PH.D. THESIS

EFFECT OF ESSENTIAL OILS AND THEIR MAJOR COMPONENTS ON BIOFILM FORMATION OF FOOD-SPOILAGE MICROBES AND ON THE CELL TO CELL COMMUNICATION

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INTRODUCTION

Due to the rapid deterioration caused by microbial contamination a large amount of food is thrown away without even being passed through to consumers. Contaminated food is not only a serious problem in the food industry, but also for our health. Food poisoning induced diseases are still present today in a high percentage. When using conventional preservatives unpleasant by-products are formed, and in many cases they do not lead to the expected results. In addition, the popularity of environmentally friendly products among consumers suggests that reforms are necessary for food preservation. Therefore, it became necessary to develop new, natural and effective antimicrobial agents that can, at least partially, replace synthetic preservatives. Bacteria form so-called biofilm communities that constitute protection against adverse environmental conditions, allow better access to nutrients and increase the genetic diversity. Compared to the free-floating cells, these communities show increased resistance to disinfectants, antibiotics. Communication between cells forming biofilms is called *quorum sensing* (QS). Synthesis of virulence factors and production of extracellular polysaccharides is regulated by QS, so an accurate understanding of the process and the possibility of blocking the process is a challenge for food scientists.

The aims of the present study were to investigate the anti-biofilm forming and anti-QS effect of selected essential oils and their major components. The essential oils and components were as follows: lemon (*Citrus lemon*), and limonene; juniper (*Juniperus communis*), and α-pinene; marjoram (*Origanum majorana*), and terpinene-4-ol; clary sage (*Salvia sclarea*), and linalool, cinnamon (*Cinnamomun zeylanicum*), and cinnamaldehyde; thyme (*Thymus vulgaris*), and thymol. Oils showing excellent antimicrobial activity were tested for their preservative effect in real foods.

OBJECTIVES

1. Effect of essential oils and their major components on food spoilage and pathogenic microbes: antimicrobial and anti-biofilm forming effect.

2. Detection of QS signal molecules from food isolates. Investigation of the effect of essential oils and their major components on the QS mechanism.

3. Use of essential oils for extension of shelf life of foods (meat, fruits, vegetables, fruit juice).

MATERIALS AND METHODS

Bacterial strains: *Bacillus subtilis; B. cereus var mycoides; Escherichia coli; Pseudomonas putida; P. fluorescens; Staphylococcus aureus; Listeria monocytogenes; Pichia anomala; Debaryomyces hansenii; Saccharomyces cerevisiae*

Biosensor strains: *Chromobacterium violaceum* 85WT and CV026*; Serratia marcescens; Rhizobium radiobacter* NTL4 (pZRL4); *Escherichia coli* JM 109 pSB401*; Escherichia coli* JM 109 pSB535*; Escherichia coli* JM 109 pSB1075.

Essential oils and components

Essential oils	**Juniper**	**Lemon**	**Cinnamon**	**Thyme**	**Marjoram**	**Clary sage**
Origin	*Juniperus communis*	*Citrus lemon*	*Cinnamomun zeylanicum*	*Thymus vulgaris*	*Origanum majorana*	*Salvia sclarea*
Main component	α-pinene	limonene	trans-cinnamaldehyde	thymol	terpinene-4-ol	linalool

DNA based techniques

- Purification of genomic DNA
- Agarose gel electrophoresis
- Polymerase chain reaction (PCR)
- DNA sequencing, sequence analysis
- Nucleotide sequence analysis (BLAST)

Antimicrobial and anti-biofilm forming effect of essential oils

- Microdilution method
- Anti-biofilm forming effect tested with crystal violet staining

Scanning electron microscopic techniques (SEM)

Investigation of the QS mechanism

- Detection of QS signal molecules with biosensor strains produced by strains isolated from foods
- Detection of QS inhibition with paper disc diffusion assay in solid cultures
- Detection of QS inhibition in liquid cultures
- Searching for a link between QS and biofilm formation

Effect of essential oils on foods

- Standard methods used for investigations of the quality of foods
- Investigation of marinating solutions and vapour phase containing essential oils
- Sensory evaluations

RESULTS

1. All essential oils and components had good antimicrobial and anti-biofilm forming effect.

We isolated 28 pure cultures from the food samples and 27 were subjected to sequencing. Besides laboratory strains the effect of EOs and components was also tested in case of two isolates: the opportunistic pathogen *P. agglomerans* and the meat-spoiler *K. gibbsonii*.

Cinnamon, and thyme EOs and their main components had the lowest MIC values. The composition of marjoram and thyme changed after a stock update in 2015. In both cases the major component changed to p- cymene and this resulted in higher MIC values for these oils.

In case of biofilm formation for G-positive bacteria the most effective oils were cinnamon and cinnamaldehyde, less effective was lemon and juniper. Food isolates were sensitive to cinnamon as well; marjoram and terpinene-4-ol had no significant effect.

In mixed-cultures of *Listeria* and *E. coli*, *Listeria* overgrew *E. coli* but after adding EOs to the culture, *Listerial* growth was inhibited and the difference in cell numbers was reduced. Mixed cultures of *Listeria* and *Staphylococcus* were more resistant; only high concentrations of EOs reduced their formation. The change in the composition of marjoram and thyme EO resulted in a less effective anti-biofilm forming effect.

We confirmed the destructive effect of EOs on the biofilm of bacteria in mono- and mixed culture biofilms with scanning electron microscopy.

2. We demonstrated the presence of AHL signal molecules from bacterial isolates. EOs and components resulted to be good anti-QS agents.

Long chain AHL signal molecules were produced by bacteria isolated from chicken meat, paprika, horse radish and packed salad. Short chain AHLs were detected from duck meat and germ mixture. We could not detect AI-2 signal molecules from our isolates, only *P. anomala* laboratory strain showed signs of AI-2 production.

Using paper disc diffusion assay we demonstrated that besides lemon all EOs and components had good anti-QS effect. In case of *S. marcescens* in most cases only growth was inhibited. The anti-QS effect of the oils was higher in liquid cultures in addition to a constant cell number even at low concentrations for both of the biosensor strains. This suggests that the oils interfere with the QS system.

We found a link between QS and biofilm formation; our treatments reduced both the pigment production and biofilm formation of *C. violaceum*. Also, it seems that QS plays an important role in the attachment of cells to surfaces, this is supported by results obtained with cinnamon EO.

3. With the aid of EOs we inhibited the formation of *L. monocytogenes* biofilms on chicken meat and increased the shelf-life of strawberries, cherry tomatoes and grape juice.

After marinating with EOs, thyme and its major component thymol inhibited biofilm formation of *L. monocytogenes*; this inhibition was more successful in marinating solutions. Pretreatment with washing of the meat did not influence total germ counts. On the contrary, *Listeria* cell numbers grew in the pretreated samples after 24 hours. This could mean that an antagonist was present on the meat which was removed by washing. The taste and odour of samples marinated with thyme and thymol was similar to the control samples; the spicy taste harmonised with the flavour of the meat.

Strawberries treated with lemon vapour remained fresh 1-2 days longer than the untreated samples. The disc can be removed after 24 hours without decreasing the shelf-life elongation effect. The taste and odour of the fruits was not negatively influenced by the EO.

Using 20µl/l thyme EO we increased the shelf life of cherry tomatoes with 4 days, as for 40µl/l concentration this time was increased with 8 days. The taste and odour of the vegetables was spicy and pleasant.

We detected higher MIC values for lemon in grape juice than in MEA broth but these were lower in case of cinnamon. Mild heat treatment increased the effect of EOs in all cases.

SUMMARY

1. We isolated 28 pure cultures from the food samples and 27 were subjected to sequencing.
2. The essential oils and components tested had good antimicrobial and anti-biofilm forming effect. A change in the composition of marjoram and thyme oils increased MIC values and anti-biofilm forming effect.
3. The destructive effect of EOs on the biofilm of bacteria in mono- and mixed culture biofilms was confirmed with scanning electron microscopy.
4. We demonstrated the presence of AHL signal molecules from bacterial isolates. EOs and components resulted to be good anti-QS agents.
5. A link was found between QS and biofilm formation; our treatments reduced both the pigment production and biofilm formation of *C. violaceum*.
6. With the aid of EOs we inhibited the formation of *L. monocytogenes* biofilms on chicken meat and increased the shelf-life of strawberries, cherry tomatoes and grape juice.

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