Membrane disruption Metal ion complexation
	Coumarins	Warfarin	Interaction with eukaryotic DNA (Antiviral activity)
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine Piperine	Intercalate into cell wall and/or DNA
Lectins and polypeptides		Mannose-specific agglutinin Fabatin	Block viral fusion or adsorption Form disulfide bridges
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyl-1,8-diol	Unknown

1.4.2.1 Phenolics and polyphenols.

1.4.2.1.1 Simple phenols and phenolic acids. Simple phenols and phenolic acids are composed of a single substituted phenolic ring. Cinnamic and caffeic acids are communal substitutes of a large group of phenylpropane-derived compounds. Tarragon and thyme both consist of caffeic acid and they are efficient against bacteria, viruses and fungi. Catechol and pyrogallol are hydroxylated phenols proved to be toxic to microorganisms (Cowan, 1999). The toxicity mechanisms comprise inhibition of the enzymes through oxidized compounds. Phenolic compounds with a C₃ side chain and having no oxygen are categorized as essential oils and generally they are called antimicrobials too (Cowan, 1999).

1.4.2.1.2 Quinones. Quinones are aromatic rings with two ketone substitutions. These quinones are responsible for the browning reaction in peeled fruits and vegetables. The compound responsible for dyeing in henna is quinone. Coenzyme Q (ubiquinone) has an important role in mammalian electron transport system. Vitamin K is a naphthoquinone. Besides providing a source of free radicals, quinones bind irreversibly with nucleophilic amino acids in proteins generally causing the protein inactivation and loss of function (Cowan, 1999).

Therefore quinones have potentially great antimicrobial effects. Possible targets in cells are surface-exposed adhesions, cell wall polypeptides, and membrane bound enzymes. Furthermore the quinones may also make the substrate unavailable for microorganism. Anthraquinones have antibacterial and antidepressant effects (Cowan, 1999).

1.4.2.1.3 Flavones, flavonoids, and flavonols. Flavones have phenolic structure containing one carbonyl group. Flavonols have extra 3-hydroxyl groups. Flavonoids are also hydroxylated phenolic compounds but there is a C₆-C₃ unit bound to the aromatic ring. They

have antimicrobial activity through their ability to form complexes with proteins and cell walls, as explained for quinones (Cowan, 1999).

Catechins inhibited *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria and microorganisms. Flavonoids show inhibitory effects against some viruses such as human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), herpes simplex virus type 1 (HSV-1), poliovirus type 1, and parainfluenza virus type 3. Hesperetin reduces intracellular replication of viruses. Galangin has antimicrobial activity against a lot of different Gram-positive bacteria, fungi, and viruses. Alpinumisoflavone stops schistosomal infection when it is administered topically (Cowan, 1999).

1.4.2.1.4 Tannins. They are a group of polymeric phenolic compounds which have the ability to tan leather or sedimentation of gelatin from solution, a feature known as astringency and they are present in bark, wood, leaves, fruits and roots of the plants. The beverages containing tannins especially green teas and red wines can cure several diseases. Their mechanism of action may be related to inactivation of microbial adhesions, enzymes, and cell envelope transport proteins. They can bind to polysaccharides as well. Tannins can be toxic to filamentous fungi, yeasts and bacteria (Cowan, 1999).

1.4.2.1.5 Coumarins. Coumarins are phenolic substances composed of fused benzene and α -pyrone rings. At least 1,300 different coumarins have been identified. Coumarins have antithrombotic, anti-inflammatory, and vasodilatory activities. Warfarin is the most popular one and it is used as oral coagulant and rodenticide. They may have antiviral effects and they are highly toxic in rodents. Some of the coumarins compounds have antimicrobial effects such as inhibitory effect against *Candida albicans* and therefore they can be used for curing vaginal candidiasis. They can induce macrophages which will effect infections negatively.

Hydroxycinnamic acids can inhibit gram-positive bacteria. Phytoalexins can be assumed to have effects against fungi (Cowan, 1999).

1.4.2.2 Terpenoids and essential oils. The essential oils have the odor of plants. The essential oils are secondary metabolites that are highly enriched in substances predicated isoprene structure on (see Figure 2). These compounds are called terpenes. Their general chemical conformation is $C_{10}H_{16}$, and their subcategories are diterpenes, triterpenes, tetraterpenes, hemiterpenes, and sesquiterpenes. When they comprise additional elements, mostly oxygen, they are called terpenoids and they are synthesized from acetate units and share their origins with fatty acids. Terpenoids are in cyclic form and extensively branched. Well-known terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenes). Artemisin is recently used as antimalarial (Cowan, 1999).

Terpenoids are effective against bacteria, fungi, viruses and protozoa. Betulinic acid can inhibit HIV. The mode of action for terpenes is not exactly understood but it is assumed that they can disrupt the membrane by lipophilic compounds. Terpenoids existent in essential oils used to control *Listeria monocytogenes*. Capsaicin is a terpenoid that is present in Chile peppers and it is involved in a series of biological activities related to nervous, cardiovascular and digestive systems of human beings and it is analgesic (Cowan, 1999).

Capsaicin can inhibit a lot of different bacteria and it has bactericidal effect against *Helicobacter pylori*. Aframodial is strong antifungal. Petalostemumol showed strong antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and less activity against gram-negative bacteria alongside *Candida albicans*. Terpenoids are useful in preventing ulcer formation and decreasing the severity of present ulcers (Cowan, 1999).

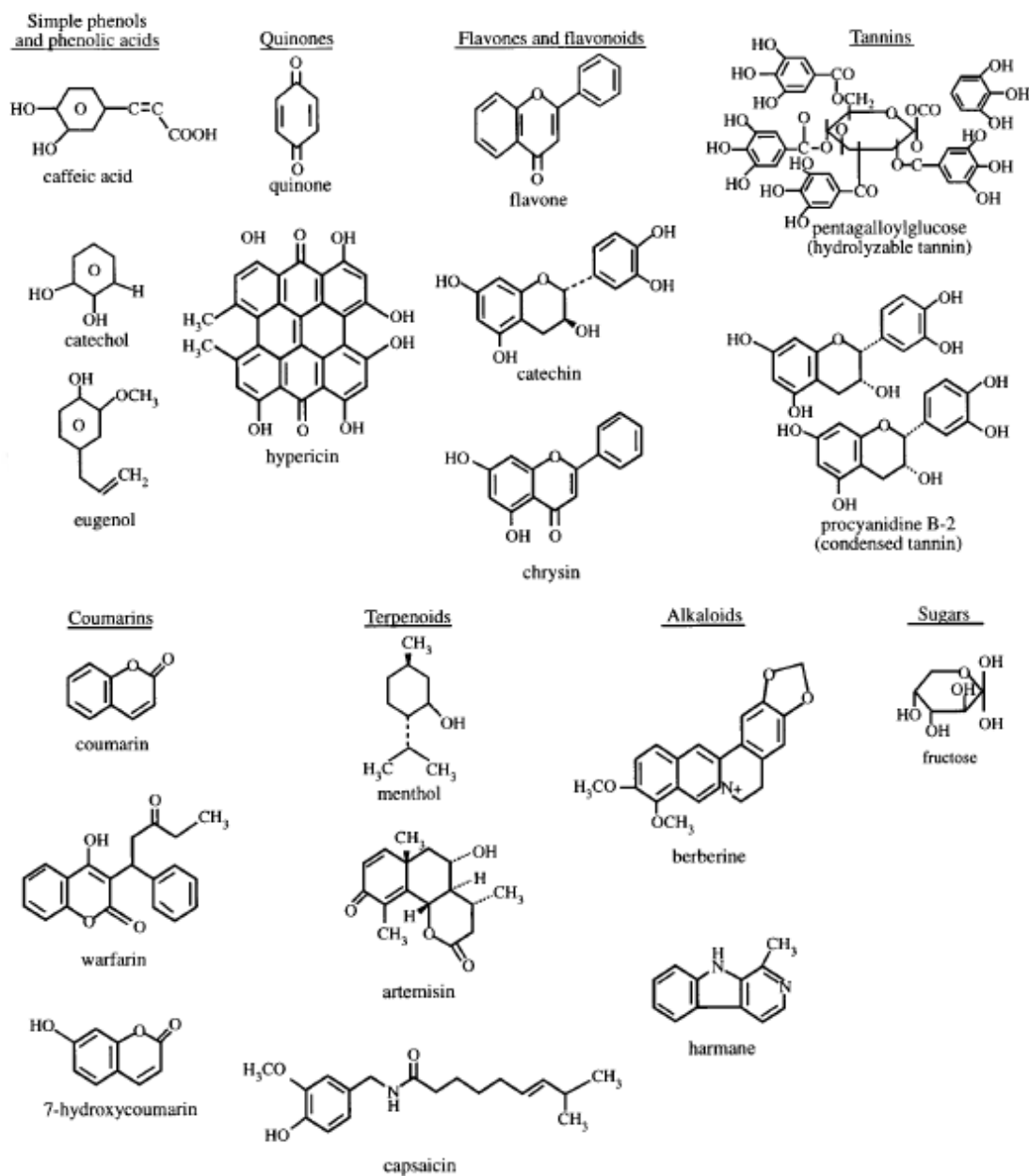


Figure 2. Structures of common antimicrobial plant chemicals (Cowan, 1999).

1.4.2.3 Alkaloids. Alkaloids are heterocyclic nitrogen compounds. Morphine was isolated from opium poppy *Papaver somniferum* in 1805 and used in medical applications. Codein and heroin are both morphine derivatives. Diterpenoid alkaloids have antimicrobial effects. Solamargine is a glycoalkaloid and it can be useful against HIV together with other alkaloids.

Berberine is a considerable group of alkaloids and they are effective against trypanosomes (Cowan, 1999).

1.4.2.4 Lectins and polypeptides. Peptides inhibiting microorganisms were first found in 1942. These peptides generally have positive charges and comprise disulfide bonds. Their mode of action can be explained by formation of ion channels in mitochondrial membrane. Thionins are commonly present in barley and wheat and they are toxic against yeasts, gram-negative and gram-positive bacteria whereas thionins AX1 and AX2 are just effective against fungi. Fabatin is a peptide isolated from fava beans and inhibits *E. coli*, *P. aeruginosa*, and *Enterococcus hirae*. The larger lectin molecules contain mannose-specific lectins from different plants. MAP30 from bitter melon, GAP31 from *Gelonium multiflorum*, and jacalin have inhibitory effects against viruses (Cowan, 1999).

1.4.2.5 Mixtures. In African countries, a chewing stick is used instead of a toothbrush for maintaining oral hygiene. These chewing sticks are obtained from a variety of species and they may contain different chemical compounds such as alkaloids. *Serinda werneckei* can inhibit periodontal pathogen microorganisms *Porphyromonas gingivalis* and *Bacteroides melaninogenicus* (Cowan, 1999).

Papaya bears a milky sap, generally called latex and it has a mixture of different chemical compounds such as papain (proteolytic enzyme), carpaine alkaloid, and terpenoids. Latex is bacteriostatic to *B. subtilis*, *Enterobacter cloacae*, *E. coli*, *salmonella typhi*, *Staphylococcus aureus*, and *Proteus vulgaris*. Ayurveda is a kind of healing practices in India and it relies on single plant or mixture preparations. The preparations such as Ashwagandha, Cauvery 100 and Livo-vet have antimicrobial, antidiarrheal, immunomodulatory, anticancer, and psychotropic effects. They might be toxic and cause an accumulation of high levels of lead in human blood.

Propolis, bee glue, is a crude extract collected from different trees by honeybees and its chemical composition is very complex. Propolis contains terpenoids, flavonoids, benzoic acids and esters, and substituted phenolic acids and esters. It has antiviral activity against HSV-1, adenovirus type 2, vesicular stomatitis virus, and poliovirus (Cowan, 1999).

1.4.2.6 Other compounds. Polyamines, isothiocyanates, thiosulfinates, and glucosides have antimicrobial effects. Cranberry and blueberry juices competitively inhibit the attachment of pathogenic *E. coli* to urinary tract epithelial cells (Cowan, 1999).

Commonly used antimicrobials in the food industry are more than 1340 plants containing clearly defined antimicrobial compounds and more than 30,000 components isolated from plant oil compounds that have phenolic groups. Nonetheless, characterization of features which are important in preservation of food is only available for several EOs (Tajkarimi et al., 2010).

In food systems more evaluations of EOs are required. The use of spices and their EOs for preservation of food as natural antimicrobials has recently caused more interest in increasing the shelf life of foods, the elimination of foodborne pathogens and the improvement in overall quality of food products (Tajkarimi et al., 2010).

The most common method for production of plant origin antimicrobials is hydro distillation, steam distillation. Supercritical fluid extraction, as an alternative method, allows higher solubility with better mass transfer rates. Additionally, the modification of parameters like temperature and pressure will allow extracting different components (Burt, 2004). Medicinal and herbal plants and spices like sage, basil, oregano, thyme, rosemary, turmeric, garlic, ginger, clove, mace, nutmeg, savory and fennel were used in food preservation either alone or in combination with other preservation techniques. They increase the shelf life of food products or have antimicrobial effects against a wide variety of Gram-negative and Gram-positive bacteria.

The factors affecting their efficiency are pH, storage temperature, the oxygen amount, the concentration and active ingredients of EOs (Tajkarimi et al., 2010).

In food manufacturing the spices and EOs are used as natural antimicrobials to increase the shelf life of foods. For the purposes of improving the food quality and removing foodborne pathogens, a lot of different type of plant and spice based antimicrobials are used. The aromatic oily liquids of flowers, seeds, leaves, bark, buds, twigs, herbs, wood, fruits and roots of plants contain plant origin antimicrobials and they are extracted with different methods. EOs are composed of the combination of various compounds. Some of them have strong antimicrobial effects such as compounds extracted from oregano, clove, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, sage and vanillin whereas ginger, black pepper, red pepper, chili powder, cumin and curry powder had lower antimicrobial effects (Holley & Patel, 2005; Tajkarimi et al., 2010).

1.4.3 Chemical components of EOs. Essential oils can contain 20-80 different constituents at different concentrations. The main group of constituents is terpenes and terpenoids (Burt, 2004). The monoterpenes constitute 90% of EOs and they have very different structures and oxygenated monoterpenes are more active than hydrocarbon ones (Carson & Riley, 1995). EOs consist of terpenoids, sesquiterpenes and diterpenes with aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters or lactones (Fisher & Phillips, 2006).

Plant extracts and EOs are mainly responsible for antimicrobial activities and they can be extracted from plants and spices with different methods (Bajpai et al., 2008; Ceylan & Fung, 2004). Main components of the EOs can be composed of 85 %, and other compounds are generally at trace levels (Burt, 2004; Grosso et al., 2008). Plant origin antimicrobials contain chemicals such as saponin and flavonoids, thiosulfinates, glucosinolates. Saponin and flavonoids

are in fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. They usually generate soapy foam after shaking in water. They have antimicrobial effects when they are obtained from roots, stem bark, leaves and wood of plants such as *Avena sativa* (oat) (Tajkarimi et al., 2010). Thiosulfinates are obtained from garlic through mild procedures and they have extreme antimicrobial effects against Gram-negative bacteria. Glucosinolates are found in broccoli, Brussels sprouts, cabbage and mustard powder and they are the reason for the sharp acid flavor of mustard and horseradish. They show a broad range of activities against bacteria and fungi with direct or synergistic effects when used with other substances.

Commonly, phenolic substances of EOs like citrus oils, olive oil and tea-tree oil, orange and bergamot have wide range of antimicrobial effects and are not classified as spices. Oils extracted from citral, garlic, clove, oregano, coriander, cinnamon, rosemary, parsley, lemongrass, sage, purple and bronze muscadine seeds have some non-phenolic compounds as well and they are effective against Gram-negative and Gram-positive bacteria (Tajkarimi et al., 2010).

1.4.4 Uses of plant-origin antimicrobials. Food spoilage can happen at any step from food processing to distribution. The sources of spoilage can be microbiological, physical and chemical. Food preservation methods for microbiological spoilage have been remarkably increased recently to eliminate pathogenic microorganisms (Gould, 1996). Research has been increased related to utilization of EOs and spices as natural food preservatives. EOs increased to improve quality and extend shelf life of food products and reduce or remove pathogenic microorganisms (Burt, 2004; Moreira, Ponce, Del Valle, & Roura, 2007; Simitzis et al., 2008). *Thymus eigii* EO showed stronger antimicrobial activity than erythromycin (15 mcg) and vancomycin (30 mcg) although the EOs of *Jalas*, *Pinus nigra* Arn subspecies *pallasiana* and *Cupressus sempervirens* possessed moderate or susceptible activity (Toroglu, 2007).

1.4.4.1 Using natural antimicrobials against pathogens. The EO from *Mentha longifolia* ssp. *longifolia* showed strong antimicrobial activity against 30 different bacteria, fungi, and yeast species (Gulluce et al., 2007). The mint EO from *Mentha piperita* at 0-1.2% v/v concentrations reduced the total viable counts of *Staphylococcus aureus* 6-7 log and *Salmonella enteritidis* about 3 log in nutrient broth (Tassou, Koutsoumanis, & Nychas, 2000).

Multiple phenolic phytochemicals from herb spices of Lamiaceae family could prohibit *Staphylococcus aureus* from evolving antibacterial resistance (Y. I. Kwon, Apostolidis, Labbe, & Shetty, 2007). Carvacrol, thymol, and trans-cinnamaldehyde had inhibitory effects on *Escherichia coli*, and *Salmonella typhimurium* at 1-3 mM, although carvone could inhibit at higher concentration (Helander et al., 1998).

Mustard EO tested against *Escherichia coli* O157:H7 and *Salmonella typhi* and it affected the membrane integrity and decreased intracellular pH and ATP concentration. The cell membranes of both bacteria were damaged with mustard EO (Turgis, Han, Caillet, & Lacroix, 2009). The antimicrobial effect of ginger, galangal, turmeric, and fingerroot extracts against foodborne pathogens, spoilage bacteria and fungi by agar dilution assay. Fingerroot extract showed stronger inhibitory activity against *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* at 0.2-0.4% (v/v) MICs. Galangal extract was most effective compound against *Lactobacillus plantarum* and *L. cellobiosus* at 4% (v/v) MIC. Fingerroot and ginger extracts had antifungal activity against *Aspergillus spp* and *Fusarium oxysporum* at 8-10% (v/v). The 8% galangal and 10% fingerroot extracts showed bactericidal effect against *Escherichia coli* O157:H7 at 36 h and 9 h, respectively (Aktug & Karapinar, 1986).

Sudjana et al. (2009) investigated the activity of a commercial extract derived from the leaves of *Olea europaea* (olive) against a wide range of microorganisms. Using agar dilution and

broth microdilution techniques, olive leaf extract was found to be most active against *Campylobacter jejuni*, *Helicobacter pylori* and *Staphylococcus aureus*. The antimicrobial activity of extracts from commonly used Indian spices, clove, cinnamon, mustard, garlic, ginger and mint, was tested against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* by using paper disc diffusion method, cup method and dilution method. The extracts of clove, cinnamon and mustard showed inhibition at 1% concentration, whereas garlic had medium bacteriostatic activity and complete bactericidal effect was shown with 3% concentration. Ginger and mint showed very low antibacterial activity against these pathogens at the same concentration (Sofia, Prasad, Vijay, & Srivastava, 2007).

Cinnamon, clove, geranium, lemon, lime, orange and rosemary oils exhibited inhibitory effect. The MIC values varied from 0.2 to 25.6 mg/ml. Cinnamon oil was effective against *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus subtilis*, and *Staphylococcus aureus* even at low concentration although aniseed, eucalyptus and camphor oils were least active against abovementioned bacteria (Prabuseenivasan, Jayakumar, & Ignacimuthu, 2006).

Sandasi, Leonard, and Viljoen (2008) investigated the effect of five common essential oil components (EOCs) (α -pinene, 1, 8-cineole, (+)-limonene, linalool and geranyl acetate against listerial biofilms. They treated biofilms with each of these components at concentration of 1mg/ml. EOCs reduced the metabolic activity of listerial biofilms as detected by the XTT reduction assay.

The antibacterial activity of essential oil from *Lonicera japonica* Thunb was investigated for controlling the growth of different food-borne pathogens. Thirty-nine major compounds were extracted and they represent 92.34% of the total oil. These compounds were trans-nerolidol

(16.31%), caryophyllene oxide (11.15%), linalool (8.61%), p-cymene (7.43%), hexadecanoic acid (6.39%), eugenol (6.13%), geraniol (5.01%), trans-linalool oxide (3.75%), globulol (2.34%), pentadecanoic acid (2.25%), veridiflorol (1.83%), benzyl alcohol (1.63%) and phenylethyl alcohol (1.25%). The oil and extracts exerted a strong antibacterial effect against *Listeria monocytogenes* ATCC 19116, *Bacillus subtilis* ATCC 6633, *B. cereus* SCK 11, *Staphylococcus aureus* (ATCC 6538 and KCTC 1916), *Salmonella enteritidis* KCTC 12021, *S. typhimurium* KCTC 2515, *Enterobacter aerogenes* KCTC 2190 and *Escherichia coli* ATCC 8739 (Rahman & Kang, 2009).

Hexanal and 2-(E)-hexanal stimulated remarkable modifications of cell membrane and volatile compounds produced during growth composition of *E. coli*, *L. monocytogenes*, *Staphylococcus aureus*, and *Salmonella enteritidis*. These two aldehydes increased some of the membrane associated fatty acids, both linear and branched fatty acids as well as unsaturated fatty acids, and released free fatty acids (Patrignani et al., 2008). The different concentrations of alcoholic extracts of thyme, mint, and bay leaves tested against food poisoning bacteria. *Vibrio parahaemolyticus* was the most sensitive and its growth was inhibited with the addition of 1000, 5000 and 6000 ppm of thyme, bay leaves, and mint, respectively (Amiri, Dugas, Pichot, & Bompeix, 2008). *Thymus pallescens* oils showed remarkable differences in antioxidant activities. The EOs from three *Thymus* species exerted antibacterial activity against Gram-positive bacteria, Gram-negative bacteria and a yeast (*Candida albicans*) but especially against *C. albicans* and *Helicobacter pylori* (Hazzit, Baaliouamer, Veríssimo, Faleiro, & Miguel, 2009).

Salmonella typhimurium has the lowest sensitivity. Thyme showed most inhibitory effect at 0.05% concentration and bay leaves was the least active spice at 0.5% concentration against *S. aureus*. The combination of methanolic extracts from medicinal herbs, *Coptidis Rhizoma*, *Mume*

Fructus, and *Schizandrae Fructus*, was tested against 26 different *Salmonella* strains. This preparation was called NP532 and animals treated with NP532 did not show any of the clinical signs and showed very little histological damage related to Salmonellosis (H. A. Kwon, Kwon, Kwon, & Lee, 2008). The phenolic contents extracted from dried fruits of cinnamon with ethyl acetate, acetone, methanol and water. Phenolic content was highest in water extract (44.5%) and it was tested for antioxidant and antimutagenic activity against *Salmonella typhimurium* TA100 strain. At 5mg/plate all the extracts showed strong antimutagenic activity and antioxidant activity was in order of water>methanol>acetone>ethyl acetate (Jayaprakasha, Negi, Jena, & Rao, 2007).

The bactericidal activity levels of 96 essential oils and 23 oil compounds were evaluated using a microplate assay against *C. jejuni*, *E. coli* O157:H7, *L. monocytogenes*, *S. enterica*. Marigold, ginger root, jasmine, patchouli, gardenia, cedarwood, carrot seed, celery seed, mugwort, spikenard, and orange bitter oils were most active against *C. jejuni*. The most active oils against *E. coli* O157:H7 were oregano, thyme, cinnamon, palmarosa, bay leaf, clove bud, lemon grass, and allspice oils; and those that were most active against *L. monocytogenes* were gardenia, cedarwood, bay leaf, clove bud, oregano, cinnamon, allspice, thyme and patchouli oils; and oils that were most active against *S. enterica* were thyme, oregano, cinnamon, clove bud, allspice, bay leaf, palmarosa, and marjoram oils (Friedman et al., 2002). The essential oils of Turkish oregano, bay laurel, Spanish lavender and fennel were tested against *E. coli* O157:H7 at doses from 0 to 80 µl/ml, *L. monocytogenes*, *S. typhimurium*, and *S. aureus*, all essential oils exerted strong antibacterial activity against all tested pathogens. The major constituents of these essential oils were carvacrol, 1, 8-cineole, fenchone, and trans-anethole in Turkish oregano, bay laurel, Spanish lavender and fennel essential oils, respectively (Dadalioglu & Evrendilek, 2004).

The carvacrol and *p*-cymene extracted from oregano and thyme were tested on protein synthesis of *E. coli* O157:H7 ATCC 43895 strain. The addition of 1 mM carvacrol during overnight incubation caused significantly high amount of heat shock protein 60 (HSP60) and inhibited the flagellin synthesis which is causing cells to be aflegallate and nonmotile (Burt et al., 2007).

The EOs of *Nandina domestica* Thunb extracted by hydrodistillation and analyzed by GC-MS. The oil has 79 different compounds such as 1-indolizino carbazole, 2-pentanone, monophenole, aziridine, methylcarbinol, ethanone, furfural, 3, 5-dimethylpyrazole and furanone. The oil (1000 ppm/disc), and different organic extracts of hexane, chloroform, ethylacetate and methanol (1500 ppm/disc) showed strong antibacterial effects with zones of inhibitions (9-18 and 7-13 mm) and MIC values (62.5 to 1000 and 250 to 2000 µg/ml), respectively against *B. subtilis* ATCC6633, *L. monocytogenes* ATCC19166, *S. aureus* KCTC1916 and ATCC6538, *P. aeruginosa* KCTC2004, *S. typhimurium* KCTC2515, *S. enteritidis* KCCM12021, *E. coli* O157-Human, *E. coli* ATCC8739, *E. coli* O157:H7 ATCC43888 and *Enterobacter aerogenes* KCTC2190 (Bajpai et al., 2008).

The antimicrobial activities of an essential oil of *Origanum minutiflorum* against 21 ciprofloxacin-resistant *Campylobacter spp* strains were tested by broth microdilution and agar well-diffusion methods. *O. minutiflorum* oil contains 29 components and major ones are carvacrol (73.9%) and *p*-cymene (7.2%) and on lower amounts of carvacrol methyl ether, heptadecanol and carvacryl acetate. The minimum inhibitory concentration (MIC) values for sensitive bacterial strains were in the range of 7.8-800 µg/ml and the essential oil of *O. minutiflorum* exhibited promising antibacterial activity against all of the tested ciprofloxacin-resistant *Campylobacter spp* (Aslim & Yucel, 2008).

The antimicrobial activity of the *Aloe vera* juice was investigated against a group of gram-positive and gram-negative bacteria by disc diffusion method. The research showed that *Aloe vera* has antimicrobial effect on *Mycobacterium smegmatis*, *Klebsiella pneumoniae*, *Enterobacter faecalis*, *Micrococcus luteus*, *Candida albicans*, and *Bacillus sphericus* (Alemdar & Agaoglu, 2009).

The antibacterial activity of ethanol, aqueous extracts, and EOs of cloves and cinnamon were analyzed for determination of antibacterial activity against 21 foodborne pathogens: *Listeria monocytogenes* (5 strains), *Staphylococcus aureus* (4 strains), *Escherichia coli* O157: H7 (6 strains), *Salmonella enteritidis* (4 strains), *Vibrio parahaemolyticus* and *Bacillus cereus* and 5 food spoilage bacteria: *Pseudomonas aeruginosa*, *P. putida*, *Alcaligenes faecalis*, and *Aeromonas hydrophila* (2 strains). Cloves and cinnamon extracts showed antibacterial activity against the test organisms with the MIC values for ethanol, aqueous extracts and EO from cloves and cinnamon ranged from 0.5 to 5.5 mg/ml and 1.0 to 5.0 mg/ml respectively. The high temperature was not affective for the antibacterial activity of cloves and cinnamon EOs. The highest antibacterial activity was at pH 5.0 for EO of cloves and cinnamon against most of the bacterial mixtures, but it was highest at pH 7.0 against *L. monocytogenes* (Hoque, Bari, M. Juneja, & Kawamoto, 2008). Terpenes, carvacrol, *p*-cymene and thymol components of oregano, savory, and thyme have antifungal and antimicrobial activities and therefore they gained interest because of their potential application in food safety (Bendahou et al., 2008; Naidu, 2000).

1.4.4.1.1 Using natural antimicrobials against pathogens in liquid food models. Clove oil and mint extract exerted strong activity at low concentration and mild heat without pulsed electric field in tomato juice (Nguyen & Mittal, 2007).

The effect of cinnamon (0% and 0.3%) in pasteurized apple juice was investigated against *S. typhimurium*, *Yersinia enterocolitica* or Enterotoxigenic *S. aureus* at 4 log CFU/ml and stored at 5°C and 20°C. Cinnamon was effective against *S. aureus* (1.2 log CFU/ml at 3 days) and *Y. enterocolitica* (0.3 log CFU/ml at 1 day). The killing effect was significantly more at 20°C for all three pathogens with ~4 log CFU/ml reductions in 1 day in *Y. enterocolitica* inoculated samples with and without cinnamon, and in *S. aureus* inoculated samples with cinnamon (Yuste & Fung, 2003).

Four EOs from clary sage, juniper, lemon and marjoram were tested against wild-type isolates of the food related yeasts *Geotrichum candidum*, *Pichia anomala*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* in malt extract medium, apple juice and milk. *S. pombe* was most sensitive yeast (MICs of 0.0625-0.125 µl/ml) and *G. candidum* was least sensitive (MICs of 0.5-2 µl/ml). Significant reduction was achieved only with highest EO concentrations (Tserennadmid et al., 2011).

The effect of a thermal treatment, applied previously, on the growth of *Bacillus megaterium* cells was investigated in a culture medium with and without presence of nisin, carvacrol and thymol. When thymol and carvacrol were combined at these high doses (0.6 mM) resulted in a further increase of the lag phase and significant decrease of the growth rate. The combination of carvacrol and/or thymol with a previous thermal treatment (killed 90 % of the population); the growth of the survivors was inhibited for at least seven days (Periago, Conesa, Delgado, Fernández, & Palop, 2006). The effect of high-intensity pulsed electric fields (HIPEF) with or without combination of citric acid or cinnamon bark oil in apple, pear, orange and strawberry juices against *Salmonella enteritidis* and *E. coli* 0157:H7 was evaluated. Both of these pathogens were reduced more than 5.0 log₁₀ units in orange juice by only HIPEF although

strawberry, apple and pear juices were pasteurized by combination of HIPEF with citric acid at 0.5, 1.5, 1.5%, respectively, or cinnamon bark oil at .05, 0.1 and 0.1%, respectively (Mosqueda-Melgar et al., 2008a).

The effects of different concentrations (0.01 to 15%) of thyme (*Thymus vulgaris*), peppermint (*Mentha piperita* L.), caraway seed (*Carum carvi*), fennel (*Foeniculum vulgar*), tarragon (*Artemisia dracunculus*), and pennyroyal (*Mentha pullegium*) EOs against *S. aureus* and *E. coli* was investigated in nutrient broth medium. Thyme EOs exerted the broadest spectrum of antibacterial effect while EOs of peppermint, caraway seed, pennyroyal and fennel showed moderate effect on tested microorganisms and in contrast, tarragon essential oil was less efficient against both of the microorganisms (Mohsenzadeh, 2007).

After inoculation of at a level of 4 log CFU/ml into unpasteurized apple juice *E. coli* O157:H7 could survive up to 3 days at 25°C and 19 days at 4°C. The addition of 1.25 mM carvacrol or *p*-cymene into juice decreased the numbers of *E. coli* O157:H7 to undetectable levels in 1-2 days for both storage temperatures (Kisko & Roller, 2005).

The effects of garlic, bay, black pepper, Origanum, orange, thyme, tea tree, mint, clove, and cumin EOs on *Listeria monocytogenes* AUFE39237, *Escherichia coli* ATCC25922, *Salmonella enteritidis* ATCC13076, *Proteus mirabilis* AUFE43566, *Bacillus cereus* AUFE81154, *Saccharomyces uvarum* UUFE16732, *Kloeckera apiculata* UUFE10628, *Candida albicans* ATCC10231, *Candida oleophila* UUPP94365, and *Metschnikowia fructicola* UUPP23067 and effects of thyme oil at a concentration of 0.5% on *L. monocytogenes* and *C. albicans* in apple and carrot juice during +4°C (first to fifth day) were investigated. Thyme, Origanum, clove, and orange EOs have the most effective antibacterial and antiyeast activities

against these microorganisms. Cumin, tea tree, and mint oils inhibited the yeasts actively (Irkin & Korukluoglu, 2009).

The EOs extracted from lemon balm, marjoram, oregano and thyme and their MICs were investigated against *Enterobacter* spp., *Lactobacillus* spp., and *Pseudomonas* spp through the use of agar dilution method and/or the absorbance based microplate assay in food model media based on lettuce, meat and milk. MICs were significantly lower in beef and lettuce media than in TSB. Thyme and oregano were most active EOs and *Listeria* strains were more sensitive than other bacteria (Gutierrez et al., 2009).

Antimicrobial effect of cinnamon was investigated against *E. coli* O157:H7 in apple juice at 8°C and 25°C. *E. coli* O157:H7 was reduced by 1.6 log CFU/ml at 8°C and 2.0 log CFU/ml at 25°C by 0.3% cinnamon (Ceylan, Fung, & Sabah, 2004).

Baskaran, Amalaradjou, Hoagland, and Venkitanarayanan (2010) investigated the antibacterial effect of low concentrations of trans-cinnamaldehyde (TC) on five *Escherichia coli* O157:H7 strains in apple juice and apple cider. Initial inoculum level for a five strain mixture was ~6.0 log CFU/ml into apple juice or cider, followed by the addition of TC at 0, 0.025, 0.075, and 0.125 %v/v levels. The inoculated apple juice samples were incubated at 23°C and 4°C for 21 days, although the cider samples were stored only at 4°C. At 23°C, 0.125 and 0.075 %v/v TC completely inactivated *E. coli* O157:H7 in apple juice on days 1 and 3, respectively. At 4°C, 0.125 and 0.075 %v/v TC decreased the counts in the juice and cider to undetectable levels on days 3 and 5, respectively (Baskaran et al., 2010).

1.4.4.1.2 Using natural antimicrobials against pathogens in solid food models. The antimicrobial activity of essential oils of oregano, thyme, basil, marjoram, lemongrass, ginger and clove was tested *in vitro* by agar dilution method and MIC determination against gram-

positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and gram-negative strains (*E. coli* and *S. enteritidis*). MIC_{90%} values were determined against bacterial strains in irradiated minced meat and against natural microbiota in minced meat samples. MIC_{90%} values varied from 0.05%v/v (lemongrass oil) to 0.46%v/v (marjoram oil) to Gram-positive bacteria and from 0.10%v/v (clove oil) to 0.56%v/v (ginger oil) to Gram-negative strains. Nevertheless, the MIC_{90%} values on minced meat and natural microbiota were 1.3 and 1.0 respectively against tested microorganisms (Barbosa et al., 2009).

The physical properties and antimicrobial activities of allspice, cinnamon, and clove bud oils against *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in apple puree film forming solutions formulated into edible films at 0.5% to 3% (w/w) concentrations. The antibacterial activities against these three pathogens were in the following order cinnamon oil > clove bud oil > allspice oil. The results showed that apple-based films with allspice, cinnamon, or clove bud oils were active against these three main foodborne pathogens either with direct contact with the pathogens or by vapors emanating from films (Du et al., 2009).

Oregano and thyme incorporated soy protein edible films (SPEF) showed similar antibacterial activity against *E. coli*, *E. coli* O157:H7, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Lactobacillus plantarum* in inhibition zone test. Although *E. coli*, *E. coli* O157:H7 and *S. aureus* were significantly inhibited by these films, *L. plantarum* and *P. aeruginosa* seemed to be more resistant bacteria. SPEF with oregano, thyme, and oregano plus thyme did not have significant effects on total viable counts, lactic acid bacteria and *Staphylococcus* spp when applied on ground beef patties although reductions ($p < 0.05$) in coliform and *Pseudomonas* spp counts were determined (Emiroglu, Yemis, Coskun, & Candogan, 2010).

The EO of cloves (10%) and cinnamon (5%) were tested against cocktail of 5 strains of *L. monocytogenes* in ground chicken meat. EOs from cloves reduced all tested strains to undetectable levels within 1 day of exposure whereas EOs from cinnamon could only reduce by 2.0 log CFU/g within 1 day with only slight reductions or no additional decline in cell numbers for 15 days of incubation (Hoque et al., 2008).

The effect of carvacrol, cinnamaldehyde, thymol, and oregano oil on *Clostridium perfringens* growth and spore germination was investigated during chilling of cooked ground beef. The addition of test compounds ($\geq 0.1\%$) into beef completely inhibited *C. perfringens* spore germination and outgrowth ($p \leq 0.05$) during cooling of the cooked beef in 12 h. Greater concentrations were required for longer chilling times. Cinnamaldehyde was significantly more effective at a lower concentration (0.5%) at the most abusive chilling rate tested (21 h) than other compounds (Juneja, Thippareddi, & Friedman, 2006).

The addition of clove and tea tree essential oils at highest concentrations (three and four times MICs) was required to inhibit the growth of *E. coli* O157:H7 in blanched spinach and minced cooked beef, respectively. The antimicrobial effect was dependent on the oil concentration, the food composition and storage temperature (Moreira et al., 2007). The ethanolic extract of cinnamon bark (2% w/v) inhibited the growth of *Escherichia coli* O157:H7 and *Listeria innocua* by 94% and 87% respectively. When it combined with commercial antibrowning dipping solution (FreshExtend), the cinnamon bark extract (1% w/v) reduced the microbial growth significantly on apple slices stored for 12 d at 6°C in comparison to the control. The major constituent of this extract was cinnamic aldehyde (Muthuswamy, Rupasinghe, & Stratton, 2008). The antibacterial effect of clove oil applied to the surface of ready to eat (RTE) chicken frankfurters was evaluated on seven strains of *L. monocytogenes* at low (2-3 log) or high

(4-6 log) inoculum level and stored 2 weeks at 5°C or 1 week at 15°C. The growth of all seven *L. monocytogenes* strains was inhibited under either storage conditions with addition of 1% or 2% clove oil (Mytle, Anderson, Doyle, & Smith, 2006).

The effect of malic acid and EOs of cinnamon, palmarosa and lemongrass and their main active antimicrobial substances attached to an alginate-based edible coating on shelf life and safety of fresh-cut “Piel de Sapo” melon was tested. Malic acid was effective in increasing shelf-life of fresh-cut melon from microbiological (up to 9.6 days) and physicochemical (>14 days) aspects when compared with non-coated melon. Microbiological and physicochemical shelf life was up to 3.6 days and <14 days, respectively. When the palmarosa oil integrated into edible coatings at 0.3% concentration it seems to be a good alternative for fresh-cut melon, because it has been accepted by panelists, preserved fruit quality properties, inhibited the native microflora growth and provided significant reduction in number of *Salmonella enteritidis* (Raybaudi-Massilia et al., 2008).

The effect of storage temperature and EOs of oregano and nutmeg on survival and growth of *E. coli* O157:H7 in ready to cook traditional Iranian barbecued chicken. The EOs of oregano and nutmeg had no significant inhibitory effect against *E. coli* O157:H7 in broth system and ready to cook barbecued chicken (Shekarforoush, Nazer, Firouzi, & Rostami, 2007). The effect of oregano EO supplementation on lamb meat was tested. The results showed that incorporation of oregano EO into dietary supplement showed promising antioxidant activities retarding lipid oxidation in lamb meat during refrigerated and frozen storage (Simitzis et al., 2008). Thyme EOs at 0.3% concentration had a weak antibacterial activity against *E. coli* O157:H7 in TSB, although at 0.9% had negative impact on organoleptic properties of minced meat, at 0.6% showed inhibitory activity against *E. coli* O157:H7 during storage at 10°C only not

at 4°C. The addition of nisin into minced beef or TSB at 500 or 1000 IU/g did not exert any activity against pathogen (Solomakos, Govaris, Koidis, & Botsoglou, 2008).

1.4.4.2 Mechanism of action for antimicrobial activity. Antimicrobial effects of EOs are due to the functional and structural damaging to the cell membrane. Hydrophobicity is also related to the toxicity of EOs (Goni et al., 2009). The antimicrobial effect of herbs and spices can be stronger at 1000 ppm concentration whereas some of them need to be used at higher concentrations. But usually they are used in 500-1000 ppm range in food models (Ceylan & Fung, 2004).

1.4.4.2.1 Biological effects.

1.) Cytotoxicity: Carvacrol and thymol caused disintegration of outer membrane and releasing outer membrane associated materials from the cells, decreased the intracellular ATP pool while they increased the extracellular ATP and disrupted the cytoplasmic membrane (Helander et al., 1998). EOs seem to have no specific cellular targets (Carson, Mee, & Riley, 2002).

They can pass through cell wall and cytoplasmic membrane and disrupt the structure of phospholipids, fatty acids, and polysaccharides and permeabilize them. Cytotoxicity seems to comprise such membrane damage. The loss of ions, reduction of membrane potential, disruption of the proton pump and ATP pool reduction is related to the membrane permeabilization in bacteria. EOs can induce depolarization of mitochondrial membranes in eukaryotic cells. Cytoplasm can be coagulated and lipids and proteins can be damaged by EOs. Cell wall damage can cause leakage of macromolecules and lysis. EOs can cause leakage of radicals, cytochrome C, calcium ions and proteins same like bioenergetic failure and oxidative stress. Cell death occurs by apoptosis and necrosis as a result of permeabilization of mitochondrial membranes.

Chain reactions occurred in cell wall access to the whole cell through the membranes of mitochondria and peroxisomes and these effects suggest a phenolic-like prooxidant activity. Ultrastructural changes in plasma membrane, cytoplasm (swelling, shriveling, vacuolations, and leakage) and nucleus, strong decrease in unsaturated and an increase in saturated fatty acids, changes in cell envelopes were observed in cell. EOs could disrupt HSV viral envelope. Cytotoxic capacity of EOs depend on colony forming ability, their chemical composition, state of the cell growth and cytotoxic activity is mostly because of the presence of phenols, aldehydes and alcohols. These cytotoxic activities of EOs can be activated by light in mammalian cells and they can induce apoptosis and necrosis. There is no any reported specific resistance to EOs (Bakkali, Averbeck, Averbeck, & Idaomar, 2008).

Cell membrane structures were damaged by active ethanol and hexane extracts of oregano, clove, sage, rosemary and celery and increase in extracellular ATP was somehow related to the intracellular leakage (Witkowska et al., 2013). The EOs extracted from catmint (*Nepeta meyeri* Benth) had strong inhibitory activity and caused a variation in band intensity, loss of bands, and appearance of new bands compared with control in random amplified polymorphic DNA (RAPD) profiles (Kekec, Mutlu, Alpsoy, Sakcali, & Atici, 2013).

Piper gaudichaudianum Kunth EO and its major component nerolidol possess strong cytotoxic effects in mammalian V79 cells and its cytotoxicity is mainly associated with reactive oxygen species (ROS) and DNA single strand breaks caused by the oxidative lesions (Sperotto et al., 2013).

A significant correlation was shown between the concentrations of *Urtica dioica* EOs and chromosomal aberrations, micronuclei frequency, apoptotic cells, necrotic cells, and binucleated cells using cytogenetic tests such as cytokinesis block micronucleus assay and chromosomal

aberration analysis in human lymphocyte cultures in vitro (Gul, Demirci, Baser, Akpulat, & Aksu, 2012).

2.) Phototoxicity: Some EOs have photoactive molecules like furocoumarins. For example, *Citrus bergamia* EO contains psoralens and they can bind to DNA and produce mono- and biadducts that are highly mutagenic and cytotoxic under ultraviolet light exposure (Averbeck, Averbeck, Dubertret, Young, & Morlière, 1990). This EO is not toxic or mutagenic in the dark. Dijoux et al. (2006) showed that *Fusinus spicatus* wood EO was not phototoxic but was very cytotoxic. Phototoxic EOs penetrate the cell without damaging the membranes or proteins and DNA and cause damage to cellular macromolecules, proteins, and membrane lipids and they cause the formation of covalent adducts to DNA (Dijoux et al., 2006). Therefore studying EOs is interesting by testing its cytotoxic as well as possible phototoxic capacity (Bakkali et al., 2008).

3.) Nuclear mutagenicity: Most of the EOs do not induce nuclear mutations but there are some exceptions that should be mentioned (Bakkali et al., 2008). The EOs of *Artemisia dracunculus* was genotoxic against *Bacillus subtilis* in rec-Assay (Zani et al., 1991), the EOs from *Mentha spicata*, *Pinus sylvestris*, *Mentha piperita*, and *Anethum graveolens* in the *Drosophila melanogaster* somatic mutations and recombination test (SMART assay) (Franzios et al., 1997; Karpouhtsis et al., 1998; Lazutka, Mierauskien, Slapšyt, & Dedonyt, 2001).

Mentone from pepper mentha was genotoxic in SMART assay (Franzios et al., 1997), anethol from fennel and anise and asarone from *Acorus calamus* were active in Ames test (Goggelmann & Schimmer, 1983; Hasheminejad & Caldwell, 1994; Nestmann & Lee, 1983). The EOs extracted from *Origanum onites* L and *Origanum minutiflorum* O. Schwarz & P. H. Davis species of oregano were tested with SMART essay in *Drosophila melanogaster*. They

have no significant genotoxic effect but they have antigenotoxic activities induced by chrome and cobalt suggesting that they are antioxidant (Demir, Kaya, Marcos, Cenkci, & Cetin, 2013).

4.) Cytoplasmic mutagenicity: In these test systems mutagenicity was assessed by defects in energy metabolism and respiration. Tests in *Saccharomyces cerevisiae* yeast were very useful. The relation between mitochondrial damage and respiratory system changes was shown after treating yeast cells with tea tree EO (Schmolz et al., 1999). The EOs of cinnamon, clove, garlic, onion, oregano, and thyme delayed ethanol production in *Saccharomyces cerevisiae* cells (Conner, Beuchat, Worthington, & Hitchcock, 1984).

5.) Carcinogenicity: The EOs extracted from *Salvia sclarea* and *Melaleuca quinquenervia* stimulate the secretion of estrogen which can increase the cancers dependent to estrogen. Some EOs comprises photosensitizing molecules like porphyrins and flavins can cause skin cancer. EOs from *Citrus bergamia* contain psoralen, a photosensitizing molecule, which can increase the incidence of skin cancer under UV light or solar light (Averbeck & Averbeck, 1998; Averbeck et al., 1990). Pulegone is a compound found in EOs of mint spices can induce cancer with the production of *p*-cresol (Zhou, Koh, Gao, Gong, & Lee, 2004). Safrole, the main compound from *Sassafras albidum* and *Ocotea pretiosa* EOs provokes cancer in rodents (Burkey, Sauer, McQueen, & Sipes, 2000; Liu, Chen, Chang, Chu, & Liu, 2000).

1.4.4.3 Antioxidant activity of EOs. Antioxidant properties and stability during the storage of hexane solutions of 14 individual EOs from black and white pepper, cardamom, nutmeg, mace, juniper berry, fennel seed, caraway, dry cinnamon leaves, marjoram, laurel, ginger, garlic and clove bud have been studied. EOs of garlic, clove bud, ginger and leaves of cinnamon have the maximal efficiency of inhibiting hexenal oxidation (up to 93%), whereas black pepper oil has the minimal activity (49%) (Misharina, Terenina, & Krikunova, 2009).

The antioxidant activity of the EOs from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and clove (*Syzygium aromaticum*) was tested. The clove EO showed highest percentage inhibition of DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical (98.74%) and the highest FRAP (ferric reducing antioxidant power) value (1.47 TEAC). The thyme EO exerted highest percentage of inhibition of TBARS (thiobarbituric acid reactive species) (89.84%). The oregano EO had the highest antioxidant activity index in Rancimat test (Viuda-Martos, Navajas, Zapata, Fernández-López, & Pérez-Álvarez, 2010).

Ozcan and Arslan (2011) tested the antioxidant effects of EOs extracted from rosemary (*Rosmarinus officinalis*), clove (*Syzygium aromaticum*) and cinnamon (*Cinnamomum zeylanicum*) at 0.25% and 0.50% concentrations on hazelnut and poppy oils. The cinnamon oil was the most effective on retarding lipid oxidation of crude oils, which was followed by clove and rosemary oils. EOs of coriander (*Coriandrum sativum* L.) and caraway (*Carum carvi* L.) assayed for their *in vitro* and *in vivo* antioxidant activity and hepatoprotective effect against carbon tetrachloride (CCl₄) damage. Free radical scavenging activity (RSC) was measured on DPPH and OH radicals and effects on lipid peroxidation (LP) in two systems of induction as *in vitro* effects. For *in vivo* effects animals were pretreated with EOs and then intoxicated with CCl₄ to assess hepatoprotective effect. EOs could reduce the stable DPPH in a specific dose and neutralize hydrogen peroxide (H₂O₂) with 50% efficiency.

Caraway EO strongly inhibited LP in both induction systems although coriander EO showed prooxidant activity. Caraway EO appeared promising for safe use in pharmaceutical and food industries. Twenty-five EOs were tested for antioxidant activities using a conjugate diene (CD) assay, the aldehyde/carboxylic acid (A/C) assay, the DPPH free RSC assay, the

malonaldehyde/gas chromatography (MA/GC) assay, and lipoxygenase inhibitor screening (LIS) assay. Thyme oil showed the greatest antioxidant effect in all assays (80-100%) except in DPPH assay (60%). Cinnamon leaf oil exhibited strong activities in A/C assay (100%) and DPPH assay (84%). Bergamot oil exhibited 100% activity in A/C assay. Aloe vera oil showed the greatest lipoxygenase inhibitory activity (96%) followed by thyme oil (86%), and bergamot oil (85%) at a concentration of 0.5 µg/ml. Thyme and clove leaf oils contained high levels of eugenol (77%) and thymol (23%) (Wei & Shibamoto, 2010).

Antioxidant and antimicrobial activities of EO and methanol extract polar and non-polar subfractions of *Stachys inflata* were tested. EO and extracts were tested for their antioxidant activities using DPPH and β-carotene/linoleic acid assays and antioxidant potency of about 22% of that of butylated hydroxytoluene (BHT) ($IC_{50} = 19.72 \mu\text{g/ml}$) for polar subfraction (Ebrahimabadi et al., 2010).

Antioxidant activity and EOs composition of peel ethanol extract were analyzed in 18 local lemon cultivars by ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) method and GC/FID (Gas Chromatography/Flame Ionization Detector) analysis respectively. The main component in peel EO was limonene (72.5-76.4%) followed by β-pinene (11.6-18.7%). Cultivars with higher EO content and antioxidant activity were identified (Di Vaio et al., 2010).

The EO composition from *Rosmarinus officinalis* var. *typicus* and var. *trogodytorum* endemic to Tunisia was determined by GC and GC-MS. The oils' antioxidant activity, determined by DPPH assay, FRAP assay and β-carotene bleaching test was relatively high and strongest activity was found in EOs from var. *trogodytorum* and in one population of var. *typicus* from the upper semi-arid bioclimate (Zaouali, Bouzaine, & Boussaid, 2010).

Antioxidant activities of EO and methanolic extract from different myrtle (*Myrtus communis* var. *italica* L.) were tested by using DPPH RSC assay, β -carotene-linoleic acid bleaching, reducing power and metal chelating assays. In all assays methanolic extract showed stronger antioxidant activity than EOs (Jimenez-Castano, Villamiel, & Lopez-Fandino, 2007).

The antioxidant activity of EOs from rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) was evaluated as a free RSC together with the effect on LP. RSC was assessed by measuring the scavenging activity of EOs on DPPH and hydroxyl radicals and $IC_{50}=3.82$ μ g/ml for rosemary and 1.78 μ g/ml for sage dependent on applied doses. Strong inhibition of LP in both systems of induction was specifically determined for the EO of rosemary (Bozin, Mimica-Dukic, Samojlik, & Jovin, 2007).

Gortzi, Lalas, Chinou, and Tsaknis (2008) tested the antioxidant and antimicrobial activity of the methanol extract of *Myrtus communis* before and after encapsulation liposomes. Liposomes improved the performance of the product by increasing the solubility of ingredients, bioavailability and *in vivo* and *in vitro* stability. The extract exerted superior activity than α -tocopherol at concentrations up to 160 ppm.

Safaei-Ghomi, Ebrahimabadi, Djafari-Bidgoli, and Batooli (2009) investigated the antioxidant activity of EO and methanol extracts of *Thymus caramanicus* Jalas and its main compound carvacrol. The highest RSC activity in DPPH test was shown by the polar subfraction of the methanol extract ($IC_{50}=43.0$ μ g/ml) which was also higher than BHT (BHT, $IC_{50}=19.7$ μ g/ml). The residue EOs (25-200 μ g/ml) extracted from the residues of *Artemisia scoparia* Waldst & Kit by hydrodistillation showed a strong antioxidant and RSC activity against hydroxyl ion ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2). The chemical composition of EO was analyzed by GC/GC-MS and 48 compounds were identified out of 49 compounds present in the

oil. The volatile oil contained 24 monoterpenoids, 19 sesquiterpenoids, 2 ketones, 1 ester, 1 chromene and a hydrocarbon compound (Singh, Mittal, Kaur, Batish, & Kohli, 2009).

1.4.4.4 Synergistic and antagonistic effects of treatments and EO components.

Combination of ethanol extracts of oregano with rosemary or sage possessed some additive antimicrobial effects against *Listeria innocua* or *Staphylococcus aureus* (Witkowska et al., 2013). The combination of rosemary and sage can increase antibacterial effects which are desirable to develop nutraceutical products and controlling bacterial growth and rancidity (Chan et al., 2012).

When carvacrol is mixed with thymol at certain amounts, total inhibition effect by oregano EO can be exerted due to membrane damage that affects the equilibrium of pH and inorganic ions (Helander et al., 1998).

The combination of eugenol (2 mg/ml) and soy lecithin (50 mg/ml) suppressed the toxic effects of eugenol on apples and decreased the disease incidence of *Penicillium expansum*, *Phlyctema vagabunda*, *Botrytis cinerea*, and *Monilinia fructigena* to less than 7, 6, 4 and 2% respectively after 6 months of storage at 2°C (Amiri et al., 2008).

Combinations of juniper and clary sage EOs resulted in additive effects against *Saccharomyces cerevisiae* and *Geotrichum candidum*. The combination of α -pinene and limonene led to synergism, while the combination of α -pinene and linalool exerted stronger anti-yeast activities (Tserennadmid et al., 2011). The effective concentration (1.25 mM) of carvacrol against *E. coli* O157:H7 in unpasteurized apple juice could be reduced even further by combining it at 0.5 mM with *p*-cymene at 0.25 mM (Kisko & Roller, 2005).

The antimicrobial effects of EOs obtained from lemon balm, marjoram, oregano and thyme against *Listeria* spp and other spoilage bacteria in food model based on lettuce, meat and

milk. The combination of lemon balm and thyme exerted additive activity against *Listeria* strains, and combined oregano and thyme showed additive activities against *Enterobacter* spp., *Lactobacillus* spp., and *Pseudomonas* spp. The greater efficiency was obtained at pH=5.0 and 2.32% sugar, but sugar concentrations above 5% did not impact EO efficacy negatively (Gutierrez et al., 2009). Inactivation of *Escherichia coli* O157:H7 by heating in citrate phosphate buffer and apple juice were investigated, and the effect of the combination of heat treatments with citral was determined. Three log units of initial *E. coli* O157:H7 population was inactivated in both media after 27 min at 54°C. The addition of 18 and 200 ppm of citral into treatment medium acted synergistically with heat at 54°C to inactivate 3×10^4 and 3×10^7 cfu / ml, respectively. The addition of citral caused reduction of time required to inactivate 1 log unit of the initial *E. coli* O157:H7 population from 8.9 to 1.7 min (Espina, Somolinos, Pagan, & Garcia-Gonzalo, 2010).

The antimicrobial efficiency of lemongrass, cinnamon leaf, and basil EOs and freeze-thaw treatment, alone or in combination, against *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Enteritidis was investigated in strawberry juice at 7°C. EOs of lemongrass or cinnamon leaf at 0.1 to 2 µL / ml and freezing at -23°C for 1 or 2 days by thawing at 7°C for 4 h all showed significant antibacterial activities against *E. coli* O157:H7 and *S. enteritidis* in strawberry juice. The increasing in EO concentration and storage time increased antimicrobial activity, but the extension of freezing time from 1 day to 2 days did not increase the antimicrobial activity of freezing. The combination of freeze-thaw treatment with EOs enhanced the overall antimicrobial effect against *E. coli* O157:H7, with adding EO before the freeze-thaw treatment showed a faster inactivation rate than when added EO after the freeze-thaw. EOs of lemongrass and cinnamon leaf at 0.1 or 0.3 µl/ml followed by the freeze-thawing resulted in a 5

$\log_{10}$ reduction in *E. coli* O157:H7 on the fifth and second day of storage respectively (Duan & Zhao, 2009). Antimicrobial effects of cinnamon, sodium benzoate, potassium sorbate, and their combinations were investigated against *E. coli* O157:H7 in apple juice at 8°C and 25°C. At 8°C, 5.2 log CFU/ml of *E. coli* O157:H7 was eliminated in 11 days by 0.3% cinnamon with 0.1% sodium benzoate, and in 14 days by 0.3% cinnamon with 0.1% potassium sorbate. At 25°C, 5.3 log CFU/ml *E. coli* O157:H7 was eliminated in 3 days by the same combinations (Ceylan et al., 2004).

The efficacy of basil, lemon balm, marjoram, oregano, rosemary, sage and thyme EOs in combination was determined and the effect of food ingredients on their efficacy was investigated against *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas aeruginosa* using the spot-on-agar test. All the oregano combinations showed additive efficacy against *B. cereus*, and oregano combined with marjoram, thyme or basil also had an additive effect against *E. coli* and *P. aeruginosa*. The mixtures of marjoram or thyme also displayed additive effects in combination with basil, rosemary or sage against *L. monocytogenes*. Starch and oils at concentrations of 5% and 10% had a negative impact on the EO efficacy. On the contrary, the EOs were more effective at high concentrations of protein, and at pH 5, by comparison with pH 6 or 7 (Gutierrez, Barry-Rian, & Bourke, 2008). The combination of thyme EO at 0.6% and 500 IU/g nisin possessed an additive inhibitory activity against *E. coli* O157:H7 and it was higher during storage at 10°C than at 4°C (Solomakos et al., 2008). The combination of carvacrol with the nisin caused an increase in reducing the growth of *Bacillus cereus* and *Listeria monocytogenes*. They added lysozyme as a third preservative component also improved the synergism between nisin and carvone (Naidu, 2000; Pol & Smid, 1999).

1.5 Nanodispersion and Nanoencapsulation of EOs for Enhanced Antimicrobial

Effectiveness

1.5.1 Challenges in applying lipophilic antimicrobials in foods. Plant-derived EOs are generally recognized as safe (GRAS) by consumers, which accept well the use of them in food preservation (Militello et al., 2011). Nevertheless, EOs are soluble in ethanol but only partially soluble in water up to 0.01 % or 1.0 g/l (Burt, 2004).

This brings out an issue when these compounds are used in complex food models as natural antimicrobials. Fat and protein content of the foods may possibly bind with EOs such as thymol and eugenol and antimicrobial efficiency will be lost or reduced. Eugenol may be required to add >1% in cheese and meat systems (Tassou, Drosinos, & Nychas, 1995).

Although EOs perform well in antibacterial assays in vitro, more EO is needed to see the same antibacterial effect in food samples. The fat, protein, water, antioxidant, preservative, salt content and pH of the food can influence bacterial sensitivity of EOs with extrinsic factors such as temperature, packaging in vacuum/gas/air, and characteristics of microorganisms. The antimicrobial effect of EOs seems to be increased with a decrease in pH and storage temperature of the food and the amount of oxygen used for packaging. High levels of fat and/or protein content of foodstuffs protect the bacteria from the action of EOs (Burt, 2004). When the EO concentrations are increased up to higher levels required to inhibit or inactivate microorganisms, they may exceed regulatory levels and change the sensory threshold levels with intense color, flavor and appearance impacts on foods. EO compounds are highly volatile and aromatic and they change the flavor profile of the food and make them compatible with the food matrix and sensory properties of the end product essential when they are added into food products. Lettuce samples treated with 1.0 g/l thymol and lemon balm were not acceptable by panelist in sensory

evaluation. Therefore there is an interest in attaching EO compounds in protective matrices as a solution (Gutierrez et al., 2008; Shah, 2011).

1.5.2 Encapsulation process. Encapsulation is used for delivery of food ingredients within capsules when the direct addition of food ingredient causes the risk for the quality of food product. These capsules are classified as nanocapsules if the sizes of these capsules are less than 100 nm while sizes of microcapsules are measured in microns. Nano- and micro-structured clusters comprising emulsion systems produced with food-grade ingredients including food biopolymers (proteins, carbohydrates), fats, low molecular weight surfactants and co-polymers (protein-carbohydrate conjugates) have been used to deliver functional ingredients into food products (Augustin & Hemar, 2009).

1.5.2.1 The design of encapsulated food ingredients. Encapsulated functional ingredients have to be designed by thinking its end application because functionality is dependent to its end application. Encapsulation is specifically used to protect and maintain quality and stability of functional food ingredients in a food product and it is required to overcome physical or chemical instability of the functional ingredients, undesired interactions of the ingredient with other components of the food matrix or early release of flavor or bioactive ingredients. It is important to know the purpose of encapsulation and the mechanism for releasing ingredients. Because this information helps to design encapsulated ingredients from the appropriate encapsulant matrix and formulation to choose the appropriate encapsulation process to produce a structure that protects the core and respond to external stimulus for release of the functional or bioactive ingredient (Augustin & Hemar, 2009).

1.5.2.2 The active core. The flavoring agents, food acids and bases, lipids, food additives, minerals, colors, and vitamins are among the traditional encapsulated food ingredients. Recently,

the encapsulation of bioactive ingredients, specifically omega-3 oils, phytonutrients, and probiotics gained attention due to their health benefits. The stability of food ingredient core in microcapsule is dependent upon the chemical structure, molecular weight, polarity and volatility of food ingredient (Augustin & Hemar, 2009).

1.5.2.3 Matrix materials for encapsulation. The materials used as encapsulant can be selected from natural biomaterials or allowed food additives with generally recognized as safe (GRAS) status. Commonly used ingredients in encapsulation are food biopolymers (proteins, carbohydrates), fats, low molecular weight surfactants and co-polymers. Their emulsifying properties and capacity to form gel networks and viscosity make them useful matrix materials (Augustin & Hemar, 2009).

1.5.2.3.1 Food proteins. Soy proteins, milk proteins-caseins and whey proteins, egg proteins, zein or hydrolysates of these proteins are used as encapsulant matrices. Due to their amphiphilic nature, proteins tend to self-assemble. Their assembly into gelled structures can be triggered by acidification which neutralizes the charge of the protein as the pH reaches the isoelectric point (pI) of the protein, or by heating which causes unfolding and exposure of hydrophobic groups. Proteins can assemble at interfaces and they may serve as an effective transporter of bioactive molecules because of their ligand binding features. Another milk protein, β -lactoglobulin, has high affinity for hydrophobic molecules such as fatty acids and retinol. Heating stimulates the production of protein-carbohydrate conjugates by the Maillard reaction. The Maillard reaction affects the solubility, gelling and emulsifying properties of proteins. The protein in emulsions is anchored at the interface and the carbohydrate protrudes into solution and this enhances the colloidal stability of the protein-based emulsion (Augustin & Hemar, 2009).

1.5.2.3.2 Food carbohydrates. Sugars (*e.g.*, glucose, sucrose, oligosaccharide, glucose syrup) and polysaccharides (*e.g.*, starch and starch products- low and high-amylose starch, dextrins; non-starch polysaccharides- alginate, pectin, carrageenan, gum Arabic, chitosan, cellulose derivatives, cyclodextrin) are generally used as components of encapsulant matrix. Food carbohydrates can produce glassy solids where they can give structural support to delivery system. Based on their structures, they are able to bind specific molecules (Augustin & Hemar, 2009).

Starch and maltodextrins have applications as encapsulant matrices. Maltodextrins are useful to produce dehydrated encapsulated systems. Maltodextrins provide structural integrity to the final product and their incorporation in place of simple sugars in formulations reduces stickiness during drying. There are two main polysaccharides in starch: amylose and amylopectin. The majority of food carbohydrates are not surface active except for gum arabic and they have to be used by combining with other ingredients with high emulsifying capacity. Gum arabic is surface active in addition to providing structural stability to both and wet dry encapsulation systems (Augustin & Hemar, 2009).

1.5.2.3.3 Lipids. Lipids (*e.g.*, natural fats and oils, monoglycerides, diglycerides, phospholipids, glycolipids, waxes-beeswax and carnauba wax) may be used for encapsulation. The majority of food fats (*e.g.*, milk fat, soybean oil, cocoa butter) have triglycerides as a major component (~98%). These non-polar lipids carry lipophilic bioactive molecules in emulsion systems. Active cores can be embedded in solid fat matrix and released with increasing temperature. Fats are protective moisture barriers as encapsulant matrices in systems. Polar lipids (*e.g.*, monoglycerides, phospholipids, glycolipids) are surface active due to their amphiphilic

character and they can be used for stabilizing emulsions including active food ingredients (Augustin & Hemar, 2009).

1.5.3 Encapsulation techniques. Several different processes can be used for encapsulation of functional ingredients. Physical and chemical characteristics of the core and the elements that control the interfacial and assembling properties of the matrix materials is required to decide on suitable encapsulation process for designing encapsulated ingredients (Augustin & Hemar, 2009).

1.5.3.1 Spray drying. Spray drying is a well-established and commonly used encapsulation process in food industry since it is more cost-effective than others. It is 30-50 times cheaper than freeze drying. Dried ingredients are valuable because of their availability and shelf life stability. To produce a spray dried encapsulated ingredient, the basic process includes dissolving the core in a dispersion of the encapsulant matrix. The dispersion is atomized and exposed heated air for rapid drying of droplets. The powder particles are then separated from the drying air (Augustin & Hemar, 2009; Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007).

Encapsulant matrix needs high solubility in water. Desirable features are forming high solids at low viscosity, and good film and emulsifying capacity. Hydrophobic core materials are usually dissolved in oil phase and oil-in-water emulsions while hydrophilic cores are dispersed in aqueous phase comprising water soluble encapsulant wall material (see Table 3). Hydrophobic core material is coated by interfacial membrane after drying and it can be released by disruption the integrity of this membrane. Spray drying is used for generation of many encapsulated ingredients such as vitamins, minerals, flavors, enzymes, polyunsaturated oils, and probiotic

microorganisms. It may be used for heat sensitive and volatile ingredients (Augustin & Hemar, 2009; Gharsallaoui et al., 2007).

Table 3

Recently Used Wall Materials for Encapsulation of Different Food Ingredients by Spray Drying

Encapsulated ingredient	Wall Materials and Stabilizers	References
Oregano, citronella and marjoram flavors	Whey proteins/ Milk proteins	Baranauskiene, Venskutonis, Dewettinck, and Verhe (2006)
Cardamom oleoresin	Gum arabic /modified starch/ maltodextrin	Krishnan, Bhosale, and Singhal (2005)
Bixin	Gum arabic /Maltodextrin /sucrose	Barbosa, Borsarelli, and Mercadante (2005)
D-Limonene	Gum arabic/Maltodextrin/Modified starch	Soottitantawat et al. (2005)
Cumin oleoresin	Gum arabic/Maltodextrin/Modified starch	Kanakdande, Bhosale, and Singhal (2007)
Paprika oleoresin	Gum arabic/Soy Protein Isolate	Rascon, Beristain, García, and Salgado (2011)
Black pepper oleoresin	Gum arabic/Modified starch	Shaikh, Bhosale, and Singhal (2006)
Short chain fatty acid	Gum arabic/Maltodextrin	Teixeira, Andrade, Farina, and Rocha-Leao (2004)
Extra virgin olive oils	Gum arabic/Maltodextrin/Sodium Caseinate/Starch/Lactose/Gelatin	Calvo, Hernandez, Lozano, and Gonzalez-Gomez (2010)
Fish oil	Maltodextrin combined with Whey Protein Concentrate or Modified Starch	Jafari, Assadpoor, Bhandari, and He (2008)
	Caseinate glycated with Glucose, Glucose syrup or Dextran	Drusch et al. (2009)
	Barley protein	Wang, Tian, and Chen (2011)
Conjugated linoleic acid	Whey Protein Concentrate / Whey Protein Isolate / Maltodextrin / Maillard reaction products of Whey Protein Isolate and Maltodextrin	K.-O. Choi, Ryu, Kwak, and Ko (2010)

Table 3

(Cont.)

Encapsulated ingredient	Wall Materials and Stabilizers	References
Flaxseed oil	Gum arabic	Tonon, Grosso, and Hubinger (2011)
	Maltodextrin / Gum Arabic / Whey Protein Concentrate / Modified Starches	Carneiro, Tonon, Grosso, and Hubinger (2013)
Polyphenols and Anthocyanins from juice and ethanolic extracts of pomegranate	Soybean protein isolates / Maltodextrin	Robert et al. (2010)
Probiotic (<i>Lactobacillus rhamnosus</i> GG) preparations	Whey Protein Isolate / Maltodextrin / Glucose / Inulin	Ying, Sun, Sanguansri, Weerakkody, and Augustin (2012)
Probiotic (<i>Lactobacillus plantarum</i>)	Regular and denatured Whey Protein Isolates with Sodium Alginate	Rajam, Karthik, Parthasarathi, Joseph, and Anandharamakrishnan (2012)
Probiotic (<i>Bifidobacterium</i> Bb-12)	Whey Protein	De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, and Sant' Anna (2012)
Chia essential oils	Whey Protein Concentrate / Mesquite gum / Gum arabic	Rodea-Gonzalez et al. (2012)
Cinnamon oleoresin	Gum arabic / Maltodextrin / Modified starch	Vaidya, Bhosale, and Singhal (2006)
Phospholipid	Maltodextrin blended with Sodium Caseinate, Gelatin, and Soy Protein	Yu, Wang, Yao, and Liu (2007)
Lycopene	β -cyclodextrin	Nunes and Mercadante (2007)
Thymol	Whey Protein Isolate / Maltodextrin conjugate	Shah, Ikeda, Davidson, and Zhong (2012)
Eugenol	Whey Protein Isolate / Maltodextrin conjugate	Shah, Davidson, and Zhong (2012a)
Lipase enzyme isolated from endophytic fungus <i>Cercospora kikuchii</i>	Maltodextrin / β -cyclodextrin	Costa-Silva, Said, Souza, and Oliveira (2010)
Elderberry (<i>Sambucus nigra</i> L.) juice	Maltodextrin / Gum Acacia / Soya Milk Powder / Soya Protein Powder / Isolated Soya Protein	Murugesan and Orsat (2011)

Table 3

(Cont.)

Encapsulated ingredient	Wall Materials and Stabilizers	References
Fresh ginger oil	Maltodextrin / Whey Protein	Toure, Lu, Zhang, and XuemingToure et al. (2011)
Thymol, Geraniol	β -cyclodextrin / Modified Starch	Mourtzinis, Kalogeropoulos, Papadakis, S. Konstantinou, and KarathanosMourtzinis et al. (2008)

Adapted from Gharsallaoui et al. (2007) and updated with recently made researches.

1.5.3.2 Spray cooling. Active core dispersed in liquefied matrix is atomized into a cool environment such as cool air. Generally high melting fats are used as the matrix material. Fat solidifies during cooling and the core is immobilized. Water soluble functional ingredients such as enzymes, flavors and food acids are incorporated into solid fat particles (lipospheres) for delaying the release of core material. These ingredients are applied in dry products (Augustin & Hemar, 2009).

1.5.3.3 Freeze drying. Freeze drying can be used for ingredients which are very sensitive to heating processes. Commercial application of freeze drying is limited for high value ingredients such as probiotics. Spray drying process is cheaper and faster than freeze drying (Augustin & Hemar, 2009).

Table 4

Recently Used Wall Materials for Encapsulation of Different Food Ingredients by Freeze Drying

Encapsulated ingredient	Wall Materials and Stabilizers	References
Turmeric oleoresin	Maltodextrin / Gelatin	Malacrda, Ferreira, Telis, and Vania (2013)
Fennel oleoresin	Gum Arabic Mixtures	Chranioti and Tzia (2013)

Table 4

(Cont.)

Encapsulated ingredient	Wall Materials and Stabilizers	References
Biofilm-like <i>Lactobacillus rhamnosus</i> probiotics	Chitosan / Alginate / Carrageenan	Cheow and Hadinoto (2013)
Garcinia fruit extract	Whey Protein Isolate / Maltodextrin	Ezhilarasi, Indrani, Jena, and Anandharamakrishnan (2013)
Potassium norbixinate and Curcumin	Maltodextrin	Sousdaleff et al. (2012)
Limonene	Gum Arabic / Sucrose / Gelatin	Kaushik and Roos (2007)
Argentine red wine (Cabernet Sauvignon) polyphenols	Maltodextrin	Sanchez, Baeza, Galmarini, Zamora, and Chirife (2011)
Lycopene extract from tomato pulp	Gelatin / Poly (γ -glutamic acid)	Chiu et al. (2007)
Flavourzyme enzyme	Chitosan / Alginate / Poly L-lysine	Anjani, Kailasapathy, and Phillips (2007)
Fish oil	β -cyclodextrin / Polycaprolactone	M.-J. Choi, Ruktanonchai, Min, Chun, and Soottitantawat (2010)
Curcumin	Hydrophobically Modified Starch (HMS)	Yu and Huang (2010)
Thymol, Geraniol	β -cyclodextrin / Modified Starch	Mourtzinis et al. (2008)
Vacuum dried pineapple pulp powder	Maltodextrin / Gum Arabic	Gabas, Telis, Sobral, and Telis-Romero (2007)
Curcumin	Pluronic tri-block copolymer micelles	Sahu, Kasoju, Goswami, and Bora (2011)
	Baker's yeast (<i>Saccharomyces cerevisiae</i>) cells / β -cyclodextrin / Modified Starch	Paramera, Konteles, and Karathanos (2011)
Probiotic lactic acid bacteria (<i>Lactobacillus spp</i>)	Bacterial cellulose (<i>nata</i>) / Skim milk / Calcium alginate	Jagannath, Raju, Bawa (2010)
Probiotics (<i>Lactobacillus</i> , <i>Bifidobacterium</i> and <i>Lactococcus</i> species)	Alginate / Lecithin / Starch	Donthidi, Tester, and Aidoo (2010)
Prebiotic and Probiotics (<i>Lactobacillus gasseri</i> and <i>Bifidobacterium bifidum</i>)	Chitosan / Calcium alginate beads	Chávarri et al. (2010)

Table 4

(Cont.)

Encapsulated ingredient	Wall Materials and Stabilizers	References
Cinnamaldehyde, Thymol	β -cyclodextrin	Cevallos, Buera, and Elizalde (2010)
Methanolic extract of <i>Hypericum perforatum</i> (St John's wort)	β -cyclodextrin	Kalogeropoulos, Yannakopoulou, Gioxari, Chiou, and Makris (2010)
Eugenol	β -cyclodextrin	Seo, Min, and Choi (2010)
Olive leaf extract	β -cyclodextrin	(Mourtzinou, Salta, Yannakopoulou, Chiou, and Karathanos, 2007)
β -carotene	Mannitol matrix	Sutter, Buera, and Elizalde (2007)
	Regular and Modified Pinhão starch / Gelatin	Spada, Marczak, Tessaro, and Noreña (2012)
Trehalose	Thermally responsive Pluronic nanocapsules	Zhang, Rong, Wang, and He (2009)
Egg Yolk Immunoglobulin (IgY)	Chitosan / Alginate	X.-Y. Li et al. (2007)

1.5.3.4 Fluidized bed coating. This technique was originally developed as a pharmaceutical technique and now it is applied in the food industry to increase efficiency of functional ingredients and additives. Food technologists should consider more about the cost of this expensive technology. Top spray film coating of food ingredients seems more feasible than other fluidized bed coating methods (Dewettinck & Huyghebaert, 1999). This process contains spraying a coating solution into a fluidized bed of solid particles. A continuous coating film formed after a few cycles of wetting-drying processes. After several cycles of wetting–drying, a continuous film is formed (Guignon, Duquenoy, & Dumoulin, 2002). The solid particles are suspended in air and matrix material for coating core materials is sprayed onto particles. The matrix material can be dispersion or concentrated solution, a hot melt or an emulsion. This technique can be used to provide extra protection for spray dried powders (Augustin & Hemar, 2009). This technology is one of the few advanced technologies have capacity to coat solid

particles with any type of shell material such as polysaccharides, proteins, emulsifiers, fats, complex formulations, enteric coating, powder coatings, yeast cell extract, etc. Thus, the controlled release possibilities are remarkably more variable than any other encapsulation methods (Poshadri & Kuna, 2010). Fluidized bed coating is used to supply encapsulated versions of vitamin C, B vitamins, ferrous sulfate, ferrous fumarate, sodium ascorbate, potassium chloride, and variety of vitamin/mineral premixes. For bakery applications, products such as vitamin C, acetic acid, lactic acid, potassium sorbate, sorbic acid, calcium propionate and salt are encapsulated by using fluidized bed coating method. Encapsulated vitamin C is used as effective potassium bromated replacer. Several food acids have been fluid-bed encapsulated to develop color and flavor systems in meat industry. These acids are used to obtain a reproducible pH in cured meat products and decrease the processing time. To prevent rancidity, fluid-bed encapsulated salt is used in meats (Dewettinck & Huyghebaert, 1999).

1.5.3.5 Extrusion. Extrusion microencapsulation has been used especially for the encapsulation of volatile and unstable flavors in glassy carbohydrate matrices. This process provides the very long shelf life to oxidation-prone flavor compounds like citrus oils. Shelf life can be up to five years for extruded flavor oils while it is one year for spray dried flavors and a few months for unencapsulated citrus oils. Carbohydrate matrices in glassy states form very good barrier and extrusion method is very convenient for encapsulation of such flavors in carbohydrate matrices. Extrusion can be used for encapsulating nutraceuticals as well. Extrusion is most suitable process for glassy carbohydrates as shell materials (Poshadri & Kuna, 2010). Syringe extrusion is used for production of alginate beads and for the formation of alginate beads an alginate solution is extruded as droplets into a calcium chloride solution at lower temperatures. In another extrusion method called spinning disk, core materials suspended in a

matrix material solution passes over a rotating cylinder to form microparticles. Double capillary is used in centrifugal extrusion method. Core material is in the inner capillary and matrix material is on the outer capillary (Augustin & Hemar, 2009).

1.5.3.6 Microencapsulation based on supercritical fluids. In microencapsulation processes, the core material is dissolved in matrix material solubilized in a supercritical fluid such as carbon dioxide. The core material is encapsulated in matrix material by removing the carbon dioxide. The dispersion is sprayed through nozzle and a particulate material containing the core material is produced in a process called rapid expansion of supercritical solutions (RESS) (Augustin & Hemar, 2009). Romo-Hualde, Yetano-Cunchillos, González-Ferrero, Saiz-Abajo, and Gonzalez-Navarro (2012) obtained bioactive natural vitamins from red pepper (*Capsicum annuum* L.) by-products using supercritical fluid extraction and stabilized them with microencapsulation method.

1.5.3.7 Coacervation. Often called as phase separation is accepted as a real microencapsulation method and it involves the precipitation or separation of colloidal phase from an aqueous phase. Water soluble or a non-solvent polymer is used in simple coacervation and this polymer competes for solubilization of gelatin solution with hydrophobic interactions. The capsule is produced by ionic interactions between oppositely charged polymers in complex coacervation method. In complex coacervation usually protein molecules and anionic macromolecules such as gelatin and gum arabic carry positive charges. Coacervation comprises the separation of liquid phase of coating material from a polymeric solution right after coating of this phase as a uniform layer around suspended core material particles (Poshadri & Kuna, 2010).

A complex is formed when the opposite charge of biopolymer solutions is mixed. Biopolymer type, pH, ionic strength, concentration and the ratio of biopolymers have impact on the affinity of biopolymers and the structure of the complex formed. One phase or two phases can occur depending on biopolymers involved and different conditions. In two phase system, one of the phases has reduced both of the biopolymers whereas the other phase is enriched in both biopolymers in a precipitated form or complex coacervate. One of the restrictions in using of coacervates is their sensitivity against pH and ionic strength. To allow the permanence of coacervates, they may be cross-linked. Enzymatic cross-linkers such as transglutaminase are more acceptable than glutaraldehyde in food industry. Plant polyphenols recently used to cross-link gelatin-based coacervates (Augustin & Hemar, 2009).

Heat-resistant nanocapsules of jasmine essential oil were obtained via gelatin and gum arabic mixture using complex coacervation encapsulation method (Lv, Yang, Li, Zhang, & Abbas, 2014). Jun-Xia, Hai-Yan, and Jian (2011) microencapsulated sweet orange oil by complex coacervation with soybean protein isolate and gum arabic. Leimann, Gonçalves, Machado, and Bolzan (2009) use simple coacervation for microencapsulation of lemongrass (*Cymbopogon citrates*) EO known because of its broad-spectrum antimicrobial activity. The size of microcapsules was in the range of 10 μm to 250 μm . No agglomeration was observed in microcapsules when SDS (sodium dodecil sulfate) at 0.03 wt% was used. Complex coacervation with transglutaminase as hardening agent used to prepare the gelatin/gum Arabic microcapsules encapsulating peppermint oil. During the 40 days of storage of microcapsules in cold water only about 7% of peppermint oil was released, showing excellent storage stability (Dong et al., 2011).

Nori et al. (2011) tried to encapsulate propolis extract by complex coacervation using isolated soy protein and pectin as encapsulant agents. They were successful to obtain the

encapsulated propolis extract in a powdered form, alcohol free, stable protecting its antioxidant and antimicrobial activities and with the possibility of controlled release in foods. Eugenol was microencapsulated using gelatin/sodium alginate complex coacervation. Eugenol microcapsules had good flow properties and therefore improved handling. Treatment with dehydrating agent caused reduction in loading and percent encapsulation efficiency of eugenol microcapsules (Shinde & Nagarsenker, 2011). Martins, Rodrigues, Barreiro, and Rodrigues (2009) developed a novel coacervation method to produce microcapsules of polylactide (PLA) to encapsulate thyme oil. The new approach in their method was dissolving PLA in dimethylformamide (DMF) which is good solvent for PLA and highly soluble in water. The homogenous solution of PLA in DMF stimulates the precipitation of PLA around the thyme oil core. They achieved to form microcapsules with a mean particle size of 40 μm , 5 μm wall thickness, spherical shape, and rough surface. To optimize and control the kinetics of gelatin-acacia complex coacervation method for encapsulation of emulsified oil droplets. This study showed the formation of nano-microparticles could be highly related to kinetics of freezing process and particle properties can be adjusted by playing with the freezing operation (Nakagawa & Nagao, 2012).

Silva, Favaro-Trindade, Rocha, and Thomazini (2012) encapsulated oily dispersion of lycopene by complex coacervation using gelatin and pectin and characterized the properties of microcapsules. Although they formed microcapsules with high values of encapsulation efficiency, they could not stop the degradation of the lycopene during storage period. Gum Arabic-chitosan microcapsules containing commercially product Miglyol 812N (blend of triglycerides) as core phase were synthesized by complex coacervation. For the emulsion step, the optimum phase volume ratio chosen was 0.10 and an emulsion time of 15 min was selected.

The results showed that the optimum formation of these complexes appears at pH 3.6 and a weight ratio of chitosan to gum Arabic mixtures of 0.25 (Butstraen & Salaun, 2014).

Ozyildiz, Karagonlu, Basal, Uzel, and Bayraktar (2013) showed antimicrobial action of red pepper seed oil (RPSO) after ozonation and they microencapsulated it for the preparation of functional textile material as a first reported material in this field. They ozonated and microencapsulated RPSO by complex coacervation method using gelatin and gum arabic as wall materials.

1.5.3.8 Liposomes. Liposomes are spherical bilayer vesicles which are formed polar lipid dispersions in aqueous media. They have been used widely used in pharmaceutical industry in delivering of drugs, vaccines, hormones, enzymes and vitamins to their targets in the body but they have limited application in food industry. Phospholipids have commonly used to prepare uni-lamellar or multi-lamellar liposomes. Liposomes can range from 25nm to several microns in diameter, are easy to make and can be stored by freeze-drying. They are carriers for both lipophilic and hydrophilic molecules. The entrapped active materials are stabilized against environmental factors such as pH, temperature and ionic strength. The core material can be released when the gel to liquid transition temperature of phospholipids is reached. Cholesterol can be added to improve the resistance of liposomal system to degradation when it is exposed to *in vitro* and *in vivo* conditions. The composition of phospholipids determines the number of layers in liposomal systems. Fatty acids can also be used to form liposomes. Small lipophilic molecules can permeate through liposomal bilayer although sugars and large polar molecules cannot permeate (Augustin & Hemar, 2009; Poshadri & Kuna, 2010).

Liolios, Gortzi, Lalas, Tsaknis, and Chinou (2009) incorporated carvacrol and thymol isolated from EO of *Origanum dictamnus* L. in phosphatidyl choline-based liposomes and were

able to increase antimicrobial activity of these compounds after encapsulation against four Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus mutans* (ATCC 31989) and *Staphylococcus viridans* (ATCC 19952), four Gram negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047) and *Klebsiella pneumoniae* (ATCC 13883), three human pathogenic fungi: *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13801) and *Candida glabrata* (ATCC 28838), as well as against the food-pathogen, *Listeria monocytogenes* (11994). The antioxidant and antimicrobial activity of *Origanum dictamnus* methanol or dichloromethane extracts was increased after encapsulation in liposomes (Gortzi, Lalas, Chinou, & Tsaknis, 2007). Varona, Martin, and Cocero (2011) studied the corporation of lavandin (*Lavandula hybrida*) EO in liposomes formed with lecithins and cholesterol. They used thin-film method and particles from gas saturated solutions (PGSS) drying process to produce liposomes. Liposomes produced by thin-film method were multivesicular or unilamellar/multilamellar with a mean diameter of 0.4-1.3 μm and encapsulation of EO was carried out at 66% efficiency. The EO of Brazilian cherry (*Eugenia uniflora* L.) leaves was incorporated into multilamellar liposomes by dry film hydration without causing phase separation in the membrane structure (Yoshida, Yokota, Foglio, Rodrigues, & Pinho, 2010).

1.5.3.9 Molecular inclusion complexes with cyclodextrins. This technique is performed at a molecular level. Cyclic oligosaccharides of α -(1, 4) linked glucopyranose units, cyclodextrins, are used as a microencapsulation method due to their capacity to form molecular inclusion complexes. The interior part of cyclodextrins is hydrophobic whereas their exterior side is hydrophilic. The size of hydrophobic internal cavity that carries hydrophobic molecules is determined based on the number of glucose units. The α -cyclodextrin is a cyclic derivative of

starch made up of seven glucopyranose units. They are prepared from partially hydrolyzed starch by an enzymatic process. The internal cavity of about 0.65 nm diameter allows the inclusion of EO compounds and can take up one or more flavor volatile molecules. In this encapsulation method, the flavor compounds are entrapped inside the center of cyclodextrin molecule.

Complex formation with a hydrophobic molecule is driven due to displacement of water from the interior of cyclodextrin is energetically favorable. The formation of inclusion complexes increases the solubility of hydrophobic molecules in water and core ingredient is protected against degradation. Unexpected odors and flavors can be masked. The hydrophobic molecule is usually displaced by heating (Augustin & Hemar, 2009; Poshadri & Kuna, 2010).

1.5.4 Encapsulation of lipophilic antimicrobials. Functional ingredients (such as drugs, antimicrobials, vitamins) are rarely used in their pure form they are usually incorporated into delivery systems. The roles of delivery systems are 1) they serve as a vehicle for carrying the functional ingredient to the desired site of action, 2) they protect it from chemical or biological degradation during processing, storage, and utilization; this helps to keep it in active phase, 3) they control the release of functional ingredient, 4) they have to be compatible with the other components in the system, as well as being compatible with qualitative and physicochemical attributes (Weiss, Takhistov, & McClements, 2006). Finally the delivery system should be prepared with food-grade ingredients using simple, cost-effective processing operations if it will be used in food industry (McClements, Decker, & Weiss, 2007).

The delivery system characteristics influence the efficiency of functional ingredients in industrial products. Different types of delivery systems has been developed for encapsulating functional ingredients including simple solutions, association colloids, emulsions, biopolymer matrices and so on (Weiss et al., 2006). Delivery systems have advantages and disadvantages for

encapsulation, protection, and delivery of functional agents in terms of their cost, regulatory status, ease of use, biodegradability, biocompatibility and so on (McClements et al., 2007).

1.5.4.1 Nano versus microencapsulation. The majority of research has been conducted on the use of emulsion based delivery systems for encapsulation of lipophilic compounds such as flavors, fat-soluble nutrients and antimicrobial EOs (McClements et al., 2007; Shah, 2011). Glenn et al. (2010) encapsulated thymol, clove, origanum and camphor white EOs in high-amylose starch-based porous microspheres. They used spray drying method oil-in-water emulsions to produce capsules at size of 5 μm . The encapsulation properties of skimmed milk powder (SMP) and whey protein concentrate (WPC) for the coating of EO of oregano and aroma extracts of citronella and sweet marjoram by spray drying method were evaluated. SMP coated products had smoother surface while WPC contains more dents and wrinkles on the surface of capsules. Particle sizes ranged from 6 μm to 280 μm and 2 μm to 556 μm for SMP and WPC, respectively for microencapsulated products (Baranauskiene et al., 2006). Farhang (2009) used maltodextrin at different ratios for microencapsulation of elemi and peppermint oils by spray drying of emulsion in a laboratory dryer. Donsì, Annunziata, Vincensi, and Ferrari (2012) used algae oil in oil-in-water emulsions and stabilized with whey protein isolate. The oxidative stability of emulsion was increased by using EDTA at concentrations $\geq 1\mu\text{M}$ at both pH 3.0 and 7.0. Biopolymer materials such as chitosan, sodium alginate, and carrageenan have been used to produce stabilized and multilayered emulsions for additional protection of EOs (Perez, Carrara, Sanchez, Santiago, & Patino, 2009; Shah, 2011; Wang & Lucey, 2003). ElShafei, El-Said, Attia, and Mohammed (2010) made environmentally friendly pesticides using water-in-oil-in-water (W/O/W) type emulsions of EOs from eucalyptus, linalool, and marjoram stabilized with

xanthan gum. Solid lipid microparticles were designed to reduce volatility of the antimicrobial compound extracted from juniper berries (Gavini et al., 2005).

1.5.4.2 Milk proteins and carbohydrates as emulsifying agents. Different carbohydrate-based matrices such as starch, maltodextrin, gum arabic and milk protein-based matrices were used to encapsulate several lipophilic ingredients like EOs and flavor volatiles. Whey protein-maltodextrin conjugates are recently used as emulsifying agents. Because of their trend to be adsorbed at fluid interfaces these amphiphilic whey proteins can be used as perfect emulsifiers during encapsulation of lipophilic compounds. Bovine serum albumin, α -lactalbumin and β -lactoglobulin form about 60 % of total whey proteins, whereas lactoferrine and immunoglobulins are some of the minor ingredients. Because of their low cost and nutritional value using whey protein isolate and whey protein concentrate gained more interest for encapsulation.

Their applications in food industry are limited by environmental conditions such as thermal processing, ionic strength and pH. These proteins are denatured, unfolded and aggregated during heating above 60°C. Negatively charged carboxyl and positively charged amine groups are present in proteins and these charged groups are important during inter-particle repulsion. It is more effective around pH 5.0, which is close to their isoelectric point (pI) and the presence of high ionic concentrations. Inter-particle affinity is increased at the isoelectric point of proteins and this causes protein precipitation resulting in increased turbidity and solid gel formation in the system. These effects are usually seen during storage and it negatively impacts consumer's acceptability. Whey proteins have some unique benefits useful for their functional properties in food such as heat resistance and increased emulsifying capacity when they are processed together with food additives such as polysaccharides. However polysaccharides do not exhibit surface activities but they are extremely hydrophilic. Thus they contribute to stabilizing

and jellifying properties and rheology of the aqueous phase. For dispersion and foam stabilizing methods heat-induced protein-polysaccharide conjugates have been suggested. Proteins are conjugated with carbohydrates by dry heating mixtures to stimulate covalent coupling of proteins and carbohydrates also known as Maillard reaction (Akhtar & Dickinson, 2003, 2007; Dickinson, 2003; Kato, 2002; Shah, 2011).

Condensation occurs between unprotonated amine group from lysine residues of proteins and carbonyl group of carbohydrates without chemical catalysis at the early stage of Maillard reaction (Wannes et al., 2010). The functional properties of whey proteins such as solubility, formation of soluble aggregates, and interfacial properties can be improved by conjugating them with alginates, carrageenans, xanthan, and pectins. Functional properties of conjugates are dependent upon hydrophilic property, viscosity, and binding number of polysaccharides. The suitable properties of conjugates can be adjusted by protein: polysaccharide ratio, size and conformation of polysaccharides and proteins based on their applications in food industry (Shah, 2011). Whey protein isolate has been conjugated with hydrolyzed polysaccharides such as maltodextrin and used for encapsulating lipophilic ingredients comprising orange oil, ginger oil, avocado oil, and conjugated linoleic acid to make them resistant to oxidative stress, volatilization and improve their bioactivity (Akhtar & Dickinson, 2003; Bae & Lee, 2008; K.-O. Choi, Ryu, et al., 2010).

1.5.5 Nanoencapsulation of EOs for enhanced antimicrobial effectiveness. The antimicrobial activity of EO is remarkably lowered in foods because of the poor solubility in water that causes non-homogenous dispersion in food matrices (Gutierrez et al., 2008). They have high affinity to lipids, proteins and other food compounds because of their hydrophobicity and chemical structure. This tendency reduces their antimicrobial activity (Burt, 2004).

To assure targeted antimicrobial efficacy same like in growth media, EOs need to be used at higher concentrations in foods and this causes changes in sensory properties of foods and decrease consumers' acceptability (Weiss, Gaysinsky, Davidson, & McClements, 2009). For this reason, EOs are encapsulated with stabilizing materials such as gum arabic and starches (Kanakdande et al., 2007), and proteins (Gbassi et al., 2009). As one of the emulsion-based systems, nanoemulsions are dispersions of nanoscale droplets less than 100 nm formed with high shear application. The separation of these droplets may be carried out by microfluidization or ultra-sonication. Nanoemulsion systems require greater amount of surfactant when compared with microemulsions. Nanoemulsion of triglyceride oils can be achieved by using microfluidizers, low molecular weight surfactants and co-solvent for stability of the oil phase. Nanoemulsions are transparent systems whereas microemulsions scatter light. This property is important in food applications because lipophilic antimicrobials can be delivered in clear emulsions. Droplets have a smaller size and potential to increase the bioavailability of core material due to increased surface area when compared with conventional emulsion systems (Augustin & Hemar, 2009). Nanoencapsulation of thymol and carvacrol in liposomes (Liolios et al., 2009), eugenol (Shah et al., 2012a), thymol (Shah et al., 2012), terpenes mixture and D-limonene (Donsi, Annunziata, Sessa, & Ferrari, 2011) in nanocapsules was performed to enhance their antimicrobial activities. These processes need high energy use, expensive materials or involve a lot of steps. Microemulsions nanoscale systems require more surfactants and co-surfactants which are very expensive and under regulation, therefore their applications in food industry are limited (Shah, 2011).

The principals behind using emulsion-evaporation system to obtain food grade nanocapsules with encapsulated antimicrobials involve dissolving natural lipophilic

antimicrobial in a volatile organic solvent to form an oil phase its emulsification into aqueous phase containing dissolved Whey protein isolate (WPI) – Maltodextrin (MD) conjugates, followed by spray drying to remove hexane. Spray drying is widely used in food industry to stabilize volatile ingredients such as EOs during processing and storage periods and to increase bioactivity of encapsulated lipophilic compounds in food matrices because it is simple, fast and low cost method. Additionally, products in a powdered form are compatible for long term storage, handling and transportation. WPI-MD conjugates are selected as emulsifiers. Amphiphilic whey proteins adsorb onto the lipophilic EO phase and hydrophilic maltodextrin moiety can be solved in aqueous phase and this will improve dispersion capacity and reduce aggregation of particles because of steric hindrance (Shah, 2011).

1.5.5.1 Physicochemical properties. Shah et al. (2012) chose the thymol as a model lipophilic antimicrobial due to its strong antibacterial and antifungal activity. They used conjugate preparation method of Akhtar and Dickinson (2007) but modified heating temperature, drying method, MD chain lengths and WPI: MD ratios. Emulsions created with different WPI: MD ratios spray dried. Spray dried powders were loaded with thymol. Nanocapsules were hydrated at 5%w/v powder concentration in deionized water at room temperature (20°C) for 14h and adjusted to pH 3.0, 5.0, and 7.0 for characterization. They showed that optimization of emulsion preparation conditions and conjugation of WPI and MD can help minimize losses and improve the encapsulation efficiency after spray drying. When spray dried capsules were hydrated, an overall 0.525 % w/v overall thymol concentration was obtained for some nanodispersions, which is much higher than the solubility limit of thymol.

They could obtain some clear dispersion with an increase in visual clarity after heating at both salt concentrations. Samples made with a WPI: MD mass ratio of 1:2 produced dispersions

with higher clarity after heating. They showed formulations need to be optimized to achieve visual clarity, improved dispersion properties and heat resistance in dispersions. Most of the formulations used to prepare nanodispersions gave less than 100 nm capsules after heating at pH 3.0 and 7.0, at both salt concentrations. The improved dispersibility and thermal stability of these dispersions are highly relevant to conjugates possibly form core-shell structures where the MD shell allows for steric hindrance stabilizing particles. The presented scalable technology in their research may be used to disperse different types of lipophilic antimicrobial compounds in clear liquid forms, making an important contribution to functional beverage industry (Shah et al., 2012).

Shah et al. (2012a) used eugenol as a model compound for encapsulation in WPI: MD40 with 1:2 ratios. Emulsifying the oil phase of eugenol in hexane into an aqueous phase with dissolved conjugates and spray drying are the two main steps of encapsulation process. Following the hydration of spray dried powders, several samples containing eugenol above its solubility limit formed transparent dispersions at pH 3.0 and 7.0 after heating at 80°C for 15 min that corresponds to smaller than ca. 100 nm mean diameters of particles.

1.5.5.2 Antimicrobial activity in growth media. Shah (2011) dispersed thymol in nanocapsules obtained with WPI: MD180 conjugates at 1:2 ratios. The antimicrobial effects of nanodispersed (ND) and free thymol were tested against *E. coli* O157:H7, *Salmonella typhimurium*, *L. monocytogenes*, and *Staphylococcus aureus* in TSB adjusted to pH at 35°C for *E. coli* and 32°C for *L. monocytogenes*. They showed that the MIC for ND and free thymol against all tested strains at pH 6.8 was 0.5 g/l, except for *E. coli* ATCC 43889 and *Lm* strains Scott A which were inhibited by 0.3 g/l free oil. *Lm* was completely inhibited at this concentration at pH 5.5 after 3h, whereas *E. coli* showed 1-3 log CFU/ml reduction after 48 h.

The effect of temperature was not statistically significant. They were able to produce clear nanodispersions of thymol with promising antimicrobial activity against foodborne pathogens.

Shah, Davidson, and Zhong (2013) investigated the antimicrobial activities of ND eugenol (NE) and free eugenol (FE) against *E. coli* O157:H7 and *Lm* strains in TSB at 35 or 32°C. The antimicrobial efficacy of NE against all tested strains was not improved with MIC and MBC values being 0.25g/l higher than those of FE. FE performed better in TSB because there was no interfering compound and the MIC and MBC were below solubility of eugenol.

1.5.5.3 Antimicrobial activity in food systems. Shah, Davidson, and Zhong (2012b) compared the antimicrobial activities of free and ND thymol (NT) against *E. coli* O157:H7 and *Listeria monocytogenes* strains in apple cider and 2% reduced fat milk. Overall, 0.5 and 1.0 g/l thymol in nanodispersions and free thymol were inhibitory and bactericidal respectively, against all tested strains at all different pH and thymol concentrations. NT at 0.5 g/l concentration has bacteriostatic activity against *Lm* and *E. coli* for up to 48h at pH 5.5. At pH 3.5, *Lm* controls could not survive after 12h but *E. coli* survived and was inhibited by 0.5 g/l NT after 12h and 48h in apple cider. *E. coli* strains were sensitive to 4°C and pH 3.5. In 2% reduced milk at 35 or 32 °C NT and free thymol inhibited tested bacterial strains at 4.5 g/l concentration.

Shah et al. (2013) investigated the antimicrobial activities of ND eugenol (NE) and free eugenol (FE) against *E. coli* O157:H7 and *Lm* strains in milk with different fat levels (whole, 2% reduced fat and skim milk) at 35 or 32°C. NE was more effective than FE, with MIC and MBC values above the solubility of eugenol. The NE was thought to be equally dispersed in food matrices at concentrations above the solubility limit of eugenol and supplied the eugenol at specific locations when the binding to food compounds caused eugenol level decrease less than required amount for inhibition.

CHAPTER 2

Evaluation for Antimicrobial Effectiveness of Certain Essential Oils (EOs) with Combination of Copper and Lactic Acid to Control *E. coli* O157:H7 in Laboratory Medium

2.1 Introduction

Due to the recent outbreaks caused by foodborne pathogens, consumers are paying more attention to food safety. The CDC reported that each year roughly 48 million people get sick, 128,000 people are hospitalized, and 3,000 people die because of foodborne diseases. *E. coli* O157:H7 is the one of the leading causes of foodborne illnesses. *E. coli* O157:H7 can cause hemorrhagic colitis; bloody, persistent, and watery types of diarrhea; and hemolytic-uremic syndrome resulting in hospitalization in the United States (CDC, 2011; Nataro & Kaper, 1998).

Different preservation methods such as pasteurization, irradiation, vacuum packaging, and chemical preservatives have been used to control foodborne pathogens (Abdulummeen, Risikat, & Sururah, 2012). Consumers are not only paying more attention to foodborne pathogens but also to the use of chemical preservatives to control pathogens in food products (Gyawali, Ibrahim, Abu Hasfa, Smqadri, & Haik, 2011). Consumers demand minimally processed, safe, and shelf-stable foods (Misharina et al., 2009), and prefer natural ingredients.

EOs are one of the natural ingredients that became more popular in food industry. EOs have antibacterial and antioxidant properties and can be used in different applications to ensure food safety (Bassolé & Juliani, 2012). Imelouane et al. (2010) showed that *Artemisia herba-Alba* Asso. EO at 0.66mg/ml concentration inhibited the growth *E. coli* O157:H7 in TSB. Clove bud EO exerted bactericidal activity against *E. coli* O157:H7 with BA50 value of 0.13 (Friedman et al., 2002).

Recently our laboratory showed that the growth of *E. coli* O157:H7 was significantly inhibited when combination of low concentrations of copper and lactic acid used and found that copper and lactic acid can inhibit the growth of foodborne pathogens at low concentrations (Gyawali et al., 2011; Ibrahim, Yang, & Seo, 2008). These combinations are water soluble and low cost compared to EOs. Insolubility in water and strong aroma of EOs negatively impact sensory properties and limit the use of EOs in food products (Misharina et al., 2009). Copper and lactic acid combinations are relatively safe and have no interaction with the food components.

There is a need to establish a comparison of the combination of copper and lactic acid with EOs to ensure safety of food products and to provide to industry with practical approach to reduce the risk for foodborne pathogens. We hypothesized that (a) low concentration of copper and lactic acid will show significant antimicrobial activity against tested strains, and (b) the antimicrobial activity of low concentrations of copper in combination with lactic acid will be at least as much as free EOs exert. Therefore, the objective of this study was to evaluate the effectiveness of certain EOs and combinations of low concentrations of copper and lactic acid to control *E. coli* O157:H7 in laboratory medium.

2.2 Materials and Methods

2.2.1 Bacterial strains. Three strains of *E. coli* O157:H7 ATCC 700599 (salami isolate), ATCC 51659 (clinical isolate), and ATCC 43895 (raw hamburger meat isolate) were used in this study. Bacterial strains were transferred to fresh tryptic soy broth (TSB) before use. Cultures of each strain were grown separately in TSB (Difco, Becton Dickinson, Sparks, MD) at 37°C and transferred at 24h intervals into 9ml fresh brain heart infusion (BHI) broth.

2.2.2 Inoculum preparation. Overnight cultures of the strains were centrifuged (8000g, 15 min) and supernatant of each strain was decanted and the cell pellet was re-suspended

(approximately 8 log CFU/ml) in 0.1% (v/v) 10 ml peptone water. Serial dilutions were made to achieve an initial inoculum level of 2-3 log CFU/ml before inoculation into broth. To determine the effect of lactic acid, copper, and EOs on bacterial population in BHI broth, three strains of *E. coli* O157:H7 were used. Strains were prepared by mixing 100µl of each strain overnight in 10ml peptone water. Several serial decimal dilutions were then made to achieve an initial inoculum level of 2-3 log CFU/ml in 7 ml of BHI in test tubes.

2.2.3 Experimental design. The list of independent and dependent variables used in the design of this experiment are shown in Table 5. Armoise (*Artemisia alba*, armoise EO, Batch No.1444, Origin: Morocco, Aromatics Inc., Ontario, Canada) and clove bud (*Syzygium aromaticum*, Organic clove bud oil, Origin: Madagascar, Simplers Botanicals) EOs were purchased from local stores in Greensboro, NC and kept at 4°C for 24h.

Table 5

List of Independent and Dependent Variables

Independent Variables				Dependent Variables
Armoise and Clove EOs % (v/v)	Copper (Cu) (ppm)	Lactic acid (LA) % (v/v)	Cu and LA combinations	
0.006, 0.0125, 0.025, 0.05, 0.1, 0.15, 0.2, and 0.25 in BHI	25	0.1	25+0.1	Absorbance (610nm)
	50	0.15	25+0.15	
		0.2	25+0.2	
			50+0.1	CFU count
			50+0.15	
			50+0.2	

Seven milliliter batches of samples from each portion were dispensed into sterile test tubes. Seven milliliters of BHI broths without EOs were used as control samples. Each set of experimental tests was conducted three times.

The antimicrobial activity of low concentration copper and lactic acid was tested according to Gyawali et al.'s (2011) method. In this procedure, seven milliliter batches of fresh BHI broth containing copper ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Thermo Fisher Scientific, Fair Lawn, NJ, USA), lactic acid (85%, Thermo Fisher Scientific, Fair Lawn, NJ, USA) and their combinations were prepared. Copper solution was filter sterilized through 0.2 μm Nalgene filtration product (Nalge Nunc International, Rochester, NY, USA).

2.2.4 Measuring bacterial growth. Bacterial growth was monitored by measuring the turbidity every two hours during incubation at 37°C for 8h using a GENESYS™ 10S UV-Vis spectrophotometer (Thermo Electron Scientific Co., Madison, WI) at the wavelength of 610 nm.

2.2.5 Bacterial enumeration. Bacterial populations were determined by plating onto BHI agar. In this procedure, samples (1 ml) were withdrawn from inoculated samples at the beginning (0h) and at the end (8h) of incubation at 37°C, serially diluted in 0.1% peptone water; then appropriate dilutions were surface-plated (100 μl) onto duplicate BHI agar. Colonies were counted after plates were incubated at 37°C for 24h.

2.2.6 Statistical analysis. Different treatments were statistically analyzed for their antimicrobial effects on *E. coli* O157:H7 by factorial analysis of variance of duplicate samples. Each experiment has three replicates to determine the effects of copper and lactic acid combination treatment, armoise EO, and clove bud EO on the survival and growth of the bacterial strains. Data from three experiments were pooled to calculate the statistical mean and standard error of the mean for each treatment. Data were verified for normality, followed by an analysis of variance ($p < 0.05$) using SAS version 9.2 (SAS Institute, Cary, NC).

2.3 Results and Discussion

2.3.1 Antibacterial activity of armoise and clove bud EOs against *E. coli* O157:H7 in BHI medium. The data are shown in following figures and tables. Figures 3–8 present survival and growth of *E. coli* O157:H7 (ATCC700599, ATCC51659, and ATCC43895) in BHI medium with different concentrations of Armoise and clove bud EOs during incubation at 37°C for 8h. In the control samples, the initial growth as determined by turbidity at 610nm was ~0.0. The bacterial strains continued to grow during incubation period and reached stationary phase within 6-8h and turbidity readings reached ~0.80 after 8h. With the addition of Armoise EO at low concentrations (0.025 and 0.05%), turbidity readings were similar to the control samples, while high concentrations of Armoise EO ($\geq 0.1\%$) inhibited the growth of all tested strains as noticed by low turbidity readings. Turbidity readings were ~0.50 with the addition of clove bud EO at low concentrations (0.006%), and ~0.0 at high concentrations (≥ 0.0125).

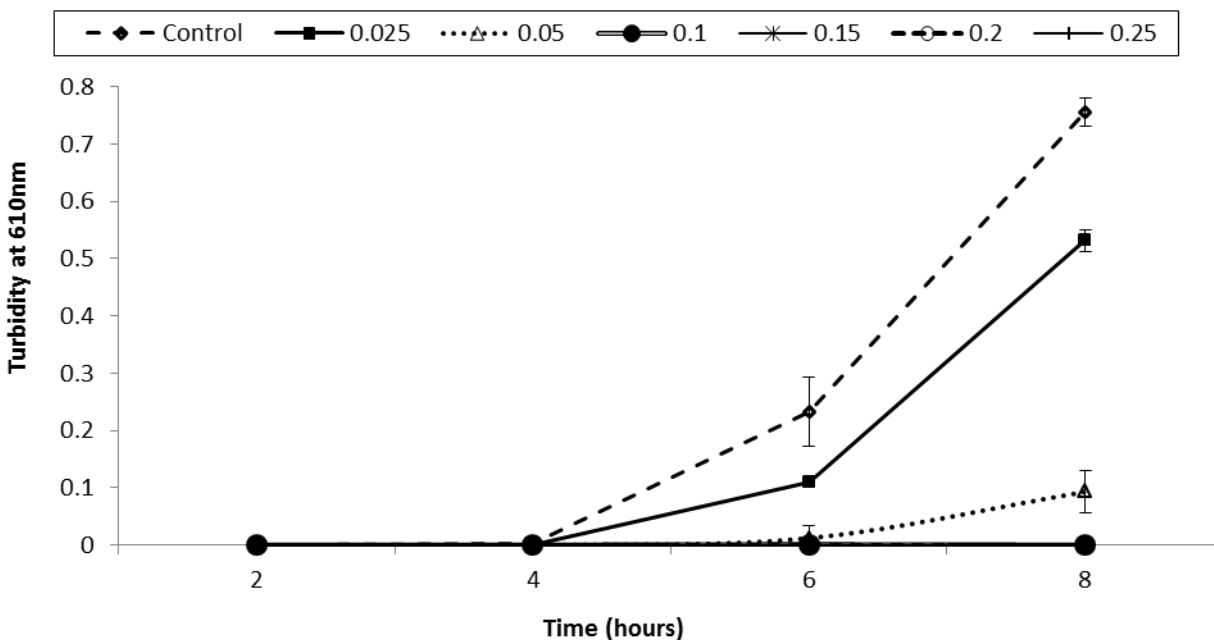


Figure 3. Survival and growth of *E. coli* O157:H7 (ATCC 700599) in BHI medium with different concentrations of Armoise EO after 8h incubation at 37°C.

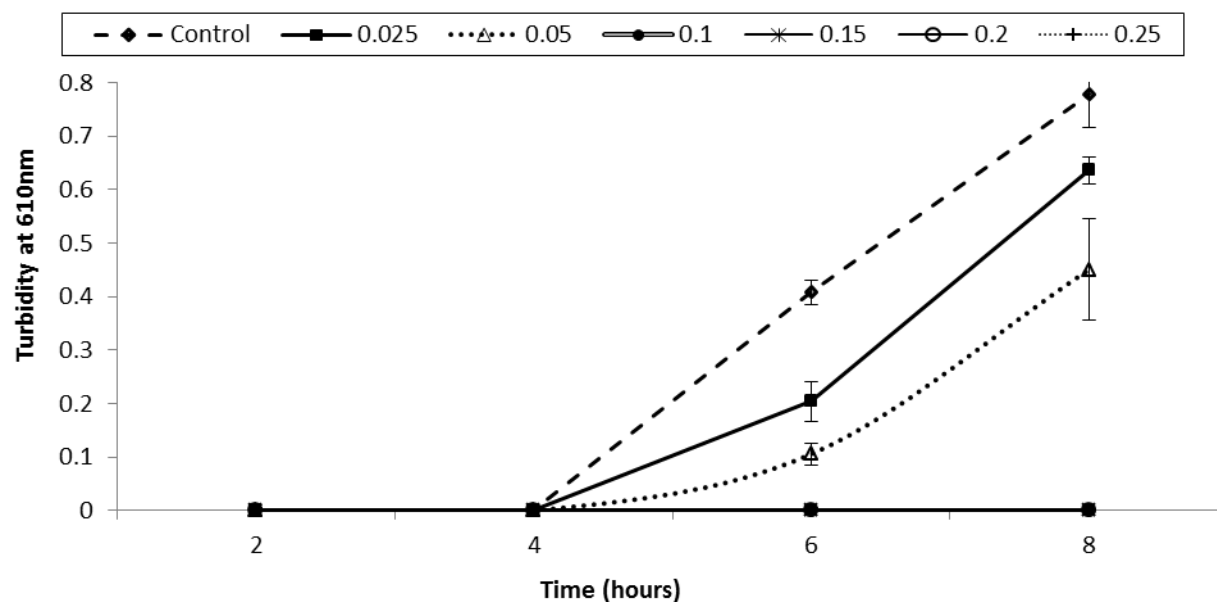


Figure 4. Survival and growth of *E. coli* O157:H7 (ATCC 51659) in BHI medium with different concentrations of Armoise EO after 8h incubation at 37°C.

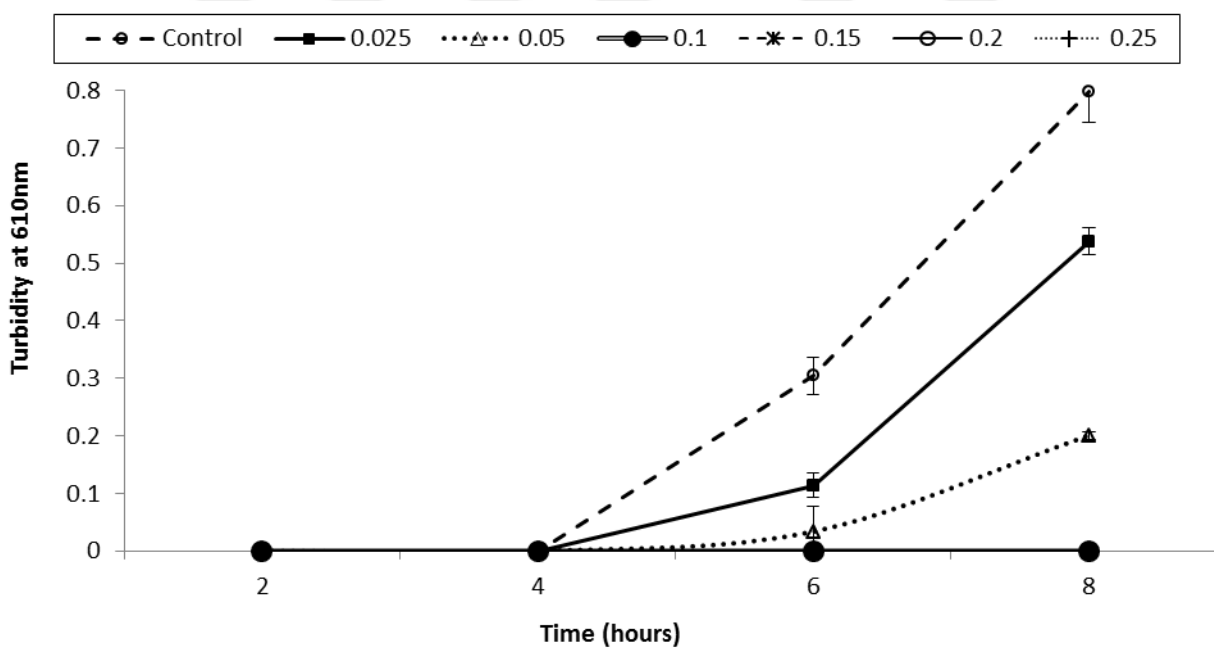


Figure 5. Survival and growth of *E. coli* O157:H7 (ATCC 43895) in BHI medium with different concentrations of Armoise EO after 8h incubation at 37°C.

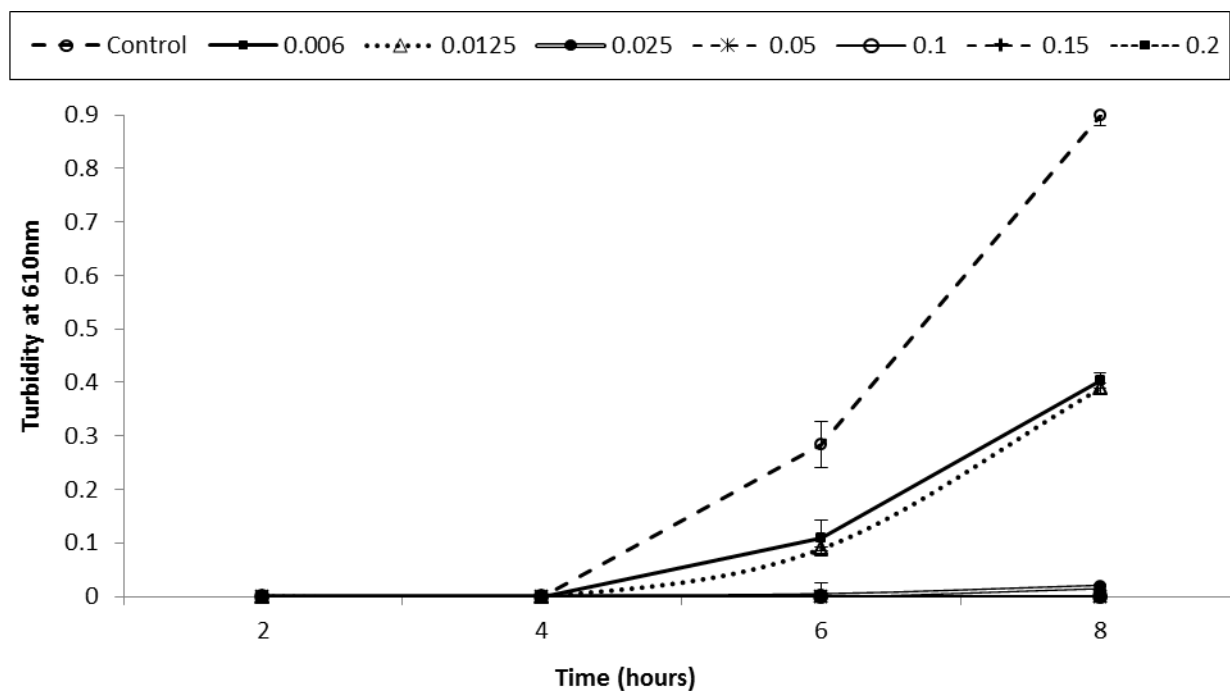


Figure 6. Survival and growth of *E. coli* O157:H7 (ATCC 700599) in BHI medium with different concentrations of Clove Bud EO during incubation at 37°C for 8h.

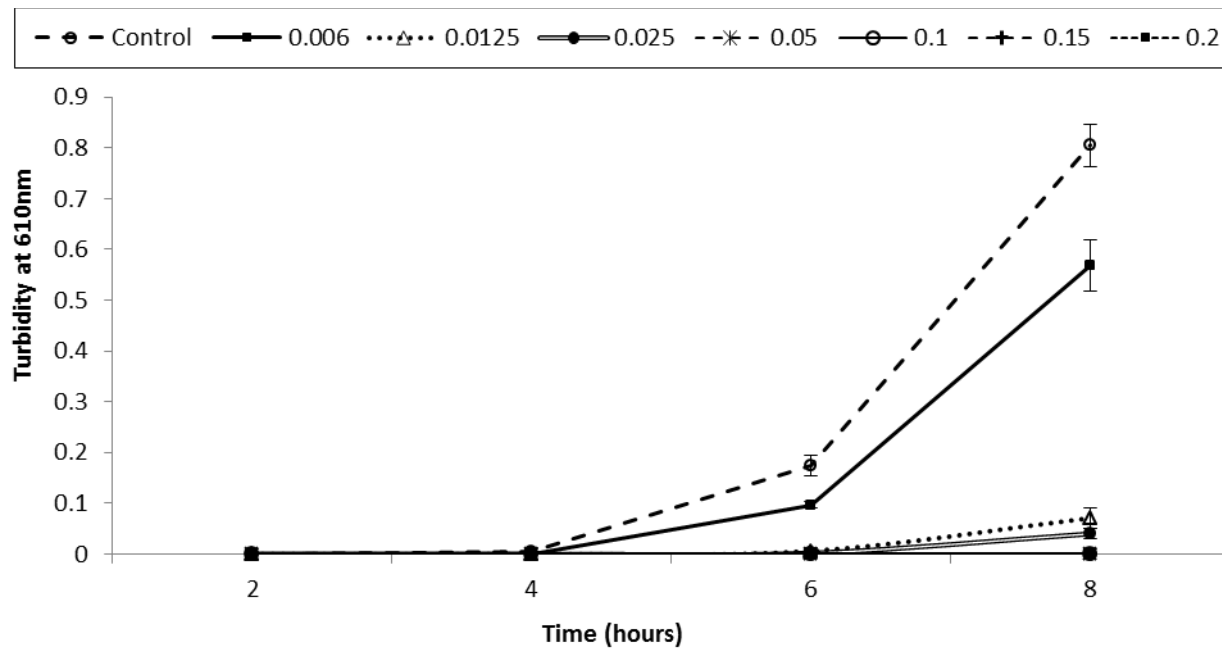


Figure 7. Survival and growth of *E. coli* O157:H7 (ATCC 51659) in BHI medium with different concentrations of Clove Bud EO during incubation at 37°C for 8h.

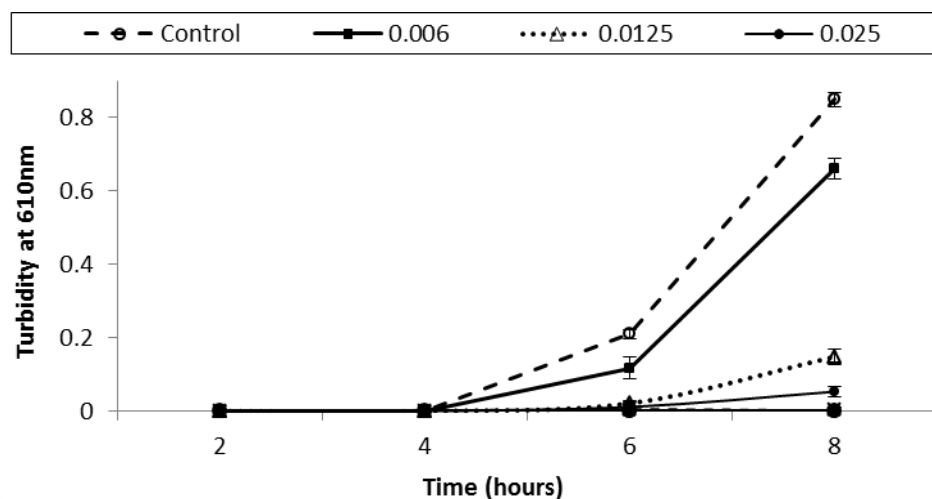


Figure 8. Survival and growth of *E. coli* O157:H7 (ATCC 43895) in BHI medium with different concentrations of Clove Bud EO during incubation at 37°C for 8h.

At the end of incubation period, the population of all tested strains was determined (see Tables 6–7). In control samples, the population of *E. coli* O157:H7 increased from ~2-3 log CFU/ml to ~8.8 log CFU/ml after 8h. With the addition of Armoise and clove bud EOs at low ($\leq 0.0125\%$) concentrations, growth was similar to the control sample; however at high ($\geq 0.15\%$) concentrations the population of all tested strains was < 1 log CFU/ml. Based on turbidity readings and population of tested strains our results indicated that the addition of armoise and clove bud EOs at certain concentrations inhibited the growth of all tested strains in BHI medium.

Table 6

Population of E. coli O157:H7 in BHI Medium In The Presence of Armoise EO at Different Concentrations after Incubation at 37°C for 8h

Treatment (% v/v)	Population of <i>E. coli</i> O157:H7 strains (log CFU/ml)		
	ATCC 700599	ATCC 51659	ATCC 43895
No EO (0)	9.01 ± 0.11^a	8.82 ± 0.11^a	8.41 ± 0.53^a
EO 0.025	8.26 ± 0^b	8.68 ± 0.26^a	8.22 ± 0.29^b

Table 6

(Cont.)

Treatment (% v/v)	Population of <i>E. coli</i> O157:H7 strains (log CFU/ml)		
	ATCC 700599	ATCC 51659	ATCC 43895
EO 0.05	8.1 ± .005 ^b	7.96 ± 0.18 ^b	6.93 ± 0.28 ^c
EO 0.1	<1 ^c	2.34 ± 0.19 ^c	<1 ^d
EO 0.15	<1 ^c	<1 ^d	<1 ^d
EO 0.2	<1 ^c	<1 ^d	<1 ^d
EO 0.25	<1 ^c	<1 ^d	<1 ^d

Notes. Initial inoculum level was 2-3 log CFU/ml. Statistically different treatments ($p < 0.05$) within same column were shown with different letters.

Table 7

Population of *E. coli* O157:H7 with the Presence of Clove Bud EO at Different Concentrations
(%v/v) in BHI Medium after Incubation at 37°C for 8h

Treatment (% v/v)	Population of <i>E. coli</i> O157:H7 strains (log CFU/ml)		
	ATCC 700599	ATCC 51659	ATCC 43895
No EO (Control)	8.48±0.05 ^a	8.52±0.47 ^a	7.23±0.26 ^a
EO 0.006	7.71±0.04 ^b	7.22±0.35 ^b	6.74±0.13 ^a
EO 0.0125	7.06±0.02 ^c	5.2±0.14 ^c	4.66±0.19 ^b
EO 0.025	3.06±0.41 ^d	4.6±0.11 ^d	4.23±0.59 ^b
EO 0.05	2.27±0.04 ^e	1.94±0.02 ^e	1.89±0.01 ^c
EO 0.1	<1 ^f	<1 ^f	<1 ^d
EO 0.15	<1 ^f	<1 ^f	<1 ^d
EO 0.2	<1 ^f	<1 ^f	<1 ^d

Notes. Initial inoculum level was 2-3 log CFU/ml. Statistically different treatments ($p < 0.05$) within same column were shown with different letters.

2.3.2 Antibacterial activity of copper and lactic acid against *E. coli* O157:H7 in BHI

medium. Figures 9–11 show survival and growth of *E. coli* O157:H7 strains (ATCC 700599, ATCC 51659 and ATCC 43895) in the presence of copper and lactic acid at different concentrations during incubation at 37°C for 8h. In the control samples, the initial growth determined as turbidity at 610nm was low (~0.0). The bacterial strains continued to grow during incubation period and reached stationary phase within 6-8h. The turbidity readings reached an absorbance of ~0.82. When copper was added to BHI broth at a concentration of 25ppm absorbance was ~0.34 and at a concentration of 50ppm absorbance was ~0.21. The growth of bacterial strains was similar to the control with the addition of lactic acid at concentrations of 0.1% and 0.15%. When copper sulfate (25 and 50ppm) was added in combination with lactic acid (0.2%), the turbidity reading was 0.0.

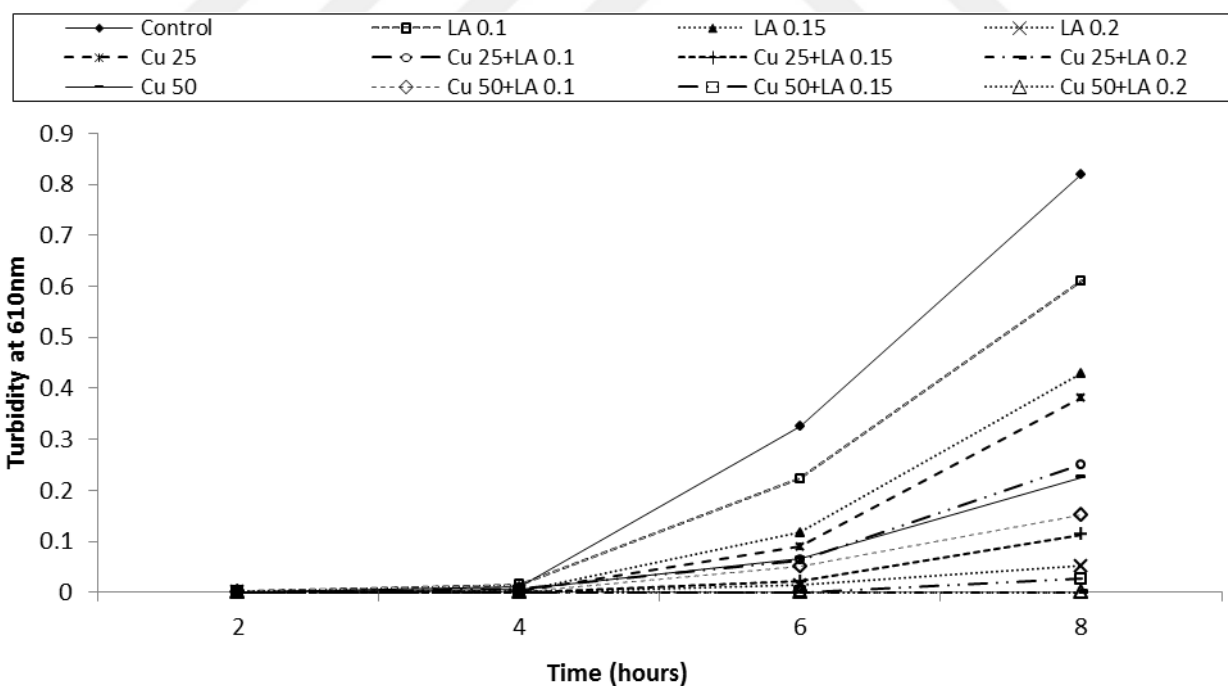


Figure 9. Survival and growth of *E. coli* ATCC 700599 in BHI broth with copper and lactic acid at different concentrations during incubation at 37°C for 8h.

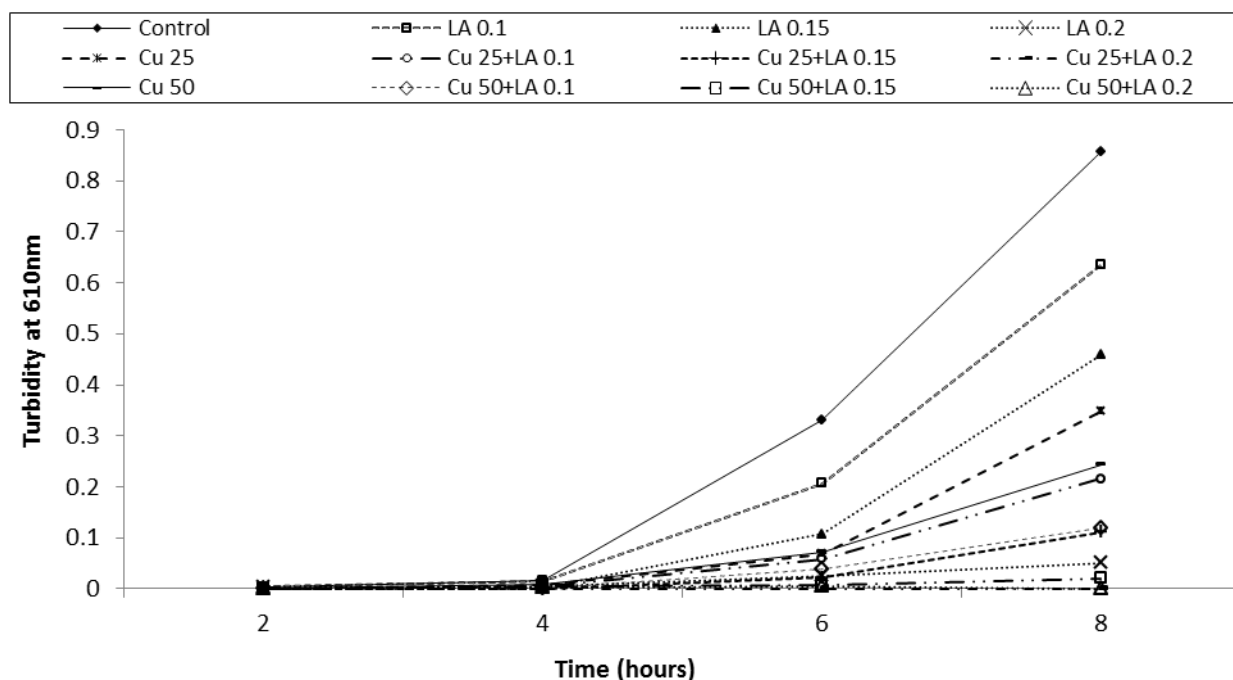


Figure 10. Survival and growth of *E. coli* ATCC 51659 in BHI broth with copper and lactic acid at different concentrations during incubation at 37°C for 8h.

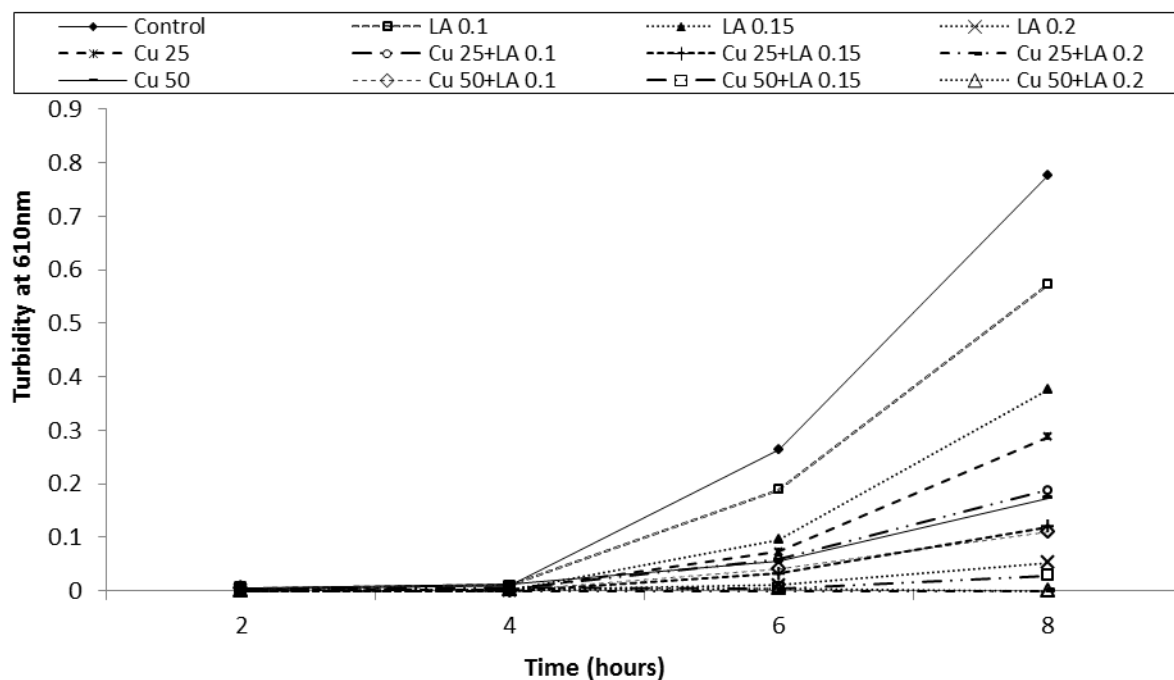


Figure 11. Survival and growth of *E. coli* ATCC 43895 in BHI broth with copper and lactic acid at different concentrations during incubation at 37°C for 8h.

Table 8 shows the population of *E. coli* O157:H7 in BHI medium in the presence of copper (Cu) and lactic acid (L.A.) at different concentrations after incubation at 37°C for 8h. In control samples, the population of *E. coli* O157:H7 increased from ~2 to 3 log CFU/ml to ~9 log CFU/ml after 8h. Copper or lactic acid alone slightly inhibited the growth of tested strains (~2 log reduction). The combination of copper (25 and 50 ppm) with lactic acid (0.1 and 0.15%) caused ~4 log reduction while copper (50ppm) with lactic acid (0.2%) caused ~7 log reduction on the growth of *E. coli* O157:H7. Our results indicated that copper or lactic acid alone had a slight effect on the growth of *E. coli* O157:H7 in laboratory medium. The combination of copper and lactic acid sulfate showed significant growth inhibition for all tested strains ($p < 0.05$).

Table 8

Population of E. coli O157:H7 in BHI Medium in the Presence of Lactic Acid (L.A.) at 0.1, 0.15, and 0.2 %v/v and Copper (Cu) at 25 and 50 ppm Concentrations after Incubation at 37°C for 8h

Treatment	Population of <i>E. coli</i> O157:H7 strains (log CFU/ml)		
	ATCC 700599	ATCC 51659	ATCC 43895
CONTROL	8.79 ± 0.21 ^a	8.70 ± 0.25 ^a	8.74 ± 0.23 ^a
L.A. 0.1	8.55 ± 0.23 ^a	8.18 ± 0.56 ^{ab}	8.35 ± 0.19 ^{ab}
L.A. 0.15	8.1 ± 0.30 ^{ab}	7.46 ± 0.27 ^{bc}	6.55 ± 0.16 ^d
L.A. 0.2	5.69 ± 0.08 ^e	5.49 ± 0.38 ^{ef}	6.10 ± 0.41 ^{de}
Cu 25ppm	7.62 ± 0.14 ^{bc}	6.98 ± 0.63 ^{cd}	7.77 ± 0.32 ^{bc}
Cu 25+L.A 0.1	6.98 ± 0.18 ^c	6.40 ± 0.20 ^{cde}	7.28 ± 0.41 ^c
Cu 25+L.A. 0.15	6.01 ± 0.48 ^{de}	6.18 ± 0.27 ^{de}	5.46 ± 0.23 ^{ef}
Cu 25+L.A. 0.2	4.58 ± 0.26 ^f	4.92 ± 0.21 ^f	4.58 ± 0.26 ^g
Cu 50	7.16 ± 0.61 ^c	6.98 ± 0.81 ^{bd}	6.47 ± 0.15 ^d
Cu 50+L.A 0.1	6.79 ± 0.40 ^{cd}	5.31 ± 1.19 ^{ef}	5.01 ± 0.47 ^{fg}
Cu 50+L.A 0.15	5.28 ± 0.49 ^{ef}	4.80 ± 0.10 ^f	3.24 ± 0.15 ^h
Cu 50+L.A 0.2	1.61 ± 0.67 ^g	1.50 ± 0.92 ^g	0.84 ± 0.20 ⁱ

Notes. Initial inoculum level was 2-3 log CFU/ml. Statistically different treatments ($p < 0.05$) within same column were shown with different letters.

The Food and Drug Administration (FDA) requires food processors to achieve at least 5-log reduction of pathogens in unpasteurized juices (FDA, 2001). Different control measures have been used to achieve 5-log reduction such as high pressure, chemicals, or combinations. The synergistic effect of copper and lactic acid in carrot juice samples were already shown in previous studies (Gyawali et al., 2011; Ibrahim et al., 2008; Tajkarimi & Ibrahim, 2011). In the present study, we have demonstrated that Armoise and clove bud oils at certain concentrations reduced the number of tested strains by at least 5-log in laboratory medium. We have shown that copper and lactic acid at low concentrations caused at least 5-log reduction of tested strains. However, EOs are expensive and not water soluble. To achieve 5-log reduction, EOs need to be added at above the solubility limit and they will be visible. Turbidity and strong aroma will negatively impact sensory properties. Copper at low concentration is not toxic for the human body and it is even an essential micronutrient and cofactor for certain enzymes. The permeabilizing properties of lactic acid could facilitate the entry of copper ions into cells and then cause toxicity on microbial cells (Ibrahim & Gyawali, 2012).

Due to the strong antimicrobial effect even used at low concentrations, low cost, no negative impact on sensory properties, no binding with lipid and protein components, and water solubility, copper in combination with lactic acid could be preferred to EOs in food to inhibit the growth of microorganisms to ensure the safety of food products. Al-Holy, Castro, and Al-Qadiri (2010) showed that a combination of sublethal concentrations of copper (50ppm) and lactic acid (0.2%) caused complete inhibition on growth of *Enterobacter sakazakii* in infant formulas. Researches are very limited on the effect of copper and lactic acid combinations in food samples. Further studies need to be conducted to better understand the effect of copper in combination

with lactic acid on growth of foodborne pathogens and food spoilage microorganisms in food samples.

2.4 Conclusion

Armoise and clove EOs at low concentrations Armoise (0.15% v/v) and clove bud (0.1% v/v) EOs caused ~7 log reduction on growth of *E. coli* O157:H7 in BHI medium. When both copper (50ppm) and lactic acid (0.2%) were added at low concentrations in laboratory medium, the growth of tested strains were significantly inhibited ($p < 0.05$). The addition of copper (50ppm) in combination of lactic acid (0.2%) at low concentrations into laboratory medium caused ~7 log reduction on the growth of tested strains. The antibacterial activity of armoise and clove EOs in BHI medium was not strain dependent. Due to the challenges in application of EOs, copper and lactic acid at low concentrations can be a practical alternative to achieve 5-log reduction in food products for industrial use. Further studies should be conducted to use combinations of Cu and LA at low concentrations to better understand their effectiveness on the growth of *E. coli* O157:H7 in food samples.

CHAPTER 3

Nanodispersion Enhanced Antibacterial Activity of Eugenol against *Escherichia coli*

O157:H7 in Laboratory Medium

3.1 Introduction

New concerns have arisen about food safety due to the increase in number of foodborne outbreaks caused by pathogen microorganisms. Consumers have strong tendencies to purchase good quality and safe food. Chemical and artificial preservatives used to inhibit the growth of pathogens are not easily accepted by consumers. Food processing techniques such as chemical preservative usage cannot totally kill foodborne pathogens or stop microbial spoilage (Gyawali & Ibrahim, 2014).

Natural antimicrobials are drawing attention since they are playing an important role in food control through preventing microbial contamination by pathogens. Great efforts have been made to establish new methods for the use of natural antimicrobials to ensure food safety (Tajkarimi et al., 2010). The use of EOs extracted from spices as natural antimicrobials has recently caused more interest for increasing the elimination of foodborne pathogens and the improvement in overall quality of food products (Burt, 2004). Eugenol, the main compound in clove EO, has strong antimicrobial activity against gram-negative and gram-positive pathogenic bacteria due to phenolic groups in chemical structures (Gortzi et al., 2007). Several studies were conducted in laboratory media to determine the antimicrobial effects of natural ingredients, including high-phenolic plant Eos, and incorporation of EOs into edible fruit and vegetable purees were tested against foodborne pathogens (Dickinson, 2003).

There are some limitations in delivery systems of eugenol and other EO compounds in foods. Poor water solubility, binding with food components, and evaporation during processing

and handling cause reduction of antimicrobial activity of EO compounds. Application of high concentrations above the solubility limit negatively impacts appearance in food samples. Strong herbal aroma will negatively impact the sensory properties of food (Misharina et al., 2009).

Shah et al. (2012a) reported an encapsulation of eugenol in nanocapsules of WPI-MD conjugates. The WPI-MD conjugates were used to prepare emulsions with the oil phase of eugenol dissolved in hexane. The emulsion was spray dried to remove hexane. Rehydration of spray-dried powders yielded with transparent nanodispersions of eugenol was prepared above the solubility limit of eugenol. We hypothesized that (a) eugenol in emulsion or nanodispersion will exert stronger antimicrobial activity than free eugenol, and (b) the mechanism of antibacterial activity for ND eugenol will inhibit the growth of tested strains by disruption of the cell wall. The objective of this study was to investigate the antimicrobial efficacy of ND eugenol against *E. coli* O157:H7 in growth (BHI) medium.

3.2 Materials and Methods

3.2.1 Materials. Eugenol (99%) was purchased from Acros Organics (part of Thermo Fisher Scientific, Morris Plains, NJ). Tween 80, polyoxyethylene (20) sorbitan mono-oleate, was purchased from Fisher (Global Chemicals, Thermo Fisher Scientific, Pittsburgh, PA). Whey protein isolates (WPI) and Maltodextrin 40 (MD40) conjugate was a gift from Dr. Qixin Zhong (Department of Food Science and Technology, The University of Tennessee, Knoxville, TN). Brain Heart Infusion (BHI) broth was product of Acumedia (Neogen, Lansing, MI). Peptone and agar (chemical grade) were products of Becton, Dickinson and Company (Sparks, MD). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

3.2.2 Bacterial strains. Three strains of *E. coli* O157:H7 ATCC 700599 (salami isolate), ATCC 51659 (clinical isolate), and ATCC 43895 (raw hamburger meat isolate) were used in this

study. Bacterial strains were transferred to fresh tryptic soy broth (TSB) before use. Cultures of each strain were grown separately in TSB (Difco, Becton Dickinson, Sparks, MD) at 37°C and transferred at 24h intervals into 9ml fresh brain heart infusion (BHI) broth.

3.2.3 Inoculum preparation. Overnight cultures of the strains were centrifuged (8000g, 15 min) and supernatant of each strain was decanted and the cell pellet was re-suspended (approximately 8 log CFU/ml) in 0.1% (v/v) 10 ml peptone water. Serial dilutions were made to achieve an initial inoculum level of 2-3 log CFU/ml before inoculation into broth. To determine the effect of free eugenol, emulsion in tween80 and nanodispersion of eugenol on the survival and growth of the bacterial strains in BHI broth three strains of *E. coli* O157:H7 were used. Strains were prepared by mixing 100µl of each strain overnight in 10ml peptone water. Several serial decimal dilutions were then made to achieve an initial inoculum level of 2-3 log CFU/ml in 7 ml of BHI in test tubes.

3.2.4 Preparation of nanodispersions of eugenol. A method described by Shah et al. (2012a) was slightly modified and used in preparation of nanodispersion of eugenol. Emulsions were prepared using homogenizer (POLYTRON® PT 2100 Homogenizer, Kinematica) at 15,000 rpm for 3 min with an oil phase and an aqueous phase with whey protein isolates (WPI) and maltodextrin (MD) conjugates. The oil phase contained 10g eugenol in 5 ml hexane and 2 ml Tween80 and was used at 5 ml/25 ml volume of the overall emulsion. The aqueous phase was dissolved with conjugates at an overall protein concentration of 3.7 g/100 g. A mixture of WPI and MD at a WPI:MD mass ratios of 1:2 was prepared by spray drying, followed by dry heating the spray dried powder at 90°C for 2 h for conjugation (Maillard reaction).

3.2.5 Experimental design. The list of independent and dependent variables used in the design of this experiment are shown in Table 9. Seven milliliter batches of samples from each

portion were dispensed into sterile test tubes. Seven milliliters of BHI broths without eugenol were used as control samples. Each set of experimental tests was conducted three times.

Table 9

List of Independent and Dependent Variables

Independent Variables			Dependent Variables
Free eugenol	Eugenol/Tween80 emulsion	Nanodispersed eugenol	
0.0125, 0.025, 0.05, 0.1, 0.15, 0.4, and 0.5% (v/v) in BHI	0.0125, 0.025, 0.05, 0.1, 0.15, 0.4, and 0.5% (v/v) in BHI	0.0125, 0.025, 0.05, 0.1, 0.15, 0.4, and 0.5% (v/v) in BHI	Absorbance (610nm)
			CFU count

3.2.6 Measuring bacterial growth. Bacterial growth was monitored by measuring the turbidity every two hours during incubation at 37°C for 8h using a GENESYS™ 10S UV-Vis spectrophotometer (Thermo Electron Scientific Co., Madison, WI) at the wavelength of 610 nm.

3.2.7 Bacterial enumeration. Bacterial populations were determined by plating onto BHI agar. In this procedure, samples (1 ml) were withdrawn from inoculated samples at the beginning (0h) and at the end (8h) of incubation at 37°C, serially diluted in 0.1% peptone water; then appropriate dilutions were surface-plated (100 µl) onto duplicate BHI agar. Colonies were counted after plates were incubated at 37°C for 24h.

3.2.8 Mechanism of action of ND eugenol against E. coli O157:H7 cell membranes.

3.2.8.1. Cell constituents' release essay. The release of cell constituents was examined according to Viuda-Martos et al. (2010). Cells from overnight bacterial suspension of tested strains were collected by centrifuge for 10 min at 5000g, washed three times and re-suspended in 0.1M PBS (pH = 7.0). One hundred milliliters of cell suspension was incubated at 37°C under agitation in an incubator shaker for 3h in the presence of ND eugenol at four different

concentrations (O(Control), MIC, 2xMIC, and 4xMIC). Then, 2ml of samples were collected and centrifuged at 12,000g for 2 min. To determine the concentration of the constituents released, 1ml supernatant was used to measure UV absorption at 260 nm. Correction was made for the absorption of the suspension with PBS (pH = 7.0) containing the same concentration of selected eugenol combinations after 2 min contact with tested strains. The untreated cells (control) were corrected with PBS (pH = 7.0) (Ozcan & Arslan, 2011).

3.2.9 Statistical analysis. Different treatments were statistically analyzed for their antimicrobial effects on *E. coli* O157:H7 by factorial analysis of variance of duplicate samples. Each experiment has three replicates to determine the effects of free eugenol, emulsion in tween80, and nanodispersion of eugenol on the survival and growth of the bacterial strains. Data from three experiments were pooled to calculate the statistical mean and standard error of the mean for each treatment. Data were verified for normality, followed by two-way analysis of variance ($p < 0.05$) using SAS version 9.2 (SAS Institute, Cary, NC).

3.3 Results and Discussion

3.3.1 Antimicrobial activities of eugenol in free, emulsified and ND forms in BHI medium. Antibacterial activities of ND eugenol against tested strains in BHI were compared to free eugenol and eugenol in emulsion with Tween 80, shown in Figures 12–14 at concentrations of 0.0125, 0.025, and ≥ 0.05 % v/v. Survival and growth of *E. coli* O157:H7 strains were determined by turbidity at 610nm after incubation at 37°C for 8h. In the control samples, the initial growth was determined by turbidity at 610nm was ~ 0.0 . The bacterial strains continued to grow during incubation period and reached stationary phase within 6-8h and turbidity readings reached ~ 0.80 after 8h. With the addition of free, emulsion, and nanodispersion of eugenol at 0.05% and higher concentrations, the growth of all tested strains was inhibited as noticed by low

turbidity readings. Turbidity readings for three different types of eugenol solutions at low concentrations were not statistically different than each other ($p \geq 0.05$). Turbidity readings for nanodispersion without eugenol (positive control) were similar to control at low and high concentrations.

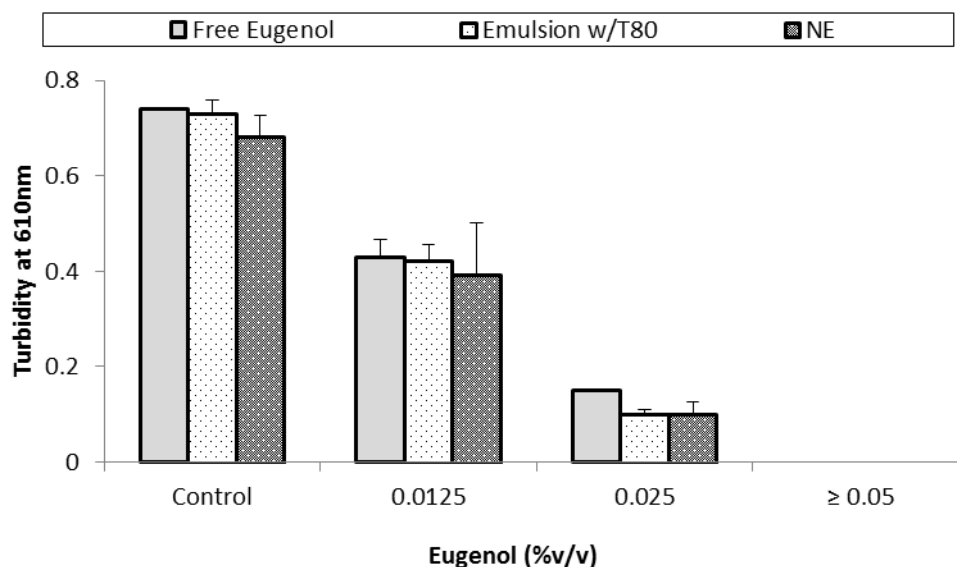


Figure 12. Survival and growth of *E. coli* ATCC700599 in BHI broth with different concentrations of free, emulsified and nanodispersed eugenol (NE) after incubation at 37°C for 8h.

At the end of incubation period, the population of all tested strains was determined (see Figure 15 and Table 10). In control samples, the population of *E. coli* O157:H7 increased from ~2-3 log CFU/ml to ~8.8 log CFU/ml after 8h. With the addition of eugenol at low ($< 0.025\%v/v$) concentrations, growth was similar to the control sample; however, at high ($\geq 0.1\%v/v$) concentrations the population of all tested strains was below the detectable level (< 1 log CFU/ml) for each treatment. When ND without eugenol at MIC (0.05%) and 10xMIC (0.5%) added population of tested strains were similar to control which indicates the inhibition effect was not due to nanodispersion components.

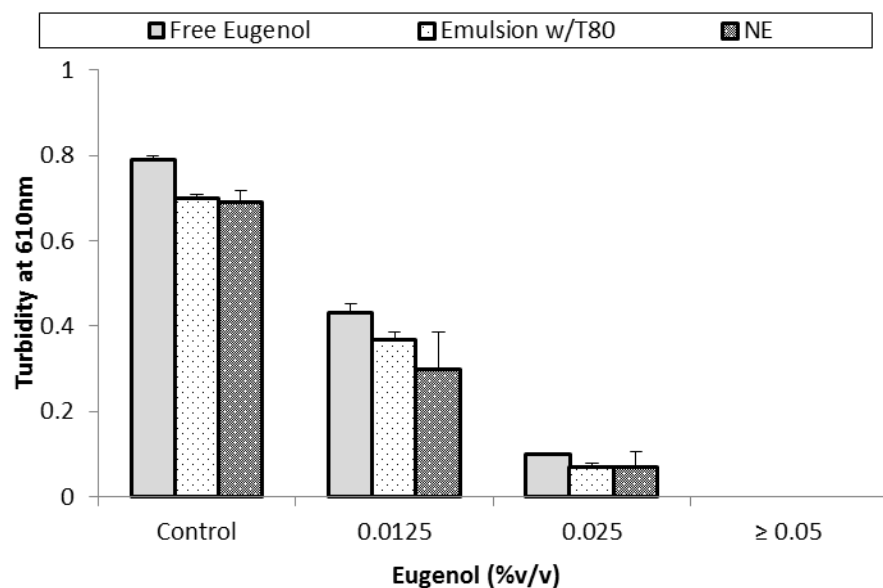


Figure 13. Survival and growth of *E. coli* ATCC 51659 in BHI broth with different concentrations of free, emulsified and nanodispersed eugenol (NE) after incubation at 37°C for 8h.

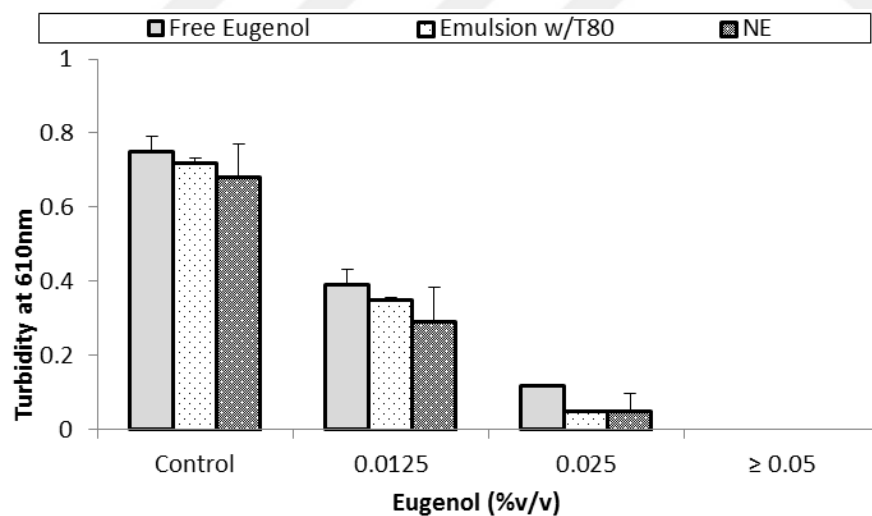


Figure 14. Survival and growth of *E. coli* ATCC 43895 in BHI broth with different concentrations of free, emulsified with T80, and nanodispersed eugenol (NE) after incubation at 37°C for 8h.

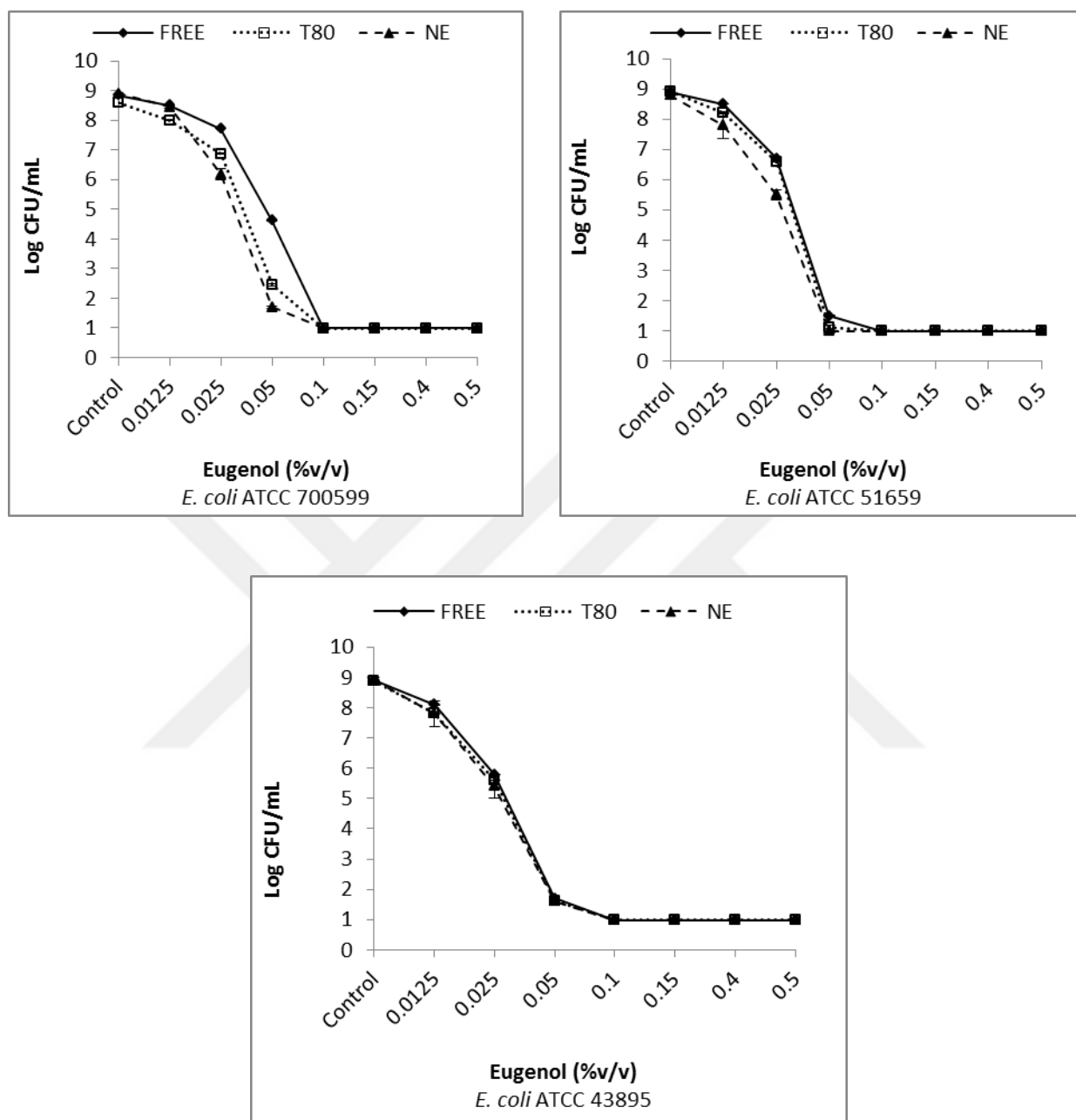


Figure 15. Population of *E. coli* O157:H7 in BHI medium with different concentrations (%v/v) of free, emulsified, and nanodispersed eugenol (NE) at after incubation at 37°C for 8h.

Table 10

Population of E. coli O157:H7 in BHI Medium in the Presence of Eugenol in Free, Emulsified (T80) and ND Forms at Different Concentrations (%v/v) after Incubation at 37°C for 8h

Eugenol concentration (%v/v)	<i>E. coli</i> O157:H7								
	ATCC 700599			ATCC 51659			ATCC 43895		
	Free	T80	ND	Free	T80	ND	Free	T80	ND
Control	8.8±0.02 ^A _a	8.7±0.02 ^A _a	8.7±0.02 ^A _a	8.9±0.00 ^A _a	8.9±0.01 ^A _a	8.8±0.14 ^A _a	8.9±0.01 ^A _a	8.9±0.00 ^A _a	8.9±0.10 ^A _a
0.0125	8.5±0.01 ^A _b	8.5±0.02 ^A _b	8.0±0.03 ^{AB} _b	8.5±0.02 ^A _b	8.2±0.02 ^A _b	7.8±0.44 ^B _b	8.1±0.01 ^A _b	7.8±0.03 ^A _b	7.8±0.41 ^A _b
0.025	7.7±0.01 ^A _c	6.9±0.01 ^B _c	6.1±0.20 ^C _c	6.7±0.01 ^A _c	6.6±0.01 ^A _c	5.5±0.16 ^B _c	5.8±0.00 ^A _c	5.6±0.00 ^A _c	5.4±0.39 ^A _c
0.05	4.6±0.01 ^A _d	2.5±0.05 ^B _d	1.7±0.03 ^C _d	1.5±0.00 ^A _d	1.1±0.10 ^A _d	1.0±0.09 ^{AB} _d	1.7±0.00 ^A _d	1.6±0.01 ^A _d	1.6±0.07 ^A _d
0.1	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e
0.15	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e
0.4	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e
0.5	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e	<1.0 _e

^B Different capital letters show treatments which are statistically different ($p < 0.05$) than control in same row.

_b Different lower case letters show treatments which are statistically different ($p < 0.05$) than control.

When eugenol was added into BHI at minimum inhibitory concentration (MIC) or lower ($\leq 0.05\%$ v/v) in emulsion with Tween 80 and nanodispersion forms, population of *E. coli* O157:H7 ATCC 700599 and ATCC 51659 were significantly ($p < 0.05$) lower when it compared with addition of the same concentrations of free eugenol. These two strains were more resistant to eugenol forms than *E. coli* O157:H7 ATCC 43895.

3.3.2 Cell constituents' release essay results. The effect of ND eugenol combinations on cell constituents' release of the tested strains are shown in Figure 16. At the beginning of the incubation period, absorbance readings at 260 nm were 0.0. After incubation at 37°C for 3h absorbance readings reached 0.1, 0.2, and 0.5 in the presence of ND eugenol at 0.05, 0.1, and 0.2% v/v concentrations, respectively.

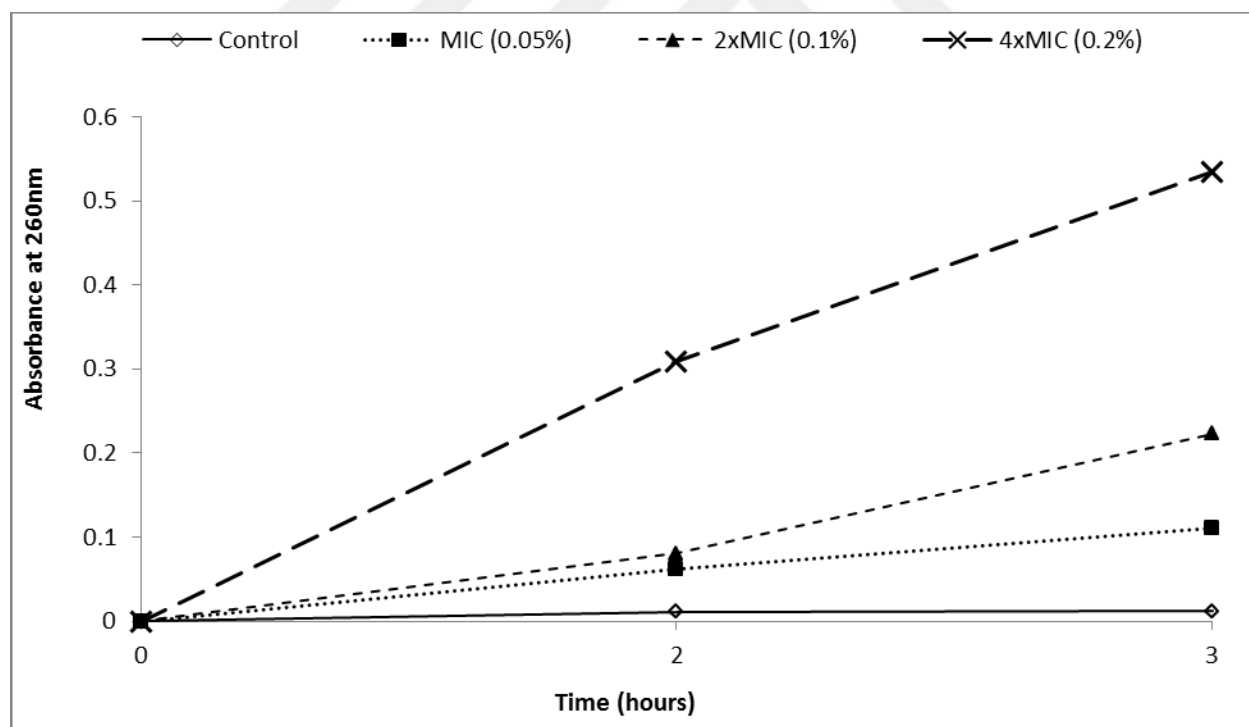


Figure 16. The effect of ND eugenol combinations on cell constituents' release of the tested strains.

ND eugenol did not improve antimicrobial activity against *E. coli* O157:H7 and *Listeria monocytogenes* in tryptic soy broth but improved in bovine milk (Gortzi et al., 2007). Based on turbidity readings and population of tested strains after incubation for 8h, ND eugenol improved the antimicrobial activity of eugenol in laboratory medium. Further studies need to be conducted in food samples to evaluate antimicrobial effectiveness of ND eugenol.

3.4 Conclusion

When eugenol was added to BHI at minimum inhibitory concentration (MIC) or lower ($\leq 0.05\%v/v$) in emulsion with Tween 80 and ND forms, populations of *E. coli* O157:H7 ATCC 700599 and ATCC 51659 were significantly ($p < 0.05$) lower when it was compared with addition of the same concentrations of free eugenol. These two strains were more resistant to eugenol forms than *E. coli* O157:H7 ATCC 43895. Cell constituents' release assay showed that ND eugenol caused cell membrane disruption based on its concentration, and the highest absorbance reading of 260nm was obtained at a concentration of 4xMIC (0.2%v/v). The antibacterial effect of free, emulsion with Tween 80, and ND eugenol was not different at concentrations $\geq 0.1\%$ (v/v), whereas emulsion and nanodispersion of eugenol improved antibacterial activity of eugenol at lower concentrations. This is a critical finding for future applications of eugenol in food samples because water solubility of eugenol and other lipophilic antimicrobials is a big challenge. Furthermore, the application of eugenol at lower concentrations will help to overcome negative impacts of eugenol on sensory characteristics of food samples.

CHAPTER 4

Antibacterial Activity of ND Eugenol against *E. coli* O157:H7 in Ground Beef and Carrot Juice Samples

4.1 Introduction

Consumers are expressing more concern about using chemical preservatives and synthetic additives to control foodborne pathogens in food and are demanding high quality, safe food. Natural ingredients have become popular as an alternative to chemical preservatives. Natural antimicrobials can be used to control foodborne pathogens but do not negatively impact sensory properties of food products. *E. coli* O157:H7 has been recognized as one of the leading foodborne pathogens due to high mortality rates in susceptible populations (Tajkarimi & Ibrahim, 2011).

Plant EOs show antimicrobial activity by disrupting cell membranes causing the release of cell constituents or inactivating genetic material (Solomakos et al., 2008). Several studies have been conducted to investigate the antimicrobial activity of EOs in meat products (Burt, 2004). Clove is one of the main spices that has shown the greatest activity against microorganisms, and its primary component is eugenol (Davidson, Cekmer, Monu, & Techathuvanan, 2014). Barbosa et al. (2009) found that clove oil at 0.1 % v/v concentration inhibited the growth of *E. coli* and *Salmonella enteritidis* in irradiated minced meat.

There are some challenges to using EOs in food such as low water solubility, strong herbal aroma, and interaction with food components. Antimicrobial activity of EOs and their primary compounds is decreased because of these issues. Addition of EO compounds at high concentrations will negatively impact sensory properties of food samples.

Recently our laboratory showed that the nanodispersion of eugenol has enhanced antibacterial activity of eugenol in BHI medium. There are limited studies on application of ND eugenol in food systems such as ground beef and fruit juices. The hypotheses of this research were (a) ND eugenol will significantly inhibit the growth of tested strains, and (b) *E. coli* O157:H7 mixture will grow slightly in the absence of ND eugenol while the growth will be strongly inhibited with the addition of ND eugenol. Therefore, the objective of this study was to investigate the antibacterial activity of ND eugenol against *E. coli* O157:H7 in ground beef and carrot juice samples during refrigerated storage.

4.2 Materials and Methods

4.2.1 Materials. Eugenol (99%) was purchased from Acros Organics (part of Thermo Fisher Scientific, Morris Plains, NJ). Tween 80, polyoxyethylene (20) sorbitan mono-oleate, was purchased from Fisher (Global Chemicals, Thermo Fisher Scientific, Pittsburgh, PA). Whey protein isolates (WPI) and Maltodextrin 40 (MD40) conjugate was a gift from Dr. Qixin Zhong (Department of Food Science and Technology, The University of Tennessee, Knoxville, TN). Brain Heart Infusion (BHI) broth was a product of Acumedia (Neogen, Lansing, MI). Peptone and agar (chemical grade) were products of Becton, Dickinson and Company (Sparks, MD). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

4.2.2 Bacterial strains. Three strains of *E. coli* O157:H7 ATCC 700599 (salami isolate), ATCC 51659 (clinical isolate), and ATCC 43895 (raw hamburger meat isolate) were used in this study. Bacterial strains were transferred to fresh tryptic soy broth (TSB) before use. Cultures of each strain were grown separately in TSB (Difco, Becton Dickinson, Sparks, MD) at 37°C and evenly mixed to make cocktails of three strains and transferred at 24h intervals into 9ml fresh brain heart infusion (BHI) broth.

4.2.3 Inoculum preparation. During the experiments, a cocktail of the three bacterial strains was used. Overnight cultures of the strains were centrifuged (8000g, 15 min) and supernatant of each strain was decanted and the cell pellet was re-suspended (approximately 8 log CFU/ml) in 0.1% (v/v) 10 ml peptone water. The bacterial cocktail was prepared by mixing overnight individual strain (10 ml). Serial dilutions were made to achieve an initial inoculum level of 4-5 log CFU/ml before inoculation into broth. To determine the effect of free eugenol, emulsion in tween80 and nanodispersion of eugenol on the survival and growth of the bacterial strains in BHI broth, a separate cocktail mixture of *E. coli* O157:H7 was used. Strains were prepared by mixing 100µl of cocktail in 10ml peptone water. Several serial decimal dilutions were then made to achieve an initial inoculum level of 4-5 log CFU/ml in 7 ml of BHI in test tubes.

4.2.4 Preparation of nanodispersions of eugenol. A method described by Shah et al. (2012a) was slightly modified and used in preparation of ND eugenol. Emulsions were prepared using homogenizer (POLYTRON® PT 2100 Homogenizer, Kinematica) at 15,000 rpm for 3 min with an oil phase and an aqueous phase with whey protein isolates (WPI) and maltodextrin (MD) conjugates. The oil phase contained 10g eugenol in 5 ml hexane and 2 ml Tween80, and was used at 5 ml/25 ml volume of the overall emulsion. The aqueous phase was dissolved with conjugates at an overall protein concentration of 3.7 g/100 g. A mixture of WPI and MD at a WPI: MD mass ratio of 1:2 was prepared by spray drying, followed by dry heating the spray dried powder at 90°C for 2 h for conjugation (Maillard reaction).

4.2.5 Preparation of carrot juice. Fresh ultra-pasteurized carrot juice was purchased from a local store (Greensboro, NC) the day before each use and stored at 4°C. As preparation

for its use as a growth media, 1 L of the carrot juice was steam-sterilized at 95°C for 10 min to inactivate natural flora and then cooled to 37°C.

4.2.6 Preparation of ground beef samples. Lean (93% lean, 7% fat) ground beef samples were purchased from a local store (Greensboro, NC) on the day of experiment and stored at 4°C. As preparation for its use as a growth medium, 100g of ground beef sample was autoclaved at 121°C for 15 min. From cocktail mixture of strains, two milliliters of bacterial suspension were transferred into the sterilized meat sample and incubated at 37°C for 2h. One hundred milliliters of sterilized peptone water (0.1%) solution was mixed and homogenized with the meat sample. Serial dilutions were made from this sludge to achieve ~5 log CFU/g inoculum level in meat samples. Separate pieces (150 grams) of inoculated ground beef samples were placed into sterile stomacher bags and kept in the refrigerator.

4.2.7 Ground beef experimental design. ND eugenol stock solution (10% eugenol) was added to each 25 g of inoculated ground beef samples to obtain 0.1, 0.2, 0.4, and 0.5% (v/w) concentrations. Ground beef samples without ND eugenol were used as control samples. Ground beef samples with or without ND eugenol were kept in the refrigerator for 7 days. Each set of experimental tests was conducted three times.

4.2.8 Carrot juice experimental design. ND eugenol stock solution (10% eugenol) was added into sterilized carrot juice to obtain 0.05, 0.1, 0.15, 0.2, and 0.4% (v/v) concentrations. Ten milliliter batches of samples with or without ND eugenol from each portion were dispensed into sterile test tubes in duplicate and kept in the refrigerator for three weeks. Ten milliliters of BHI broths without eugenol were used as control samples. Each set of experimental tests was conducted three times.

4.2.9 Bacterial enumeration. The meat and samples with ND eugenol at different concentrations were kept at 4°C for 7 days. Twenty-five gram samples were withdrawn at 2, 3, 5, and 7 days and well mixed and homogenized with 225 ml sterile peptone water (0.1%). Carrot juice samples including ND eugenol at different concentrations were kept at 4°C for 3 weeks. Samples (1 ml) were withdrawn from inoculated samples at one-week intervals up to three weeks. Serial dilutions were made in 0.1% peptone water and then appropriate dilutions were surface-plated (100 µl) onto duplicate BHI agar, Eosin Methylene Blue (EMB) agar, and MacConkey agar. Plates were incubated at 37°C for 48h. Colonies were counted to determine bacterial population.

4.2.10 Statistical analysis. Different treatments were statistically analyzed for their antimicrobial effects on *E. coli* O157:H7 by factorial analysis of variance of duplicate samples. Each experiment had three replicates to determine the antimicrobial effect of ND eugenol on the survival and growth of the bacterial strains in ground beef and carrot juice samples. Data from three experiments were pooled to calculate the statistical mean and standard error of the mean for each treatment. Data were verified for normality, followed by two-way analysis of variance ($p < 0.05$) using SAS version 9.2 (SAS Institute, Cary, NC).

4.3 Results and Discussion

During refrigerated storage, the population of mixture of strains in ground beef samples was determined (see Figure 17). In control samples, the population of *E. coli* O157:H7 increased from ~5 log CFU/g to ~6.1 log CFU/g after 7 days. With the addition of ND eugenol at low (< 0.2% v/w) concentrations, growth was similar to the control sample; however, at high (0.4 and 0.5%) concentrations the populations of the mixture of strains were ~4.0 and 2.8 log CFU/g, respectively. When ND eugenol was added to ground beef at minimum inhibitory concentration

(MIC) or higher ($\geq 0.4\%$ v/w), population of *E. coli* O157:H7 mixture was significantly ($p < 0.05$) lower when it was compared with the addition at lower concentrations ($\leq 0.2\%$ v/w).

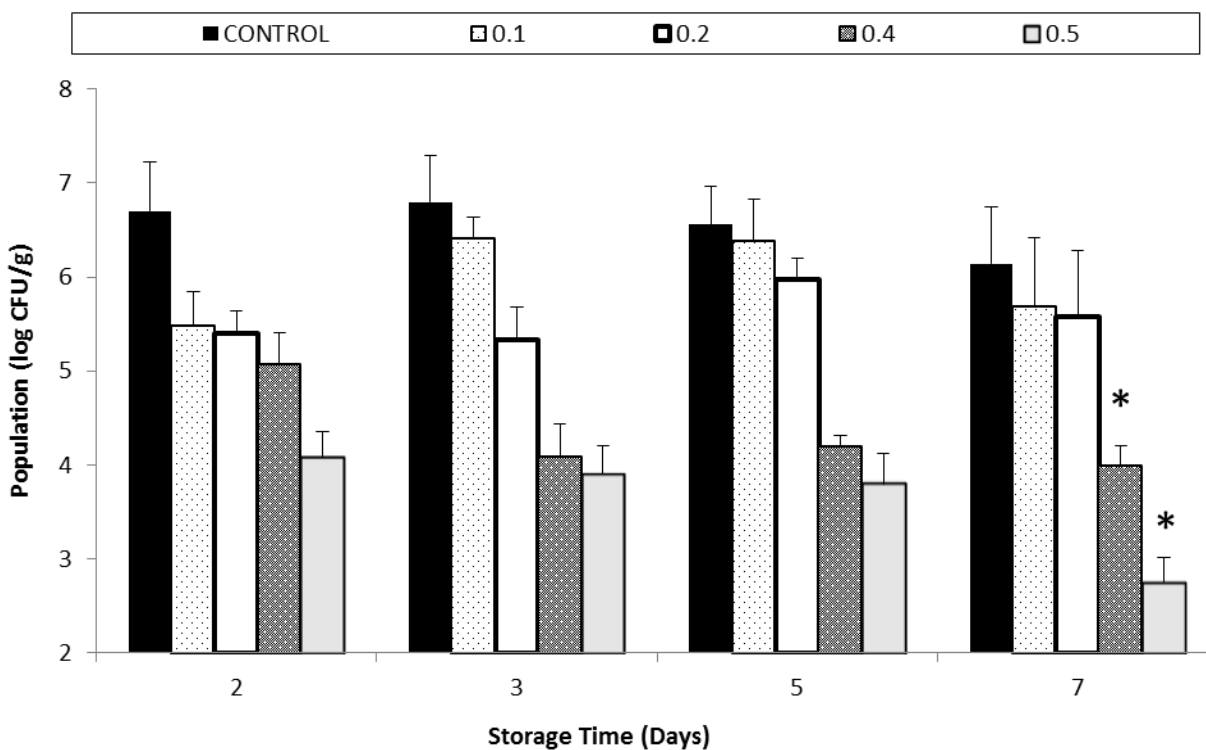


Figure 17. Survival and growth of mixture of *E. coli* O157:H7 strains in ground beef samples with different concentrations of ND eugenol during refrigerated storage at 4°C for 7 days. Statistically different ($p < 0.05$) treatments after 7 days of incubation were shown with asterisk (*).

Figure 18 shows the population of mixture of strains in carrot juice during refrigerated storage for 3 weeks. In control samples, the population of mixture of strains remained similar to initial population (4.2 log CFU/g) after 3 weeks of storage at 4°C. With the addition of ND eugenol at MIC (0.05%v/v), growth of mixture was reduced by 1.0 log CFU/g, while ND eugenol at 0.1%v/v and higher concentrations reduced the growth of tested mixture of strains to below detectable level (< 1 log CFU/g).

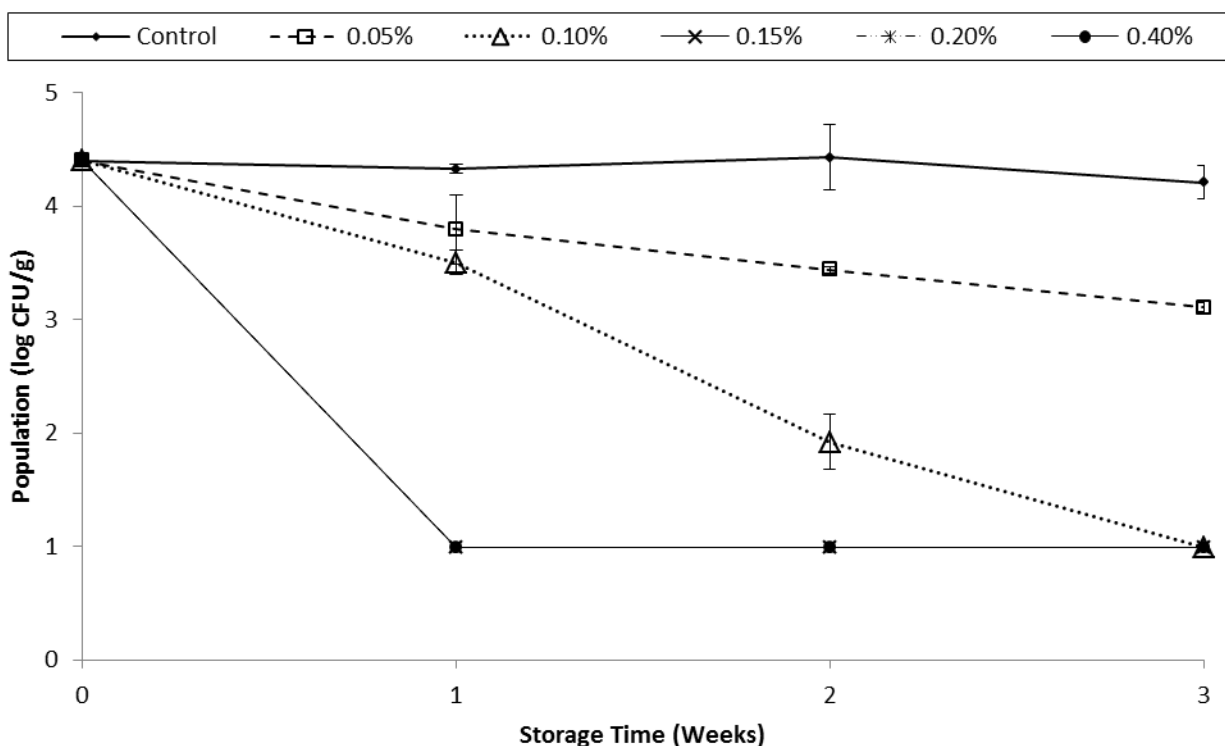


Figure 18. Survival and growth of mixture of *E. coli* O157:H7 strains in carrot juice with different concentrations of ND eugenol during refrigerated storage at 4°C for 3 weeks.

In the present study, ND eugenol ($\geq 0.1\%$ v/v) was found to significantly inhibit the growth of *E. coli* O157:H7 in carrot juice during refrigerated storage. Due to its strong antimicrobial activity, ND eugenol can cause at least a 5-log reduction in carrot juice at low concentrations. Researches evaluating antibacterial activity of EOs on survival and growth of *E. coli* O157:H7 in fruit and vegetable juices are limited. Different preservation methods were tested to inactivate pathogens in carrot juice to meet FDA 5-log reduction standard; High hydrostatic pressure alone at different temperatures (Van Opstal, Vanmuysen, Wuytack, Masschalck, & Michiels, 2005), in combination with nisin (Pathanibul, Taylor, Davidson, & Harte, 2009) and mild heat treatment (Valdramidis et al., 2007), pulsed electric fields (Rodrigo, Martínez, Harte, Barbosa-Cánovas, & Rodrigo, 2001; Selma, Salmerón, Valero, & Fernández, 2004), pepsin-digested lactoferrin (Chantaysakorn & Richter, 2000), ultrasound (Knorr, Zenker,

Heinz, & Lee, 2004; Zenker, Heinz, & Knorr, 2003), UV light (Lopez-Malo & Palou, 2005), and gamma irradiation (Song et al., 2006). All of these preservation methods cause undesirable changes in organoleptic properties and nutritional values in carrot juices. ND eugenol as a natural ingredient can be used to avoid all of these changes.

Clove bud oil caused 1.6 log CFU/g reduction in the growth of *E. coli* O157:H7 in cooked beef patties (Rounds et al., 2012). Zengin and Baysal (2014) found that MIC of clove oil was 0.5% in minced meat samples. The combination of thyme EO at 0.6% and nisin at 500 or 1000 IU/g showed an additive effect against *E. coli* O157:H7 in minced meat during storage at 10°C more than at 4°C (Solomakos et al., 2008). Based on their findings, free clove oil at high concentrations (0.5-2.0%) could reduce population of *E. coli* O157:H7 by less than 2 log CFU/g in meat samples. Our results showed that the addition of ND eugenol at 0.5% v/w caused 3.3 log CFU/g reduction on population of tested strains. Although it was not enough to cause 5-log reduction on growth of tested strains, ND eugenol can be used in combination with other EO compounds or natural ingredients to achieve 5-log reduction in ground beef samples.

4.4 Conclusion

Our results showed that ND eugenol at $\geq 0.1\%$ v/v concentration significantly ($p < 0.05$) inhibited the growth of *E. coli* O157:H7 in carrot juice during refrigerated storage for three weeks. Due to its strong antimicrobial activity, ND eugenol can cause at least 5-log reduction in carrot juice even at low (0.1% v/v) concentrations. Low concentrations of eugenol will not change the general appearance, color, or turbidity of carrot juice. Further studies need to be conducted to evaluate antibacterial activity of ND eugenol on survival and growth of *E. coli* O157:H7 in fruit and vegetable juices. The addition of ND eugenol at 0.4% and 0.5% v/w concentrations caused 3.3 log reductions on growth of tested strains in ground beef samples

during refrigerated storage for 7 days. The lower concentrations of ND eugenol ($\leq 0.2\%v/w$) did not show significant antibacterial activity against tested strains in ground beef. ND eugenol can be used in combination with other natural ingredients to achieve 5-log reduction in ground beef samples.



CHAPTER 5

Conclusions and Future Research

Armoise (0.15% v/v) and clove bud (0.1% v/v) EOs caused ~6 log reduction in growth of *E. coli* O157:H7 in BHI medium. When both Cu (50ppm) and LA (0.2%) were added at low concentrations into laboratory medium, the growth of tested strains were significantly inhibited ($p < 0.05$). Cu and LA at low concentrations can be a practical alternative to EOs to achieve 5-log reduction in food products for industrial use. Further studies should be conducted to use a combination of Cu and LA at low concentrations to better understand the effectiveness of the growth of *E. coli* O157:H7 in food samples. When eugenol added into BHI at minimum inhibitory concentration (MIC) or lower ($\leq 0.05\%v/v$) in emulsion with Tween 80 and ND forms, population of *E. coli* O157:H7 ATCC 700599 and ATCC 51659 were significantly ($p < 0.05$) lower compared to the addition of the same concentrations of free eugenol. These two strains were more resistant to eugenol forms than *E. coli* O157:H7 ATCC 43895. Based on turbidity readings and the population of tested strains after incubation for 8h, ND eugenol improved the antimicrobial activity of eugenol in laboratory medium. Further studies need to be conducted in food samples to evaluate antimicrobial effectiveness of ND eugenol. In the present study, ND eugenol ($\geq 0.1\%v/v$) was found to significantly inhibit the growth of *E. coli* O157:H7 in carrot juice during refrigerated storage. Due to its strong antimicrobial activity, ND eugenol can cause at least 5-log reduction in carrot juice at low concentrations. Low concentrations of eugenol will not negatively impact the sensory characteristics of carrot juice. Further studies need to be conducted to evaluate antibacterial activity of ND eugenol on survival and growth of *E. coli* O157:H7 in fruit and vegetable juices. ND eugenol can be used in combination with other natural ingredients to achieve 5-log reduction in ground beef samples.

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