UTILITY OF PHYTOPHENOLIC COMPOUNDS IN FOOD SYSTEMS- THE IMPACT OF TEMPERATURE ON ANTIMICROBIAL ACTIVITY

by

GRISHMA KOTWAL

(Under the Direction of Faith Critzer)

ABSTRACT

The objective of this study was to quantify the antimicrobial activity of cinnamaldehyde, carvacrol and eugenol after exposure to various temperature conditions, against *Escherichia coli* O157:H7 and *Salmonella enterica* serovars. Compounds were subjected to temperature exposure for 0 h, 0.5 h, 1 h, 4 h, at 60°C and 70°C, and for 0 h, 12 h, 24 h, 48 h, 72 h, 7 d, 14 d and 21 d at 4°C and 25°C. After temperature treatments, concentrations of 1, 2, 5, and 10 mM of each antimicrobial were evaluated in order to determine the minimum inhibitory concentration (MIC) through microbroth dilution assay. Afterwards, antimicrobials were evaluated in milk, which served as a model food system. Carvacrol was the most effective *in vitro* with an MIC of 2 mM, followed by cinnamaldehye and eugenol with MICs of 5 mM and 10 mM, respectively. The bactericidal concentration in milk increased to 60 mM for carvacrol and eugenol while cinnamaldehyde was found to be 10 mM.

INDEX WORDS: Carvacrol, Cinnamaldehyde, Eugenol, Temperature, MIC, Milk

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For my mother who never believes in me

and

For my father who always does…

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Food safety is one of the most important factors affecting public health and well being. The development of safer methods for the procurement of raw material, preparation of the product, storage, transportation and distribution of the finished commodity, ending at the plate of the consumer, has been a continuous research process for food microbiologists around the world. Even after tremendous improvements in the field, work is still needed. While it is difficult to quantify the impact foodborne illnesses have in the United States, it has been estimated that foodborne diseases cause 76 million illnesses annually and approximately 5,000 deaths *(37)*. These estimates clearly demonstrate the need for more work towards designing better methods of food protection and preservation.

The use of preservatives in the form of food additives is an important intervention in the determination of product safety. These preservatives may slow down microbial growth, inactivating microorganisms, or preventing their entry into the food systems *(23, 36, 15)*. At the same time it is important to know that the introduction of a new additive into the food industry is a very long, cumbersome, and expensive process. This makes the option of using antimicrobial compounds which are present naturally in foods more feasible from an economic standpoint.

There has also been an increase in consumer awareness about healthy diet through fresh food intake. The consumer demand for „high quality fresh foods" with minimum processing and less synthetic additives and less impact on the environment is on the increase (*10*). The 2010 FDA report stating they would work in collaboration with different agencies to support the

reduction in salt content in food supply also calls for an additional preservation technology to ensure the safety of these foods (*18*).

A technology which has a potential to address to all the above mentioned conditions is the use of phytophenolic essential oil components. These essential oils are aromatic oily liquids present in the essential oil fractions of various parts of a plant body. These compounds have a long history of use in foods in the form of spices and flavoring agents. Studies have been conducted to determine their antimicrobial activity, and they have been shown to be antibacterial *(13, 41)*, antimycotic *(40)*, antiparasitic *(46)* and antiviral (*6*). However, it is important to understand the properties and composition of these essential oils, the antibacterial nature of their components, mechanism of action, present usage in food, and the need to study their activity with other processing parameters in order to effectively incorporate them into foods as an antimicrobial compound.

Historical use of essential oils

The term essential oil has been thought to be derived from the word „*Quinta essentia*", which was coined by a Swiss physician, Paracelsus, in the 16th century for the oils obtained after distillation of various parts of a plant body. He believed these to be the most active parts of a tree, plant, shrub or a flower. Historically, these oils have been used as spices, flavoring agents, perfumes, preservatives and embalming antiseptics *(3)*. India, Persia and Egypt started the use of distillation as an extraction procedure for these oils, but the first authentic written account of the process was given by Villanova (1235-1311), a Catalan physician. In the 17th century the use of essential oils was largely for pharmaceutical purposes *(24)*. Although believed to be medicinal in nature, it was not until 1881 that De la Croix studied the bactericidal properties of essential oil vapors *(7)*.

The reintroduction of essential oils into modern medicine began during the late 19th and early 20th centuries. The current use of essential oils or their components ranges from flavorings in food, pharmaceuticals, aromatherapy, dental surgeries, antiseptics, feed supplements and insect repellents.

Chemical composition

Essential oils are commercially recovered by steam distillation, pressing, or solvent extraction. The nature and concentration of their components determine their use in the food industry *(12)*. The composition of the oil varies with the method of extraction, which is shown by the difference in the organoleptic profile of the resultant, which may influence the antimicrobial properties of the oil *(10)*. The compositional analysis of essential oil is determined with gas chromatography-mass spectrometry (GC/ MS) *(14)*. An essential oil can be made of up to 60 components *(51)*, out of which the main components responsible for antimicrobial properties are the phenolic compounds. The composition of essential oils varies with geographical sources and with harvesting seasons *(11)*. The composition of the essential oils also differs for different parts of the same plant *(14)*.

Antimicrobial activity of natural phenolic compounds

Phenolics are the derivatives of phenol, which consist of a hydroxyl group (OH^-) that is directly bonded to a hydrocarbon group. They have relatively high acidity due to the strong bond between oxygen and the aromatic ring and along with a loose bond between oxygen and hydrogen. These phenolic compounds are classified as simple phenols and phenolic acids, hydroxycinnamic acid derivatives, flavonoids and tannins *(12)*. The antimicrobial activity of phenolics depends on several factors such as, the microbial species to be studied, the type of phenolic compound and concentration, combination effects with other antimicrobials,

temperature, other food additives, and the food components (*e.g.*, proteins, lipids, and minerals) *(48)*. The phytophenolics possess varying degrees of antimicrobial capacities. They may be obtained from several parts of the plant body such as, stems, barks, leaves, flowers and fruits. As mentioned above, the chemical composition of phytophenolics is influenced by the geographic origin and also the crop to crop variations, which may consequently lead to changes in the antimicrobial activity of the whole spice or the essential oil *(42)*. It has also been seen that a change in the method of evaluating antimicrobial capacity can alter the results when evaluating the same compound *(26)*.

Plant phenolics are biosynthesized through two major pathways. The shikimic acid pathway is the primary synthesis mechanism in plants. While the malonic acid pathway is an important mode of synthesis for these compounds in fungi and bacteria and less significant in higher plants. The shikimic cycle derives carbohydrate precursors from glycolysis and pentose phosphate pathway and converts them into aromatic amino acids. Three phytophenolic compounds will be discussed in greater detail: cinnamic aldehyde (3-phenyl-2-propenal), carvacrol (5-isopropyl-2-methylphenol) and eugenol (4-allyl-2-methoxyphenol).

Mechanism of action of phytophenolics

The fact that essential oils are made of a large number of different groups of chemical compounds clearly indicates that they do not follow a single mechanism of antimicrobial action. Several mechanisms and site of action on bacterial cells have been discussed to date, namely the damage to cell membrane leading to leakage of cellular contents *(28)*, damage or denaturation of membrane proteins *(60)*, damage to the cytoplasmic membrane *(62)*, coagulation of cytoplasm *(25)*, and depletion of proton motive force *(61)*.

It has also been postulated that most of the active antimicrobial components of an essential oil are phenolic compounds, and their mechanism of action should be similar to phenols. The antimicrobial activity of phenols has been shown to be concentration dependent; at lower concentrations they may inhibit enzyme activity while at high concentration they cause protein denaturation. Phenolic compounds also have the ability to alter the bacterial cell membrane permeability leading to the loss of macromolecules thereby negatively effecting the microbial growth and energy production, leading to cell death *(12)*.

Hydrophobicity is another important property of essential oils that may affect their mode of action. This property enables them to partition in the cell membrane lipids, disturbing the membrane structure and increasing permeability *(55)*. In addition to disturbing the cell membrane, this could also play a role in depletion of the proton motive force.

Cinnamic aldehyde

Cinnamic aldehyde or cinnamaldehyde (Fig. 1) is scientifically known as 3-phenylpropenal. It constitutes 65 to 75% of the oil of the bark of cinnamon plant. It is a yellow liquid at room temperature and possesses strong odor characteristics of cinnamon. It belongs to the group phenylpropenoids of the essential oils, which have an aromatic ring of six carbons attached to a chain of three carbons. It has a benzene ring substituent acrylic aldehyde having a double bond, which makes the geometry planar. This may occur in *cis* form, but is more commonly found in the *trans* configuration. It is derived from phenylalanine which is synthesized by the shikimate metabolic pathway. It was first isolated in 1834 by Dumas and Peligot. Today it is commercially produced through steam distillation of cinnamon bark oil.

The properties of cinnamaldehyde that have been widely studied are its antioxidant, antifungal and antibacterial potential. The phenolic coefficient has been determined as 7.1. This

coefficient is the ratio of the dilution of a given compound being tested, to the dilution of phenol that is required to kill the same microorganism, under controlled time and temperature conditions. A value of 7.1 is an indicator of the strong antimicrobial properties of this phenolic compound.

Bullerman (1974) observed that a 1% to 2% ground cinnamon in broth allowed some growth of *Aspergillus* but reduced the production of aflatoxin up to 99% *(8)*. Later Bullerman studied the concentration of cinnamon essential oil which would be inhibitory to growth and aflatoxin production by *Aspergillus parasiticus* in yeast extract sucrose broth as substrate *(9)*. The concentration which delayed growth of *Aspergillus* was 200 ppm for cinnamon oil, while the concentration of its component cinnamic aldehyde producing the same effect was 150 ppm. When the concentration of cinnamic aldehyde was increased to 200 ppm complete inhibition of growth was observed. Cinnamon essential oil fraction has also shown inhibitory activity against both Gram-negative and Gram-positive bacteria such as, *Salmonella*, *Escherichia coli, Clostridium perfringes, Staphylococcus aureus, Streptococcus* and *Yersinia (1)*.

A single mechanism of action has not been identified for cinnamaldehyde. In a study conducted by Becerril et al. *(4)*, in 2007, transmission electron microscopy (TEM) was used to determine the action on the bacterial cell of active packaging containing cinnamon. The results showed that the periplasmic space of the bacteria was altered, becoming larger and uneven. Cell wall damage was also seen, followed by reduction in cytoplasmic material along with formation of blebs. An extraction procedure was conducted on the dead cells and GC/ MS identified the presence of cinnmaldehyde in cells. The bactericidal action of cinnamic aldehyde against *Listeria monocytogenes* has also been tested. The mechanism of action against the Gram - positive bacteria was studied and identified as the depletion of adenosine 5´-triphosphate in cells,

thereby making inhibition of energy metabolism in the bacteria an important mechanism of action *(22)*.

Carvacrol

Carvacrol (Fig. 2) is a monoterpenoid phenol, with a hydroxyl group and delocalized electron system that contribute to its antimicrobial effect *(63)*. It forms a major part of the essential oil fraction of oregano and thyme. It forms 40 to 70% of oregano oil (*Origanum vulgare*) which is a perennial plant member of the *Labiatae* family. It may also comprise up to 45% of the thyme essential oil. However, these percentages may vary with climate, time of harvest and storage conditions.

The antimicrobial activity of carvacrol has been studied in the past years along with its precursor p-cymene, and the biological mechanisms which influence its antimicrobial nature have been of much interest. Its effect has been studied over a wide range of bacteria and has been found to be strong alongside thymol which has a similar structure with just the difference of having hydroxyl group on the meta position.

The mechanism of action of carvacrol has been hypothesized by Ultee et al. in 2002 *(63)*. This study illustrated the major role played by the hydroxyl group in the events leading to cell death of *Bacillus cereus*. Carvacrol and its precursors destabilize the cell membrane by accumulating in the cell membrane causing spacing of phospholipids leading to expansion and consequent dissemination of ions out of the cell. The carvacrol molecule in its undissociated form passes through the cytoplasmic membrane of the bacteria. Inside the cell it dissociates thereby releasing a proton, leading to a decrease in internal pH. It then attracts a potassium ion and migrates out of the cell undissociated whereby it is again protonated and led through the

cytoplasmic membrane. This futile cycle caused by delocalized electrons leads to efflux of potassium ions and reduction in ATP generation in cell resulting in cellular death.

Another study investigated the effect of carvacrol, menthol, eugenol and two carvacrol derivative compounds against *E. coli* and several other bacteria such as *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *Bacillus subtilis*, a yeast *Saccharomyces cerevisiae* and one fungi *Botrytis cinerea (5)*. The study proved carvacrol to be the most inhibitory amongst evaluated compounds against *E. coli* and other organisms studied. The increased antimicrobial efficacy was attributed to its hydrophobicity along with the above mentioned properties. The toxicity of a hydrophobic compound is most observed on the cytoplasmic membrane *(54)*. The lipophilic nature of this compound attracts it to the cell membrane and the physical and chemical properties of the membrane are affected due to this intrusion leading to reduction in bilayer stability and enhanced proton passive flux, overall compromising the cellular integrity *(5)*.

The activity of carvacrol against *Salmonella enterica* was studied by Olasupo et al. (2003) to determine the minimum inhibitory concentration (MIC) which is defined as the lowest concentration of an antimicrobial that results in no visible growth *(43)*. The MIC of carvacrol was determined to be 1.0 mM, which shows its high activity against this microorganism. Another study conducted for *in vitro* assessment of its activity against *Salmonella* serovars through measurement of optical density at 600 nm showed a reduction in counts up to 6 $\log_{10}$ units *(54)*. In a 2005 study, twenty eight essential oils were evaluated on their antimicrobial potential against four pathogenic bacteria (*E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus*). *Corydothymus capitatus* (Spanish oregano) which

contains 76% carvacrol was found to be the most effect natural antimicrobial against both Gram-negative and Gram-positive bacteria.

The activity of carvacrol against *E. coli* in different food complexes has been studied recently. Friedman et al. (2004) studied the effect of 17 plant essential oils on *E. coli* O157:H7 and *Salmonella* in apple juice *(20)*. The assay determined the percentage of the compound that resulted in 50% reduction in bacterial population. They found that at 5 min and 21˚C carvacrol was the most effective compound against *E. coli*. Here cell death was shown to be due to disruption of the proton-motive force followed by depletion of intercellular ATP pool. This was attributed to increased membrane permeability of ions through the phenol-damaged membrane *(28, 61)*. The increased efficacy of carvacrol at low pH due to increased hydrophobicity allows it to interact more easily with the lipids present in the cell membrane *(35)*.

In a recent study, carvacrol was tested for its ability to reduce heat resistance of *E. coli* O157:H7 in ground beef *(30)*. The experiment illustrated how a D-value of 63.9 min at 55˚C reduced to 24 min when carvacrol was incorporated at 0.5%, and to 18.16 min with 1% carvacrol. Similar tests were performed for cinnamic aldehyde which led into similar results.

The results indirectly suggest that not only do phenolic antimicrobial compounds possess strong antibacterial properties at ambient conditions but their activity may remain intact at higher temperatures as well, thereby suggesting activity under high temperature processes. The phenomenon of retention of antimicrobial capacity under different set of temperature conditions over varying time periods needs to be studied extensively in order to determine the feasibility of incorporating natural antimicrobials under various processing conditions. This would satisfy today"s consumers need for food products with more fresh-like attributes with the acceptable natural ingredients but at the same time microbiological safety as well.

Eugenol

Eugenol (Fig. 3), commonly called clove oil, is an alkyl chain-substituted guaicol. It is a pale yellow liquid sparingly soluble in water but soluble in organic solvents. As the name suggests, it is a major constituent of the essential oil fraction of cloves (*Syzygium aromaticum, Eugenia aromaticum* and *Eugenia caryophyllata*) comprising 72 – 90% of the constituents. Other than clove, lower amounts may be found in cinnamon, nutmeg and bay leaf. It has also been evaluated to determine its antioxidant and antimicrobial properties.

One of the early studies was carried out to study eugenol"s inhibition against *Salmonella* Typhimurium, *Staphylococcus aureus* and *Vibrio parahaemolyticus.* Eugenol was very effective against all three pathogens with a MIC of 100 µg/ml and remained so at inoculum levels of ~7.0 $\log_{10}$ CFU *(31)*. This study showed eugenol to be the most effective antimicrobial when compared to thymol, anethole and menthol. Eugenol was not as effective at inhibiting fungal growth which established its status as more of a bacteriostat/bacteriocide rather than fungistat/fungicide. Eugenol was also one of the ten most effective compounds against *E. coli* O157:H7 with a range from 0.018%-0.093% of the compound to get a 50% reduction in the bacterial population after 60 min of exposure *(20)*.

In a recent study, the mechanism of action of eugenol against *Salmonella* Typhi was discussed. The treatment with 0.0125% eugenol resulted in reduced viability and with 0.025% resulted in complete inhibition of the organism *(17)*. The effect of eugenol on the cytoplasmic membrane was studied through crystal violet assay and showed increased permeability and confirmed disruptive action on cytoplasmic membrane. Fourier transform infrared spectroscopy (FT-IR) further verified the deformation of macromolecules in the membrane after exposure to eugenol.

Activity against Gram-negative vs. Gram-positive bacteria

Many studies have shown the difference in action of antimicrobials against Gram-negative and Gram-positive bacteria, showing that their effect is more pronounced against Gram-negative organisms. Differences in cell membrane have been attributed to these differences since Gram-negative organisms possess an outer membrane which surrounds the cell wall. This membrane is the major site of action of these compounds, against Gram-negative bacteria, as was illustrated by Kim et al. (2004) when the action of cinnamic aldehyde (cassia shoot) was evaluated against *E. coli* O157:H7 *(33)*. *E. coli* O157:H7 cells were observed under scanning electron microphotograph (SEM) after incubation at 37°C for 2 h and showed prominent outer membrane disintegration, which lead to an increase in the permeability causing depletion of intracellular ATP *(33)*. However, several studies have demonstrated opposite or no difference in susceptibility *(13, 16)*. The degree of activity against Gram-positive and Gram-negative bacteria may be variable depending on the individual components of an essential oil *(16)*. The variability in the composition of the essential oil of the same plant species due to different geographic origin and harvesting period is enough to cause a varying degree of susceptibility against Gram-negative and Gram-positive bacteria *(10)*.

Antimicrobial activity *in vitro*

In vitro studies have established strong activity of these antimicrobials between levels of 0.2 and 10 µL/ ml against major bacterial pathogens like *Listeria monocytogenes, Salmonella* Typhimurium*, E. coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus.* The absence of standardized tests confounds comparisons between various studies. Researchers try to adapt experimental methods to better represent future applications. The National Council of Clinical Laboratory Services (NCCLS) method for the determination of antibacterial

susceptibility of antibiotics has been modified for testing essential oils. But since the results of a test depends on several factors like the pH, incubation time, temperature, the method used in extraction of essential oil and the culture medium used *(49)*, even with the same method different results may be obtained thereby making comparison difficult. Therefore there is an immense need to not only standardize test methods, but also regulate other parameters during the test so that comparable data may be obtained. An important step towards standardizing can be the use of the pure essential oil components for the experiment to nullify the effect of the anomaly caused by changing chemical compositions of the essential oil extract as a whole.

Current test methods used for determining various aspects of antibacterial activity range from assays which screen for activity, like disk diffusion and agar wells, time-kill assays for determining the rapidity and duration of activity, and scanning electron microscopy for observing the effects of the activity on target cells. The strength of the antimicrobial is primarily tested through two methods, agar dilution method and the broth dilution method. The broth dilution method itself has been tested on different parameters namely, visible growth *(45, 2)*, optical density *(52, 60, 56, 38)*, colorimetric *(21)*, conductance *(58)*, and viable count *(19)*. The microbroth dilution method helps in the determination of the MIC. It can be carried out on a microscale as well with volumes in the range of 300-200 µl.

Antimicrobial activity in food systems

Ultimately, it is important to establish the antimicrobial capacity of an antimicrobial in foods. A large number of studies have been carried out in foods. Generally, much higher concentrations of the antimicrobial are needed in food than in the microbiological medium to produce the same effect *(53)*. A number of reasons have been proposed to explain this change in activity. Gill et al. *(21)* in 2002 explained that the reduction in activity may be due to the

presence of higher amount of nutrients in food systems when compared to broth, which may help the bacteria have faster cellular repair. Another reason could be the effect of the intrinsic parameters of the food component which inhibit the reaction between the oil in the lipid phase and the bacterial cell in the water phase. The essential oil dissolves in the lipid phase of the food, thereby reducing the amount available for action against bacterial cell *(38)*. It was proposed that the proteins and fats in the milk interfere in the antimicrobial action by sequestering antimicrobial constituents. The essential oil attaches to the fats due to their hydrophobic nature, and also to the proteins which possess hydrophobic side chains. Protein was determined to be the limiting factor for antimicrobial action of clove oil against *Salmonella* Enteriditis in cheese *(57)* and carvacrol against *Bacillus cereus* in milk *(47)*. Other inherent parameters of the food complex like the water content, presence of preservatives, salt, antioxidants and other additives along with the pH can also affect the activity of the essential oil component. Temperature and the packaging of the food are extrinsic parameters that may also affect the MIC of microorganism in food and may vary depending on the microorganism investigated (*10*).

The activity of cinnamic aldehyde, carvacrol, and eugenol have been tested in foods, namely: carvacrol in vacuum packaged minced pork *(29)*, red grouper *(34)*, cod and salmon fillets *(38)*, semi-skimmed milk *(32)*, boiled rice *(62)*, and kiwi fruit *(50)*, cinnamic aldehyde in shrimp *(44)* and alfalfa seeds *(64)*, and eugenol in minced mutton and cheese *(39)*, cooked chicken breasts *(27)*, and cooked pork *(58)*. The results showed strong antimicrobial action of these compounds against a range of bacteria with variable reduction in final population.

Conclusions

The antimicrobial effect of natural phenolic compounds has been well studied. At the same time it is important to realize that determining of their antibacterial activity is not enough on its own. The main aim is to be able to put this knowledge to use in the real world. It is essential to study the retention of their antimicrobial activity in different set of processing conditions so that some practical usage of these components in the food industry can be established. There are very few studies which look at this aspect, and there is a crucial need for more knowledge on this front to be able to make correct use of these naturally available compounds. The aim of this study is to determine the effect of different temperatures that are commonly used during food processing on the antimicrobial activity of three major phytophenolic components to provide relevant information to food processors when they seek to incorporate these compounds in foods.

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Chapter 2

UTILITY OF PHYTOPHENOLIC COMPOUNDS IN FOOD SYSTEMS - THE IMPACT OF TEMPERATURE ON ANTIMICROBIAL ACTIVITY

Food preservation is the process of slowing down growth or complete inactivation of microorganisms in foods in order to maintain their safety, quality, nutritive value and palatability. Several different methods of preservation are in use today, ***e.g.,*** drying, addition of salt or sugar, freezing, vacuum packaging, spray drying and thermal processing. An important method of preservation is the addition of food additives which may be natural (salt, sugar and vinegar) or synthetically derived (calcium propionate, sodium nitrate and sodium benzoate). Preservation of food through the use of natural compounds which possess antimicrobial properties in order to achieve high quality foods with fresh like attributes is one of the major areas of focus today *(5)*. The consumer demand for additives that are perceived to be "natural", and the long and expensive process for approval of new chemical compounds, has increased the need for naturally available compounds tremendously.

These phytophenolic compounds are inherently present in plants and have been used traditionally in their natural form as preservatives. The antimicrobial effect of these essential oils has been primarily attributed to the phenolic compounds present in them.

Several studies have reported the strong antimicrobial activity of these compounds but further research is needed to determine the effect of variable processing conditions on their activity. The determination of the effect of these different parameters on their antimicrobial capacity will help food processors to effectively incorporate them under suitable processing conditions and in appropriate foods.

One of the most important processing parameter is temperature and its effect on the activity of these compounds is crucial for the determination of their efficacy in foods. This parameter has not been studied well in the past, but with the growing need of natural antimicrobials in foods useful information is needed on this front.

There are two studies to date which have studied the effect of temperature on the efficacy of natural antimicrobials. One study evaluated the effect of temperature on the antimicrobial activity of *Origanum vulgare* essential oil *(4)*. They determined the strong antimicrobial activity of the essential oil against *Escherichia coli, Staphylococcus aureus, Yersinia enterocolitica, Salmonella enteriac* and *Serratia marcencen*s and showed that the activity of the antimicrobial was maintained at higher test temperatures. Another study determined the effect of thermal oxidation on pure vanillin and its antimicrobial activity. The results demonstrated increased efficacy due to conversion to vanillic acid, which is a stronger antimicrobial compound *(7).* These studies provide pertinent information, but a lot of work is still needed to establish efficacy of other important phenolic compounds to allow appropriate usage.

The objective of this study was to determine the effect of temperature on the antimicrobial activity of three major phytophenolic compounds: carvacrol, cinnamic aldehyde and eugenol. These compounds have already been established as strong antimicrobial agents and this study will provide important information on the effect of different processing conditions on the retention of their efficacy, which if positive shall allow effective incorporation in pertinent foods.

Material and Methods

Cultivation of microorganisms

Five *Salmonella enterica* serovars (Typhimurium, Enteriditis, Gaminara, Agona, and Montevideo) and *Escherichia coli* O157:H7 strains (EDL933**,** 932, E0019, H1730, and F4546) were obtained from Dr. Larry Beuchat, University of Georgia Center for Food Safety culture collection. The strains were cultured in trypticase soy broth with 0.5% yeast extract (TSBYE; Difco Becton Dickinson Microbiology Systems Sparks, MD) overnight at 37°C in a shaking incubator. All cultures were transferred a minimum of three consecutive times in 24 h intervals prior to use.

Preparation of inoculum for microbroth dilution assay

Five *Salmonella enterica* serovars and five *E. coli* O157:H7 strains were diluted with TSBYE and 0.05% (w/v) agar (Difco Becton Dickinson Microbiology Systems Sparks, MD), to approximately 5 $\log_{10}$ CFU/ml for the microbroth dilution assay.

Natural antimicrobials

Three antimicrobials, cinnamaldehyde (99%; MP Biomedicals; Solon; OH), eugenol (99%; Acros organics; Morris Plains, NJ), and carvacrol (98+%; SAFC; St. Louis, MO), were utilized for all antimicrobial assays.

Temperature treatment of antimicrobials for microbroth dilution assay

One point four milliliter of each antimicrobial was placed in a 1.5 ml Argos black microcentrifuge tube (Argos Technologies; Elgin, IL) for temperature treatment. Tubes were placed in 4 and 25°C incubators for 12 h, 1, 2, 3 7, 14, and 21 d. For higher temperatures, tubes were placed on a heating block for 0.5, 1 and 4 h at 60 and 70°C. The tests for antimicrobial efficacy were carried out immediately after the temperature treatments.

Microbroth dilution assay

The cultures were diluted with TSBYE and 0.05% agar to 5 $\log_{10}$ CFU/ml. The untreated (control) and temperature treated antimicrobials were tested at four concentration levels of 0, 1, 2, 5 and 10 mM. The stock solutions of were prepared in 100 ml TSBYE and 0.05% agar in 200 ml screw capped bottles in a biological safety cabinet. The function of agar was to serve as a stabilizer preventing the separation of essential oil from the broth phase facilitating better interaction with bacterial cells *(2)*. Ninety-six microwell plates (Nunc; Thermo Fisher Scientific; New York, NY) were used for the assay. The antimicrobial stock solutions in TSBYE and 0.05% agar were poured in 50 ml sterile polystyrene disposable pipette basins (Fisherbrand; Pittsburgh, PA), followed by dispensing 216 µl into the sample wells by eight channel pipettes (Finnipipette; Thermo Scientific), and finally addition of 24 µl of 5 $\log_{10}$ CFU/ml of each strain individually in separate wells, making the total volume 240 µl and a final bacterial population of 4 $\log_{10}$ CFU. Two hundred and sixteen microlitre of TSBYE and 0.05% agar with 24 µl of bacterial inoculum served as the positive control, and for the blank 240 µl of TSBYE with 0.05% agar was used. Three samples of each treatment combination were evaluated per replication.

The absorbance was read at 630 nm using an ELx800 Absorbance Microplate Reader (BioTek; Winooski; Vermont) for 0, 6, 12, 24, and 48 h. The plates were incubated at 37°C between the readings. The entire experiment was conducted in duplicate. The absorbance data obtained over five time periods was used to analyze the inhibitory effect of the temperature treated antimicrobials on the individual strains.

The average of the six data points, and the blanks, for a particular sampling time were calculated. The blank average was subtracted from the sample reading and the resultant absorbance values were plotted on XY scatter graphs, and the minimum inhibitory concentration

(MIC) was determined. The MIC was the concentration at which no increase in absorbance occurred over the 48 h sampling time.

Preparation of inoculum for milk assay

Bacterial cultures were cultivated as previously described. Five serovars or strains of *Salmonella* or *E. coli* O157:H7, respectively, were combined in a 50 ml centrifuge tube (Corning Incorporated; Corning, NY) in 5 ml volumes to yield a five serovars/strain cocktail with a volume of 25 ml. The bacterial cells were harvested by centrifugation at 8000xg for 10 min at 4°C. After this the supernatant was discarded and the pellet was resuspended in 25 ml of 0.1 M phosphate buffer (PB; Becton Dickinson Microbiology Systems; Sparks, MD) solution. The washed inoculum was diluted in 0.1% peptone water (PW; Difco; Sparks, MD) to a population of approximately 6 $\log_{10}$ CFU/ml.

Temperature treatment of antimicrobials for the milk assay

One point four milliliter of each antimicrobial was placed in a 1.5 ml Argos black microcentrifuge tube for temperature treatment. Tubes were placed in 4 and 25°C incubators for 21 d. For higher temperatures, the tubes were placed on a heating block for 4 h at 60 and 70°C. The tests for antimicrobial efficacy in milk were carried out immediately after the temperature treatments.

Incorporation of antimicrobial to milk

Grade A ultra high temperature pasteurized whole milk (Parmalat; Farmland Dairies; Wallington, NJ) was used for the assay. The milk was transferred into 99 ml aliquots in 200 ml sterile screw capped bottles in a biological safety cabinet. The cinnamic aldehyde was added at 0, 5, 10 and15 mM in the UHT milk, and 0, 10, 20, and 40 mM concentrations were evaluated for eugenol and carvacrol in UHT milk.

Inoculation of milk

One ml of the 6 $\log_{10}$ CFU/ml *Salmonella* or *E. coli* O157:H7 cocktail was added to evaluate the effect of the antimicrobial, making the final inoculum level approximately 4 $\log_{10}$ CFU/ml. The inoculated milk bottles were kept at 37°C in a static incubator (Isotemp; Fisher Scientific; Pittsburgh, PA) between sampling times.

Enumeration of bacteria for milk assay

The sampling of the microbial population occurred at 0, 12 and 24 h. Serial dilutions were made in 0.1% peptone broth. Hundred microlitre of the dilution was plated on tryptic soy agar (TSA; Difco Becton Dickinson Microbiology Systems) and xylose lysine tergitol 4 agar (XLT4; Difco Becton Dickinson Microbiology Systems) for *Salmonella*, and TSA and cefixime tellurite sorbitol MacConkey agar (CT-SMAC; Difco Becton Dickinson Microbiology Systems) for *E. coli* O157:H7 in duplicate, using a WASP 2 Spiral plater (Microbiology International, Frederick, MD). The enumeration of the bacterial population occurred after incubation for 24h at 37°C using an automated plate counter (Acolyte; Frederick, MD).

Data Analysis for milk assay

All experiments were replicated two times and three samples were analyzed for each replication. The statistical model consisted of a randomized block design, blocking on replication due to variations in inoculum population. Statistical analysis was conducted using the mixed models procedure (PROC MIXED) of SAS® 9.2 (SAS Institute Inc.; Cary, NC) and significance of factors set at $P<0.05$. Analysis of variance was used to determine statistical differences in survival of pathogens.

Results and Discussion

Overview

The results of the microbroth dilution assay provided important information about the retention of the antimicrobial properties of the three essential oil components: carvacrol, cinnamic aldehyde and eugenol over varying time temperature treatments. The milk assay also allowed insights to the effects of antimicrobial capacity in conjunction with the components of a food complex (*e.g.,* lipids, proteins, divalent cations).

Carvacrol was found to be the most effective natural antimicrobial in the microbroth dilution study. Its monoterpenoid phenolic structure, with a hydroxyl group and delocalized electron system contributes to its antimicrobial effect *(10)*. At same time, it was also found that the MIC was wavering with the efficacy reducing over time at lower test-temperatures while at higher temperatures the reduction occurred within 30 min. Nevertheless, it still maintained a low MIC of 2 mM even after reduction in activity.

Cinnamaldehyde was the second most effective compound in the microbroth dilution assay. It maintained activity until 21d exposure at lower temperatures (Table 1 and 2), and up to a 1 h exposure at 70°C (Table 3), thereby showing the compound to retain its effectiveness at all the test-temperatures.

Eugenol was found to be the least effective in the microbroth dilution assay, but at the same time had the most stable MIC of all the compounds with a value of 5 mM (Table 1). It maintained its MIC at all the test-temperatures over all the time periods (Table 1 and 2), increasing to 10 mM only after 1 h exposure at 70°C (Table 3).

These results were further evaluated in a model food system. UHT pasteurized whole milk is a complex food matrix with many compounds known to interfere with the activity of

antimicrobials. UHT milk was evaluated with incorporated antimicrobials treated at each test temperature with maximum exposure time. Overall the effect of the food complex was more pronounced on carvacrol and eugenol. Their bacteriostatic/bactericidal concentration increased several folds in the milk assay as compared to the microbroth dilution study. This clearly shows the impact of the food complex as a major factor affecting the antimicrobial activity of these natural phytophenolic compounds. Cinnamic aldehyde was second in antimicrobial strength in the microbroth dilution study, while the food complex had minimal effect on its activity, thereby making it effective at low concentrations and more acceptable as an antimicrobial in foods.

***In vitro* assay in broth**

The results of the *in vitro* assay determined the MIC of untreated phytophenolic compounds against all the test strains (Table 1). Carvacrol was the most effective with an MIC of 1 mM, followed by cinnamic aldehyde with an MIC of 2 mM and eugenol with the MIC 5 mM.

Carvacrol

The MIC of carvacrol was maintained at 1mM for 4°C test temperature up to an exposure period of 14 d. At 14 d, the antimicrobial activity reduced slightly and the MIC of 2 mM was obtained (Table 1). Similar results were obtained with exposure to 25°C test-temperature where the MIC increased to 2 mM by day 14 (Table 2). The reduction in activity here may be attributed to the oxidation of the compound over time and not because of the effect of the temperature, as the same effect was observed at both test temperatures. So it can be established that 4°C and 25°C do not have any negative effect on the activity, and carvacrol can be used as an effective antimicrobial at these test temperatures in the food processing industry. The effective shelf life extension provided by the compound would be approximately two weeks.

On the other hand, the high test temperatures of 60 and 70°C did have an effect on the antimicrobial activity of the compound as an increase in the MIC was observed after a 30 min exposure to 60°C (Table 3). However, it was also seen that though the MIC increased to 2 at exposure to 60°C it remained as such even up to 4 h at 70°C. This suggests that there is no ongoing reduction at high temperatures and though the effective value increases slightly it retains the same activity even after an exposure of 4 h at high temperatures. This fact also shall allow food processors to incorporate carvacrol as an antimicrobial for high temperature processed food products, but attention should be given to the impact of a high temperature process followed by storage at lower temperatures.

Cinnamic aldehyde

Cinnamic aldehyde was the second most effective compound in the *in vitro* assay. The initial MIC of cinnamic aldehyde was maintained up to 21 d at the test temperatures of 4°C and 25°C (Table 1 & 2). However, at 21d exposure the MIC was increased to 5 mM (Table 2). Here again the effect can be attributed to prolonged exposure and not to the temperature because a similar increase in MIC was observed for both test temperatures at the same exposure time. At higher temperatures the compound maintained its initial MIC up to 1 h exposure at 70°C and after that the MIC increased to 5 mM (Table 3).

Eugenol

The MIC of eugenol was the most stable among the three tested and was maintained at 5 mM throughout the exposure period of 21d at 4 and 25°C (Table 1 and 2). This indicates that neither oxidation nor degradation of the eugenol is seen over extended periods of time, and the activity is maintained. At higher temperatures the compound behaved similarly to cinnamaldehyde and the activity was reduced only after 1h exposure at 70°C (Table 3).

***In vivo* milk assay**

The results of effect of whole milk on the bactericidal concentration of the antimicrobials against five strain cocktails of *Salmonella enterica* and *E. coli* O157:H7 have been given in Table 4. The intention of using whole milk for the study was to determine the effect in one of the most complex food systems. We know that milk is a colloid or emulsion of fat globules in a water based fluid. The fat globules have an outer membrane which consists of proteins and phospholipids that prevent the individual fat globules from sticking together. The fluid portion has a large amount of the protein casein and whey proteins which are more water soluble are also present. Milk also is the source of the carbohydrate lactose (composite of glucose and galactose), which is responsible for the inherent taste of milk and is an important constituent. These components of milk make it a very complex food and pronounced effect on the effective concentrations of the three antimicrobials was observed.

Carvacrol and eugenol

The effect of milk components greatly increased the bactericidal concentration of these two components individually (Table 4). The preliminary studies conducted with the concentration of 5, 10, and 15 mM of these components proved ineffective in milk and there was pronounced bacterial growth even at 15 mM of the compound (data not shown). Therefore, higher concentration of 20, 40, and 60 mM were evaluated.

For carvacrol and eugenol, the combined effect of treatment and concentration demonstrated statistically significant difference in the population of *E. coli* O157:H7 and *Salmonella* due to the difference in concentrations (0, 20, 40, 60 mM) over time of exposure ($p \leq 0.05$). This pattern was followed by all the temperature treatments (4, 25, 60, and 70°C). The population of *E. coli* O157:H7 or *Salmonella* were not found to be different ($p \geq 0.05$) at the

highest concentration evaluated, 60 mM, for all the temperature treatments at 12 and 24 h of exposure.

For *Salmonella*, it was observed that the response to 0 mM and 20 mM concentration of carvacrol remained similar at 12 h incubation until 24 h, suggesting that 20 mM concentration allowed growth of organisms up to 12 h, similar to the absence of antimicrobial, and became effective after that time period.

The bactericidal concentration of untreated carvacrol which led to a 4-$\log_{10}$ reduction in the bacterial population after 24 h was 40 mM (Fig. 4). Similar results were observed for the carvacrol subjected to 4°C for 21 d where again the concentration which produced 4 $\log_{10}$ reduction was 40 mM (Fig. 5). At all the other test temperature, 21 d at 25°C (Fig. 6), 4 h at 60°C (Fig. 7) and 4 h at 70°C (Fig. 8), the effective concentration increased to 60 mM. This increase in concentration may be attributed to a combined effect of prolonged exposure and food complex for 25°C and high temperature and food complex for 60 and 70°C which may be driving changes in the chemical structure of these compounds such as oxidation.

A similar effect was observed for untreated eugenol where the bactericidal concentration increased to 60 mM to achieve a 4-$\log_{10}$ reduction of *Salmonella* and *E. coli* O157:H7 (Fig . 9). The effective antimicrobial concentration for all the temperature treatments remained at 60 mM (Fig. 10, 11, 12, 13). This increase may be attributed to the highly hydrophobic/ lipophillic nature of carvacrol and eugenol. A large part of the added antimicrobial might be dissolved in the lipid phase of milk leaving a small amount available for action against the bacteria which are present in the water phase. The presence of the hydrophobic protein, casein, might also reduce the amount available for antimicrobial action as it would sequester a part of the added compound. This could lead to an increase in the amount of antimicrobial required to produce the

same antimicrobial effect as was observed in the *in vitro* study. It needs to be realized that though the level increased immensely in the whole milk medium, similar increases may not be seen in all food complexes. In food products with lower fat content, lower concentration may provide the required antimicrobial effect.

Cinnamic aldehyde

Cinnamic aldehyde was the most effective antimicrobial in the *in vivo* assay. The untreated compound had the bactericidal concentration of 10 mM which gave a 4-$\log_{10}$ reduction against both pathogens evaluated (Fig. 14). The effective concentration giving 4-$\log_{10}$ reduction remained the same, 10 mM, after 21d exposure at 4°C (Fig. 15) and after 4 h exposure at 60°C (Fig. 16). Ten mM concentration resulted in a 3-$\log_{10}$ reduction at the other test temperatures of 21d at 25°C (Fig 17) and 4 h at 70°C (Fig 18).

The effect of treatment, concentration and duration of exposure was studied and the results obtained demonstrated that there was a significant difference in the response (survival of pathogens) due to the difference in concentration (0, 5, 10, 15 mM) over increasing time of exposure (0, 12 and 24 h; $p \leq 0.05$). The population of pathogens significantly decreased as antimicrobial concentration and time of exposure increased for *Salmonella* and *E. coli* O157:H7. There was no difference ($p \geq 0.05$) in the effective antimicrobial concentration (10 mM) between different temperature treatments (4, 25, 60, and 70°C) after 24 h of exposure to cinnamic aldehyde.

The maintenance of the activity of cinnamaldehyde in the milk medium may be attributed to it structure which shall be discussed below. Also the oxidation of cinnamaldehyde results in the formation of cinnamic acid which also possesses antibacterial properties, so it is possible that it may also be contributing to the overall antimicrobial activity against the evaluated pathogens.

Comparison of the activity of carvacrol, eugenol and cinnamic aldehyde in milk

The study of the antimicrobial activity of the heat-treated antimicrobials in the *in vitro* assay gave the MIC of all the three compounds with the maximum value as 2 mM for carvacrol, 5 mM for cinnamic aldehyde and 10 mM for eugenol. The *in vivo* milk assay showed major changes in these values. The effective concentration for cinnamaldehyde increased 2-fold and was maintained at 10 mM for all the test temperatures, but on the other hand, the activity of eugenol and carvacrol reduced by 6-fold for eugenol and a 30-fold for carvacrol. Previous studies have shown this to be a common occurrence where the effective concentration of an antimicrobial *in vitro* increases several fold in a food complex *(1)*. Explanation for this phenomenon has not been offered other than the fact that it is the effect of the food complex.

The question that arises here is the vast difference in the effective concentration for cinnamic aldehyde compared to eugenol and carvacrol. The explanation to this phenomenon can be proposed, on evaluating the structure of the three compounds. Carvacrol has a hydroxyl group on the first carbon, and two side chains, a methyl group on the second carbon and a propyl group on the fifth carbon. Similarly, eugenol has the hydroxyl group on the first carbon, and two side chains, a methoxy group on the second carbon and an allyl group on the fourth carbon. Cinnamaldehyde has only one side chain with the phenyl group attached to the unsaturated aldehyde *(3)*. The structure of the previous two phenolic compounds with several branches provide several points for interaction with the lipid phase and the hydrophobic proteins in the milk, and the interaction with any one functional group may contain the compound in the lipid phase or be sequestered by the protein, and so, only a fraction of the amount being added would be available for interaction with the bacteria in water phase. Cinnamic aldehyde having only one side chain may be less prone to interaction with lipids and proteins and may be able to interact

with the target microorganisms more effectively when compared to eugenol and carvacrol in milk. This may be given as a reason for the vast difference in activity in the *in vivo* assay.

Proposed usage of these compound in the food industry

The effect of the food complex on the activity of antimicrobials has been studied extensively. The components of the food complex react with the phenolic compounds and the amount left for action against bacteria is reduced, thereby raising the amount of antimicrobial needed for the same activity as in an *in vitro* assay *(1)*. Although the amount needed is increased, the effective concentration is so low; it does not pose a safety issue. The property that becomes an issue is the aromatic properties of the compound. The aroma of these phenolic compounds is strong and even a small increase in the concentration can have a high effect on the organoleptic property of the compound which in turn effects the overall acceptability of the food product *(6)*. Although this can pose a problem, there are solutions that the food industry can seek out to effectively incorporate these compounds to foods. The solution lies in the use of these compounds in food products where their aroma complements the entire product and not contradicts the inherent flavor profile.

The taste and smell of the ingredients combine to create a flavor profile of the food. The analysis of a particular food product through gas chromatography-mass spectrometry (GC/ MS) helps in the identification and quantitation of semi-volatile and the volatile organic compounds present, which together give the food its inherent smell and taste *(8)*. So it becomes imperative that if any new compound with a strong aromatic profile is added to the food product it should match the flavor notes already present in the food and also prevent development of any odd aroma in the product. For example, carvacrol, the main component of oregano, is a savory compound and eugenol, the main component of cloves, has both sweet and savory applications

(9). As shown through the study the concentration of 60 mM had a bactericidal effect on both *Salmonella* and *E. coli* O157:H7 in whole milk, but at this concentration the aroma of the compound was strong enough to be detected by olfaction compromising the organoleptic acceptability.

It is clear that these concentrations do not pose a safety issue, but due to the effect on aroma these cannot be incorporated in milk. Carvacrol and eugenol could be incorporated in primarily those products which are traditionally associated with herbs and spices, like meat, fish, vegetable dishes, soups, sauces and cheese *(2)*. At the same time, cinnamaldehyde whose oragnoleptic profile complemented milk and gave it a pleasant aroma can be used in milk and other dairy products, desserts and other foods high in sugar content.

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Conclusion

The antimicrobial properties of essential oils and their components have been studied widely. Today it is realized that the knowledge about their antimicrobial properties alone cannot be put to use on the commercial level. There are several factors they may act to alter their activity. If these compounds are to be effectively used by the food industry to replace or supplement current antimicrobials, extensive studies must be conducted to determine what factors play a role in altering their activity. So, the focus has shifted towards understanding the effect of different processing parameters on the antimicrobial activity of these phenolic compounds. One of the most important processing parameters that need to be studied with respect to their activity is temperature. This will help in making correct decisions about the temperature and antimicrobial combinations that will ensure maximum safety of food product and at the same time maintain fresh attributes.

This study conducted on three major essential oil components was able to provide useful data about the antimicrobial activity retention when these compounds were subjected to variable processing temperatures. Carvacrol was proven to be the most effective antimicrobial with the lowest MIC value *in vitro,* but had the least stable MIC among the group, losing activity after seven days exposure at lower temperatures and also within 30 min at high temperatures. Nevertheless, its increased MIC was still low (2 mM). Cinnamic aldehyde maintained the MIC of 2 mM up to 14 d at 4°C and 25°C, and also up to 1 h at 70°C. The initial MIC of eugenol was maintained even at 21 d exposure over lower temperatures and until 1 h at 70°C showing high retention of antimicrobial activity over variable time temperature conditions.

The milk assay also gave important results and demonstrated the strong effect of a food complex on the activity of untreated and treated compounds, with the bactericidal concentration rising to 60 mM for eugenol and carvarol. Cinnamic aldehyde proved to be the most effective in this assay. Interestingly, the food complex did not lower its activity, and a concentration of 10 mM was found to inhibit bacterial growth.

It needs to be realized that this study was aimed towards the determination of the effect of temperature on the activity of these compounds and also towards determination of the effect of the food complex in order to give meaningful and applicable data to the food industry. The sole purpose of using whole milk was to study the effect of a very complex food on the activity of these antimicrobials but was not aimed towards studying the feasibility of these compounds in milk on the commercial level.

On the other hand the results of heat treated cinnamic aldehyde which proved to be the second most effective compound *in vitro* with a maximum MIC of 5 mM, and the most effective *in vivo* with minimal effect on the antimicrobial activity in whole milk (maximum MIC 10 mM), gave a sweet cinnamon flavor to milk thereby enhancing its organoleptic properties. This study not only showed cinnamaldehyde to be the overall strongest antimicrobial among the three, but was able to indicate a food group where the antimicrobial property of this compound can be utilized.

Future study

The antimicrobial study of these compounds has determined their affectivity on the food safety front and has also established the effectiveness pattern of these compounds under different set of temperature conditions over an extended period of time. However, in order to incorporate them into food, further studies on other aspects of food science are necessary. Firstly, this study

has established the effect of different temperature treatments on the antimicrobial activity of these compounds, but to get a clear understanding of chemical changes that occur in these compounds at different temperatures, analysis through GC/ MS is needed. Secondly, research is needed on the product development front to conduct studies to incorporate these compounds in food products with similar flavor profile so that in addition to ensuring safety of the food product the organoleptic acceptability can also be maintained.

Table 1.The Minimum Inhibitory Concentration (mM) of carvacrol, cinnamic aldehyde and eugenol against five *Salmonella enterica* serovars and five *E. coli* O157:H7 strains after incubation at 37°C for 48 h, when the three antimicrobials were subjected to 4°C temperature treatment.

	Control[1]	4°C 12h	4°C 24h	4°C 48h	4°C 72h	4°C 7d	4°C 14d	4°C 21d
Carvacrol								
Salmonella	1	1	1	1	1	1	2	2
***E. coli* O157:H7**	1	1	1	1	1	1	2	2
Cinnamaldehyde								
Salmonella	2	2	2	2	2	2	2	2
***E. coli* O157:H7**	2	2	2	2	2	2	2	5
Eugenol								
Salmonella	5	5	5	5	5	5	5	5
***E. coli* O157:H7**	5	5	5	5	5	5	5	5

[1]The control is the MIC obtained through the microbroth dilution method against *Salmonella enterica* and *E. coli* O157:H7 strains for newly procured carvacrol, cinnamic aldehyde and eugenol.

Table 2. The Minimum Inhibitory Concentration (mM) of carvacrol, cinnamic aldehyde and eugenol against five *Salmonella enterica* serovars and five *E. coli* O157:H7 strains after incubation at 37°C for 48 h, when the three antimicrobials were subjected to 25°C temperature treatment.

	Control[1]	25°C 12h	25°C 24h	25°C 48h	25°C 72h	25°C 7d	25°C 14d	25°C 21d
Carvacrol								
Salmonella	1	1	1	1	1	1	2	2
***E. coli* O157:H7**	1	1	1	1	1	1	2	2
Cinnamaldehyde								
Salmonella	2	2	2	2	2	2	2	5
***E. coli* O157:H7**	2	2	2	2	2	2	2	5
Eugenol								
Salmonella	5	5	5	5	5	5	5	5
***E. coli* O157:H7**	5	5	5	5	5	5	5	5

[1]The control is the MIC obtained through the microbroth dilution method against *Salmonella enterica* and *E. coli* O157:H7 strains, for newly procured carvacrol, cinnamic aldehyde and eugenol.

Table 3.The Minimum Inhibitory Concentration (mM) of carvacrol, cinnamic aldehyde and eugenol against five *Salmonella enterica* serovars and five *E. coli* O157:H7 strains after incubation at 37°C for 48 h, when the three antimicrobials were subjected to 60°C and 70°C temperature treatment.

	Control[1]	60°C 0.5h	60°C 1h	60°C 4h	70°C 0.5h	70°C 1h	70°C 4h
Carvacrol							
Salmonella	1	2	2	2	2	2	2
***E. coli* O157:H7**	1	2	2	2	2	2	2
Cinnamaldehyde							
Salmonella	2	2	2	2	2	2	5
***E. coli* O157:H7**	2	2	2	2	2	2	5
Eugenol							
Salmonella	5	5	5	5	5	5	10
***E. coli* O157:H7**	5	5	5	5	5	5	10

[1]The control is the MIC obtained through the microbroth dilution method against *Salmonella enterica* and *E. coli* O157:H7 strains, for newly procured carvacrol, cinnamic aldehyde and eugenol.

Table 4. The bactericidal concentration (mM) of carvacrol, cinnamic aldehyde and eugenol, against two five strain cocktails of *Salmonella enterica* serovars and *E. coli* O157:H7 in UHT pasteurized whole milk on TSA after incubation at 37°C for 24 h, when the antimicrobials were subjected to various temperature treatments.

	Control[1]	4°C 21d	25°C 21d	60°C 4h	70°C 4h
Carvacrol					
Salmonella	40	40	60	60	60
***E. coli* O157:H7**	40	40	60	40	60
Cinnamaldehyde					
Salmonella	10	10	10	10	10
***E. coli* O157:H7**	10	10	10	10	10
Eugenol					
Salmonella	60	60	60	60	60
***E. coli* O157:H7**	60	60	60	60	60

[1]The control is the bactericidal concentration (mM) of newly procured carvacrol, cinnamic aldehyde and eugenol, against two five strain cocktails of *Salmonella enterica* serovars and *E. coli* O157:H7 in UHT pasteurized whole milk on TSA.

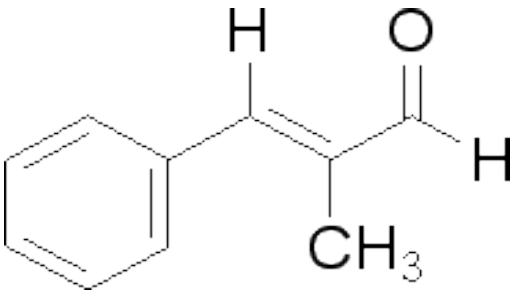

Fig 1. Chemical structure of cinnamic aldehyde.
Source: http://www.sigmaaldrich.com/thumb/structureimages/76/mfcd00006976.gif

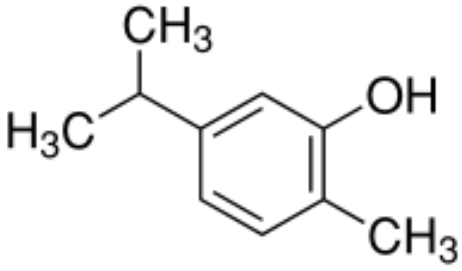

Fig 2. Chemical structure of carvacrol.

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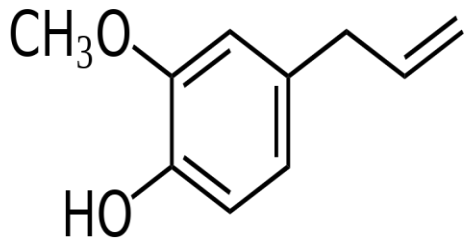

Fig 3. Chemical structure of eugenol.
Source: http://upload.wikimedia.org/wikipedia/commons/thumb/7/7e/Eugenol_acsv.svg/800px-Eugenol_acsv.svg.png

Fig 4a.

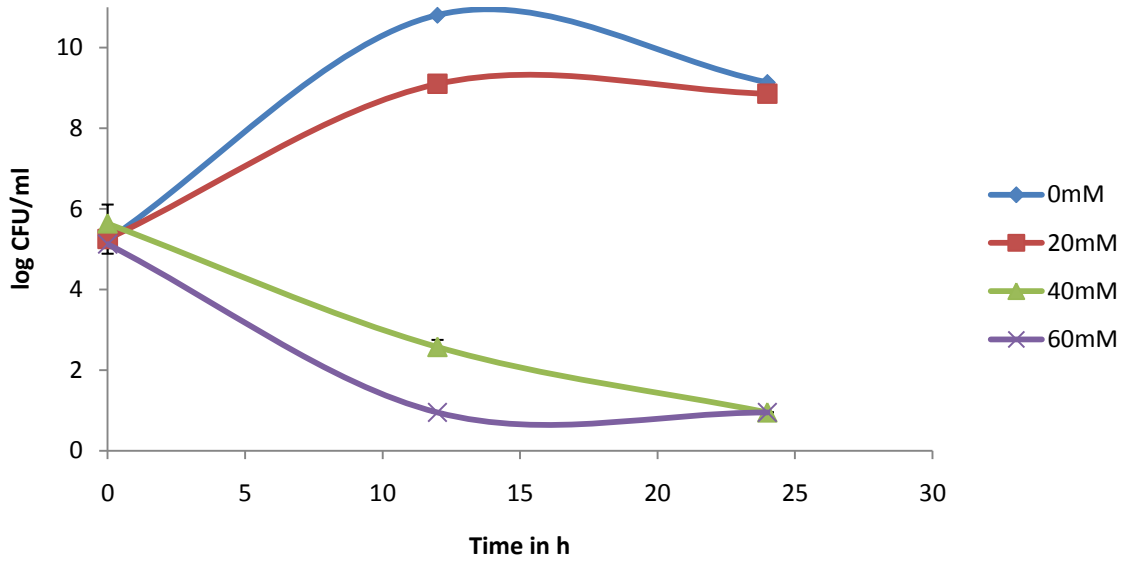

Fig 4b.

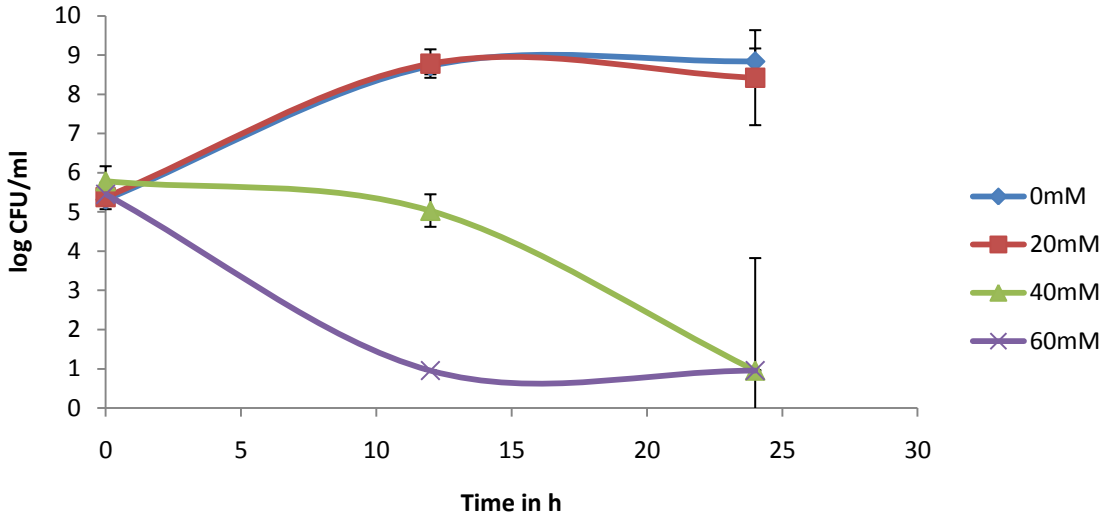

Figure 4. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of untreated carvacrol.

Fig 5a.

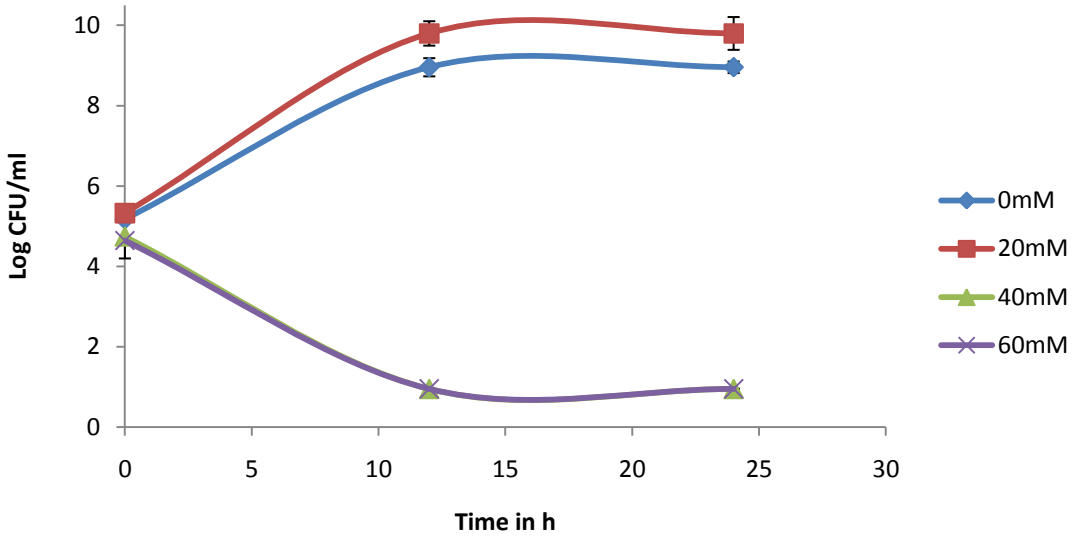

Fig 5b.

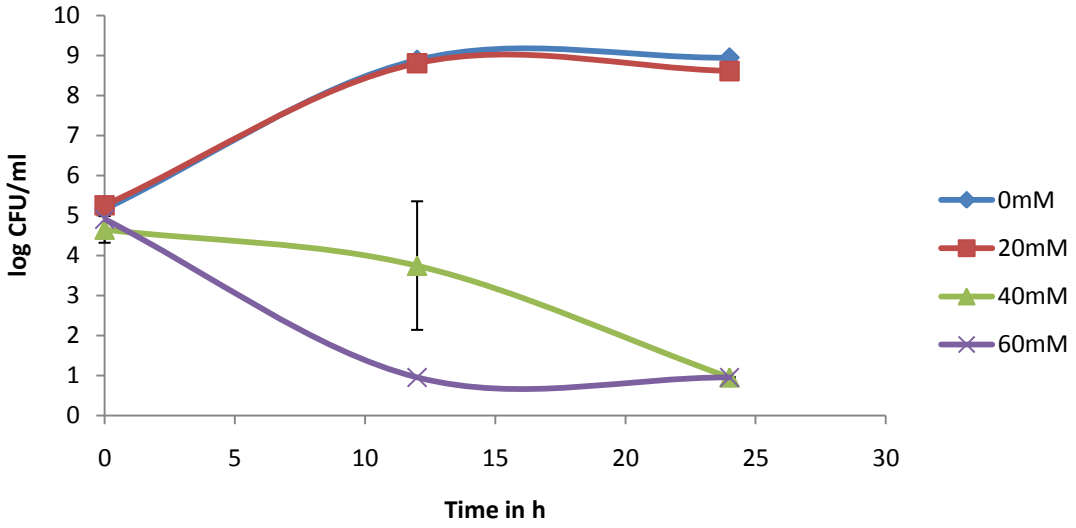

Figure 5. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of carvacrol subjected to 4°C for 21 d.

Fig 6a.

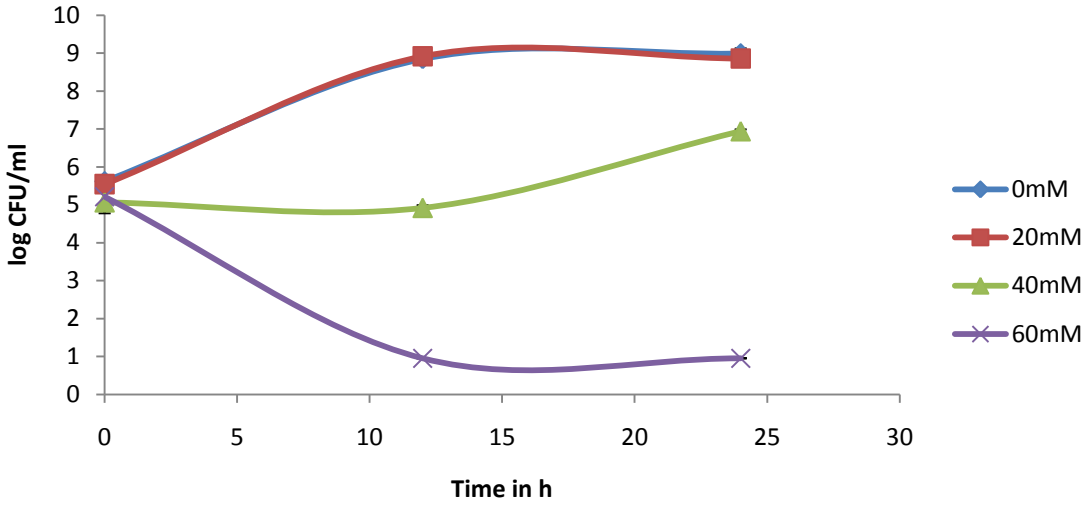

Fig 6b

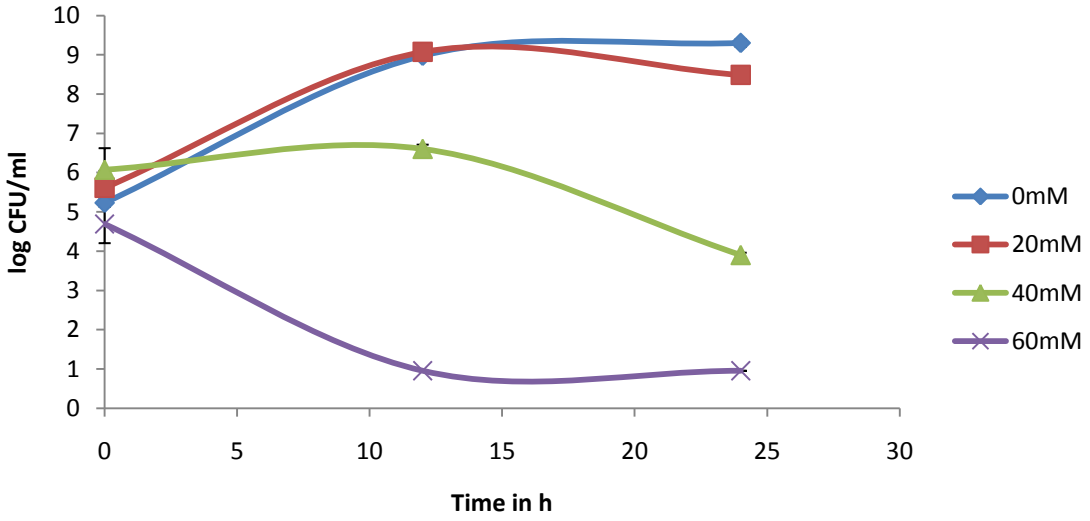

Figure 6. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of carvacrol subjected to 25°C for 21 d.

Fig 7a.

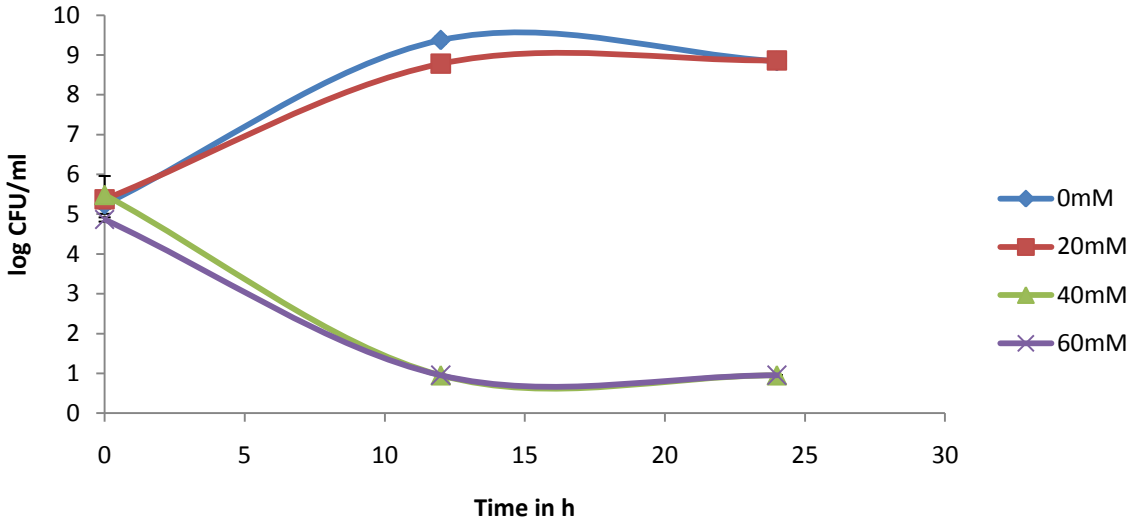

Fig 7b

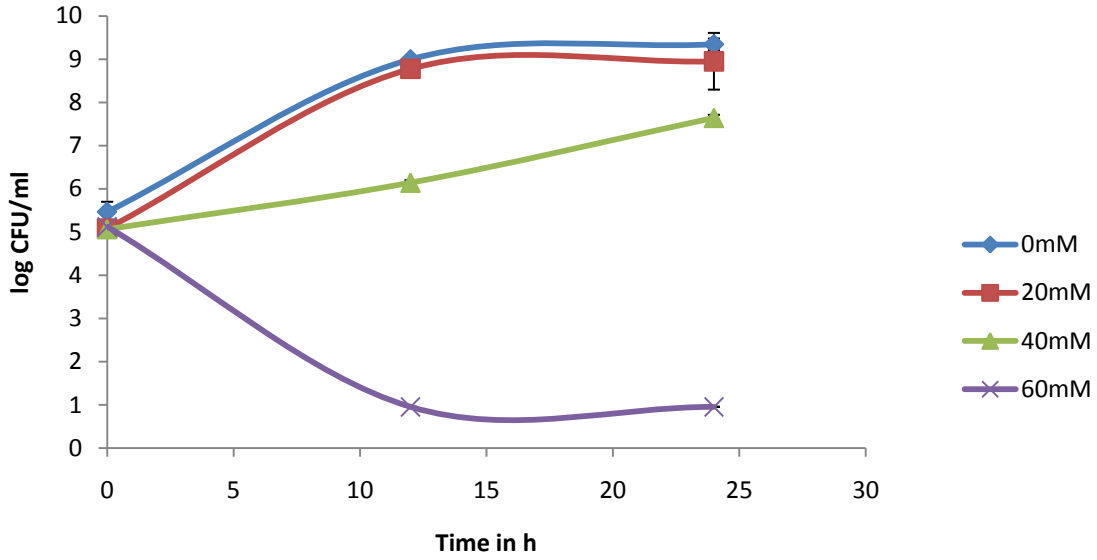

Figure 7. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of carvacrol subjected to 60°C for 4 h.

Fig 8a.

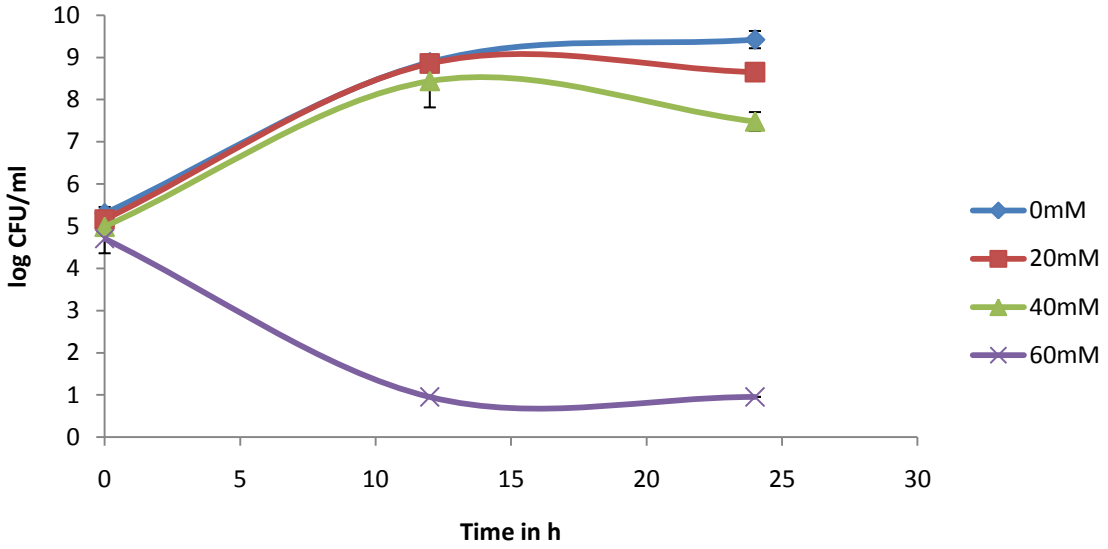

Fig 8b.

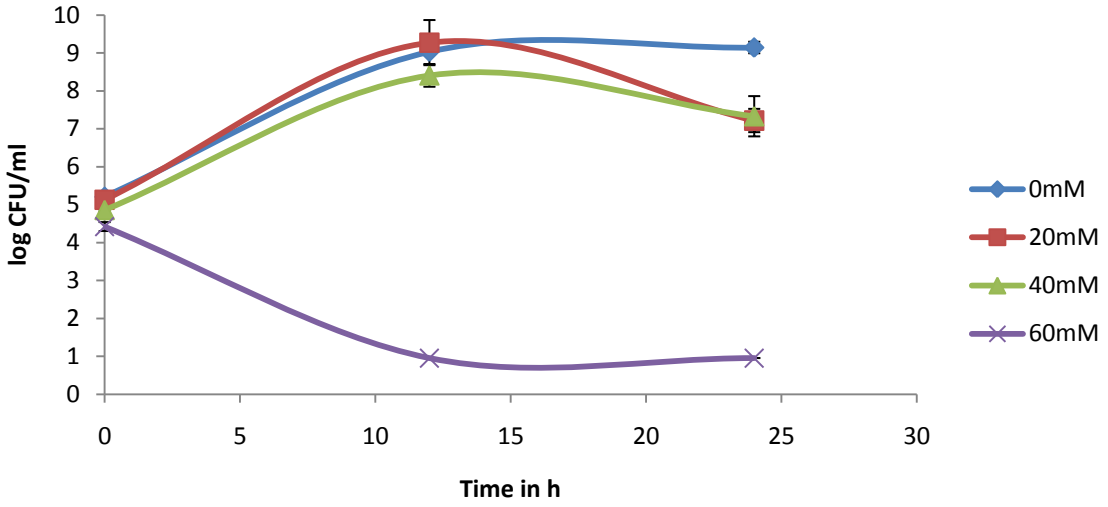

Figure 8. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of carvacrol subjected to 70°C for 4 h.

Fig 9a.

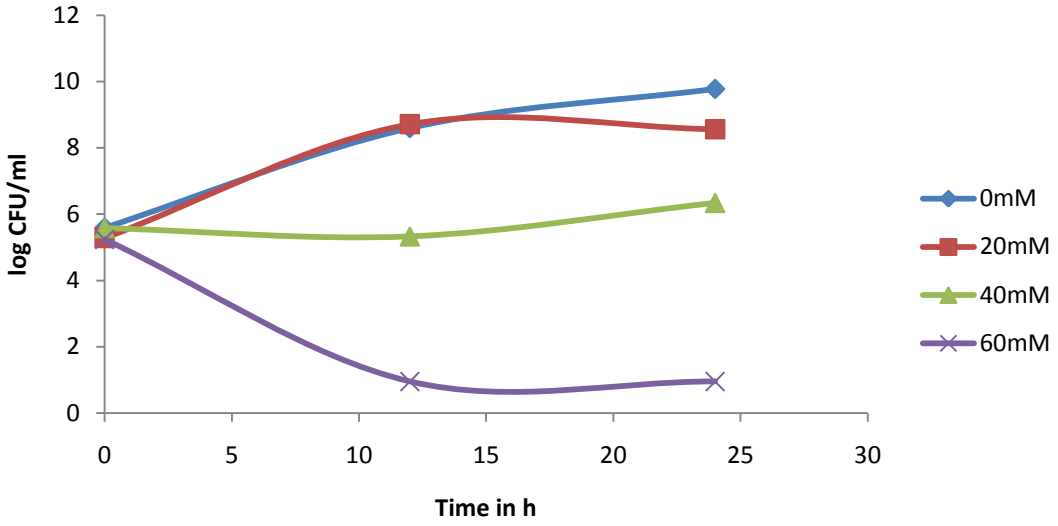

Fig 9b.

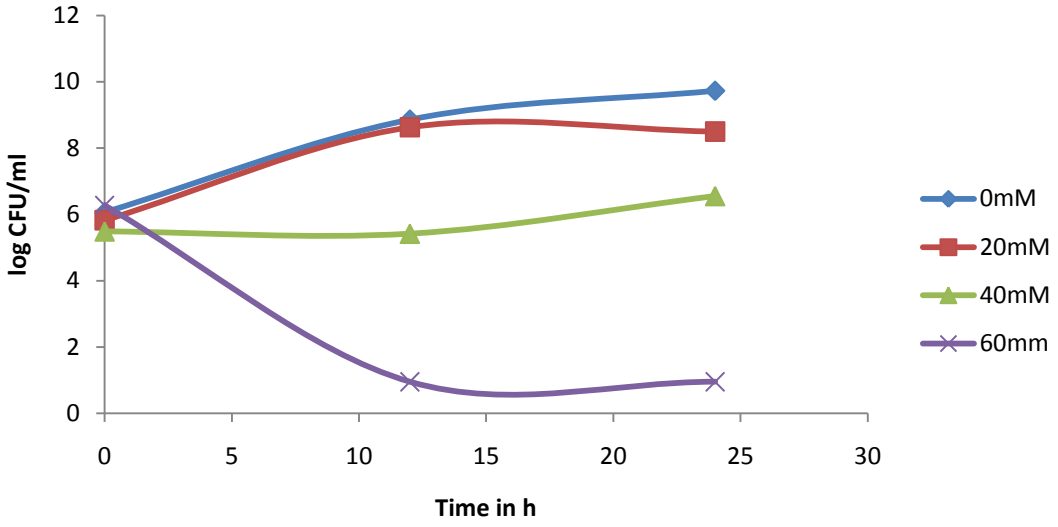

Figure 9. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of untreated eugenol.

Fig 10a.

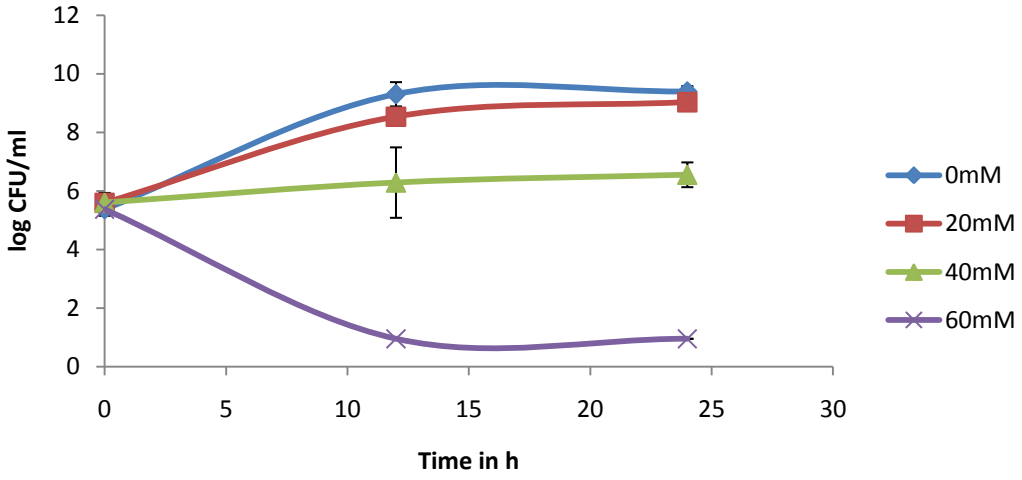

Fig 10b.

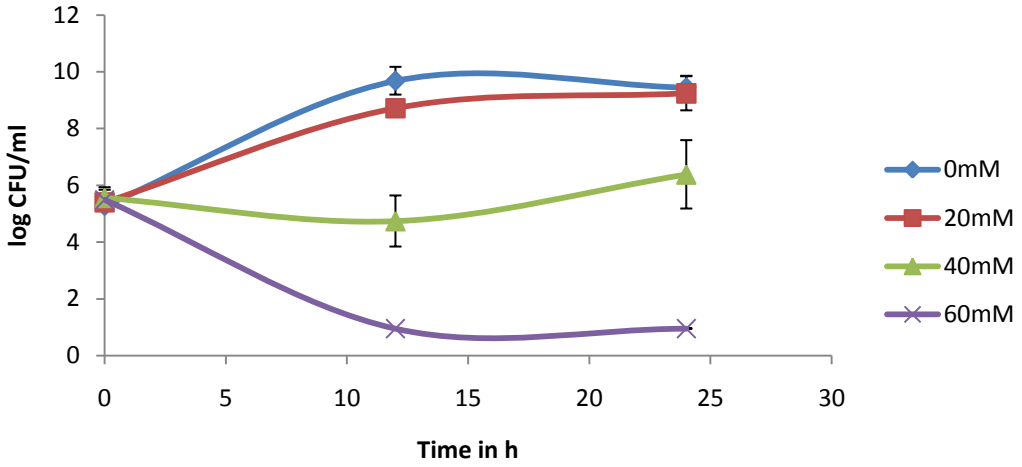

Figure 10. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of eugenol subjected to 4°C for 21 d.

Fig 11a.

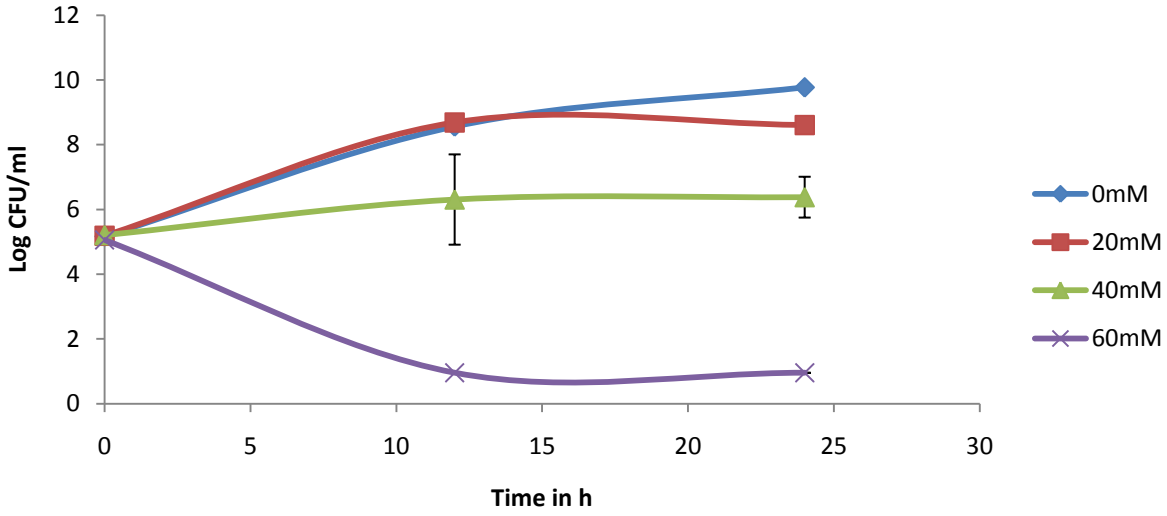

Fig.11b.

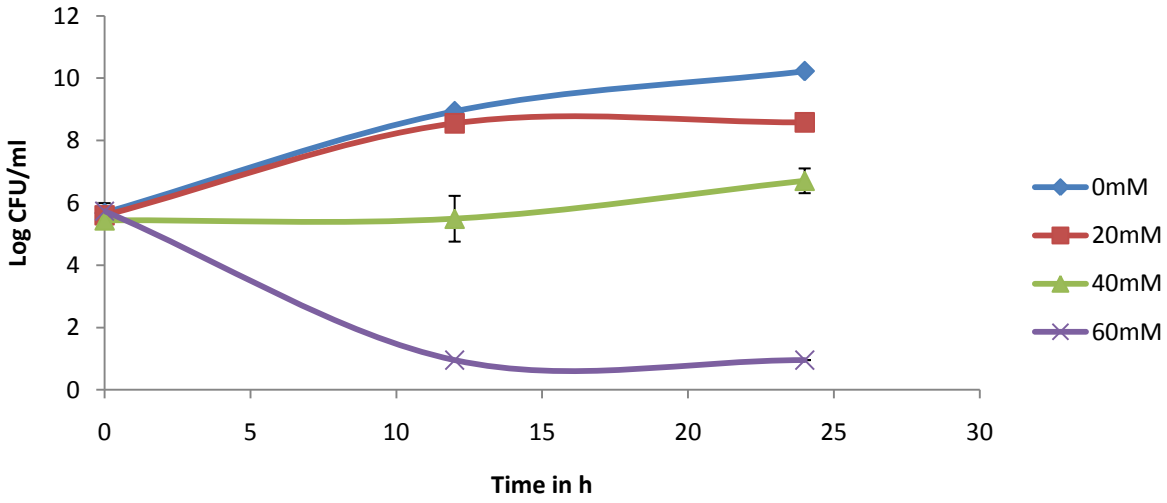

Figure 11. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of eugenol subjected to 25°C for 21 d.

Fig 12a.

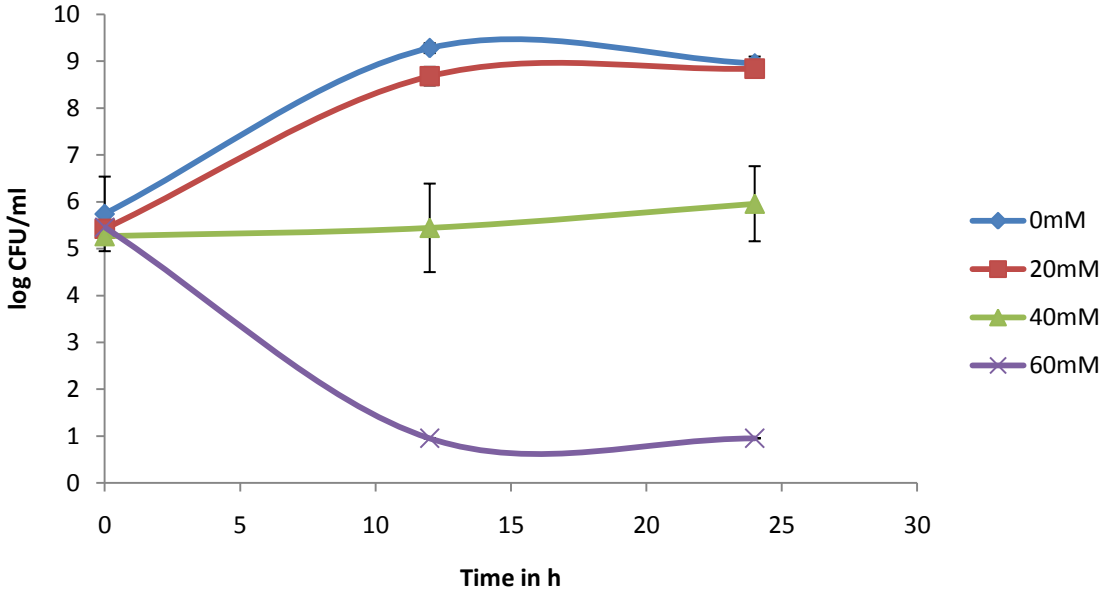

Fig 12b.

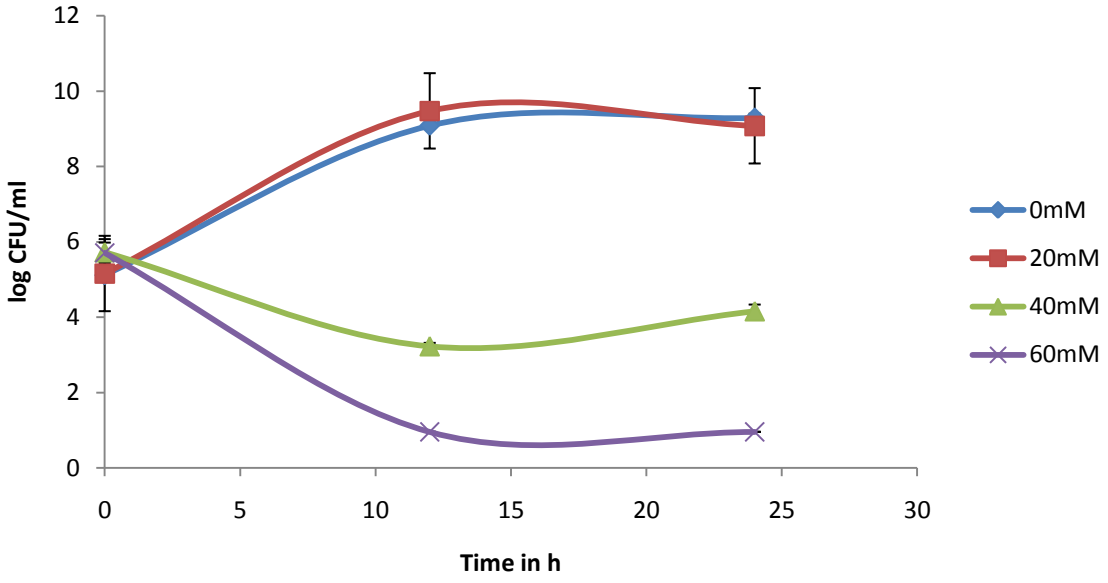

Figure 12. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of eugenol subjected to 60°C for 4 h.

Fig 13a.

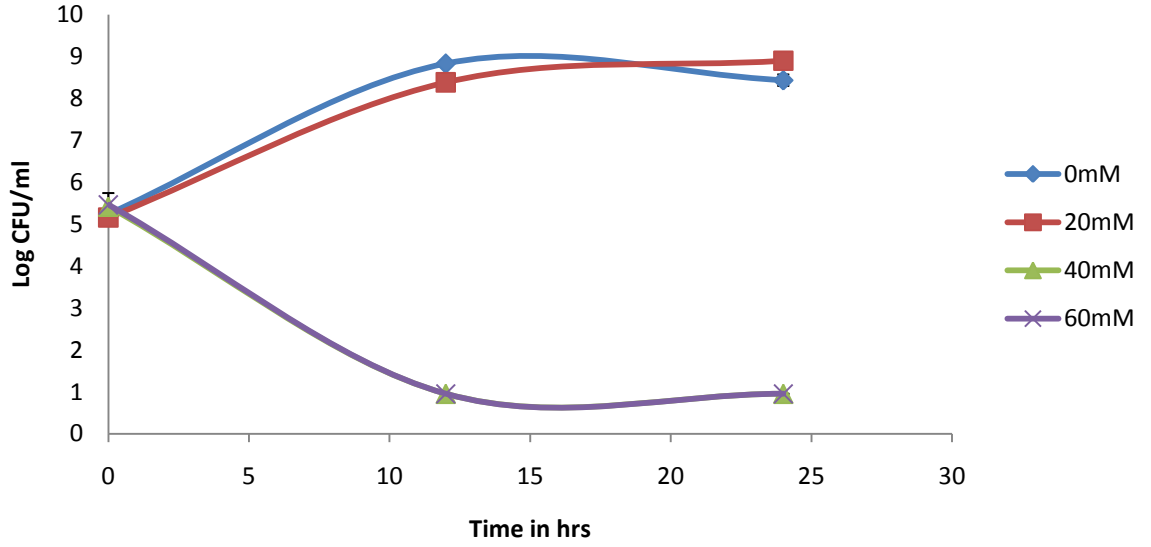

Fig 13b.

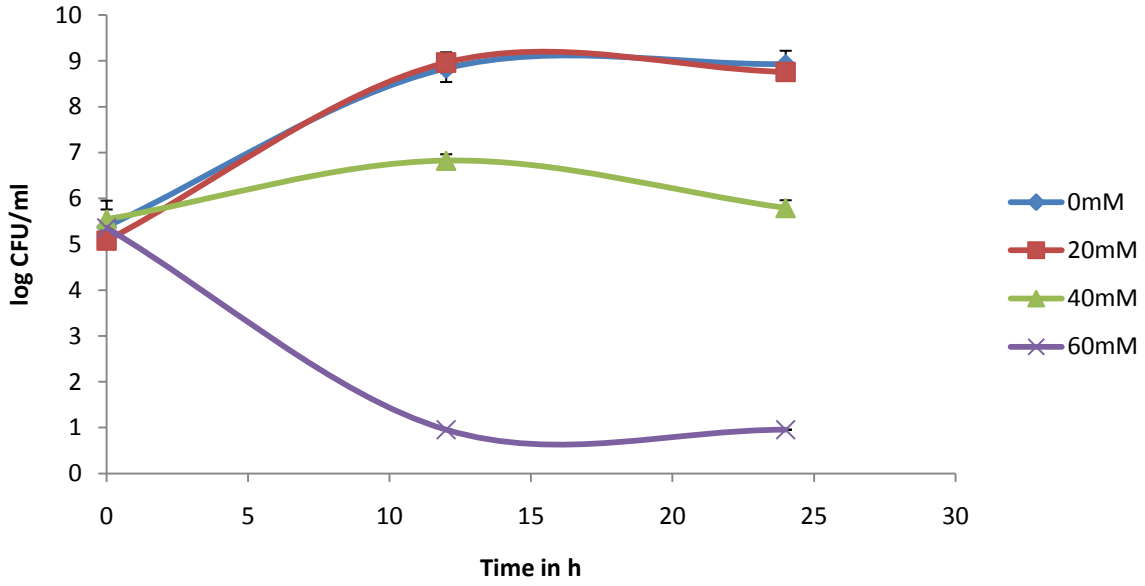

Figure 13. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of eugenol subjected to 70°C for 4 h.

Fig 14a.

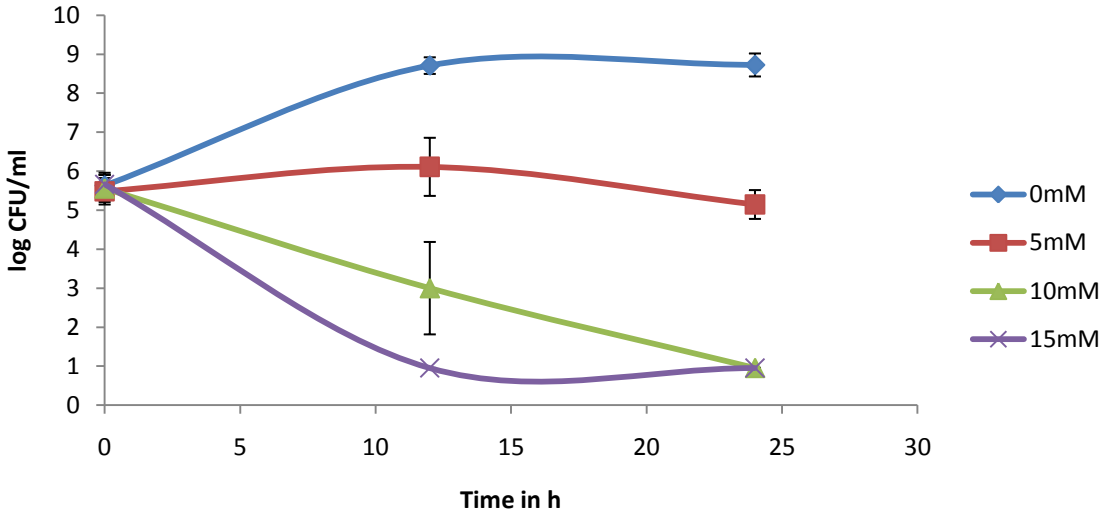

Fig 14b.

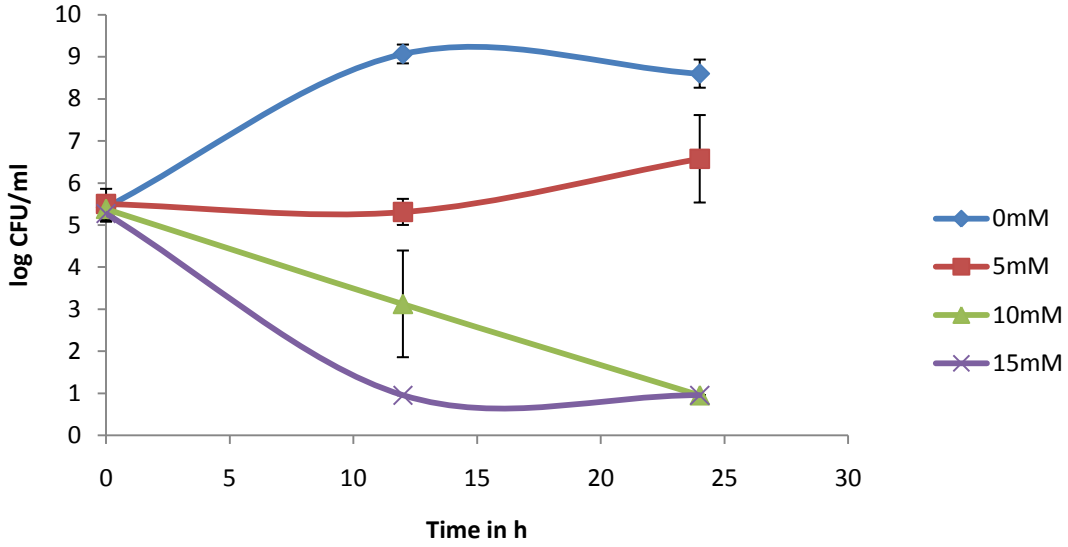

Figure 14. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of untreated cinnamic aldehyde (TSA).

Fig 15a.

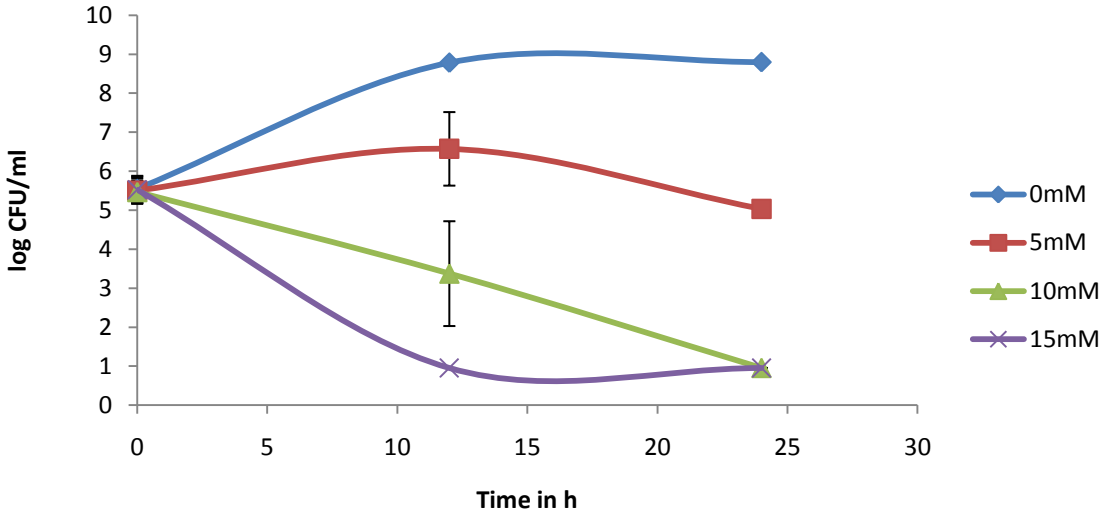

Fig 15b.

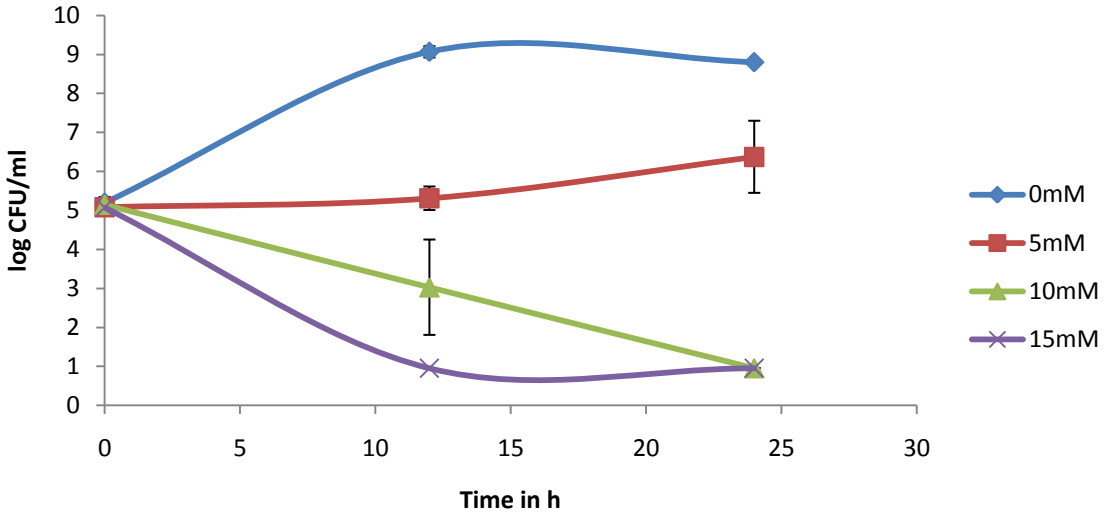

Figure 15. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of cinnamic aldehyde subjected to 4°C for 21 d.

Fig 16a.

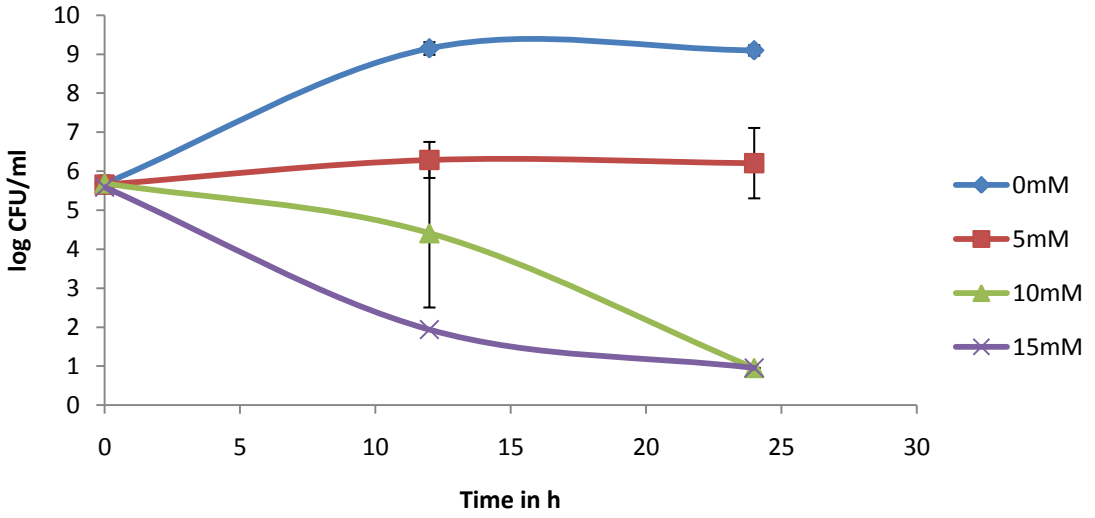

Fig 16b.

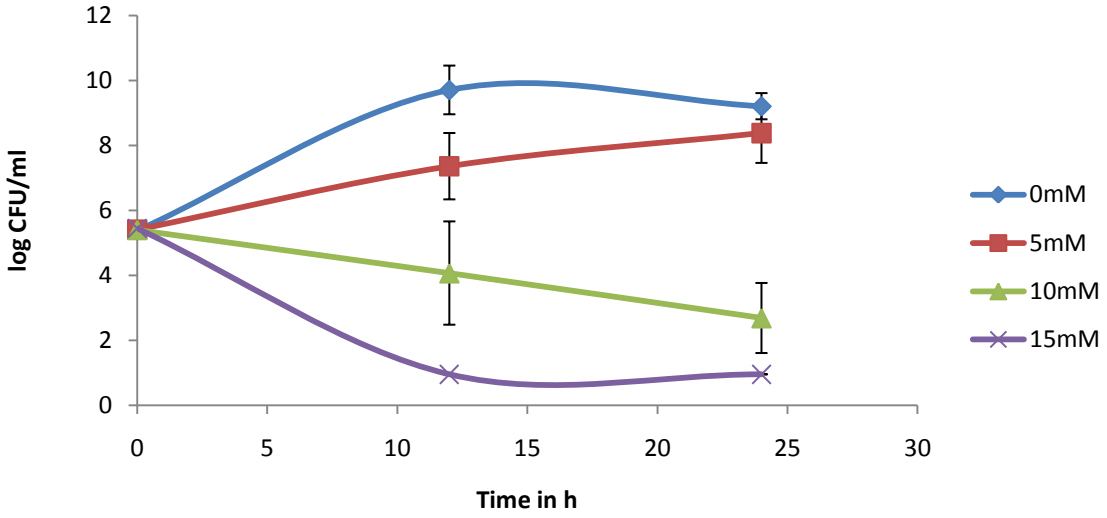

Figure 16. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of cinnamic aldehyde subjected to 25°C for 21 d.

Fig 17a.

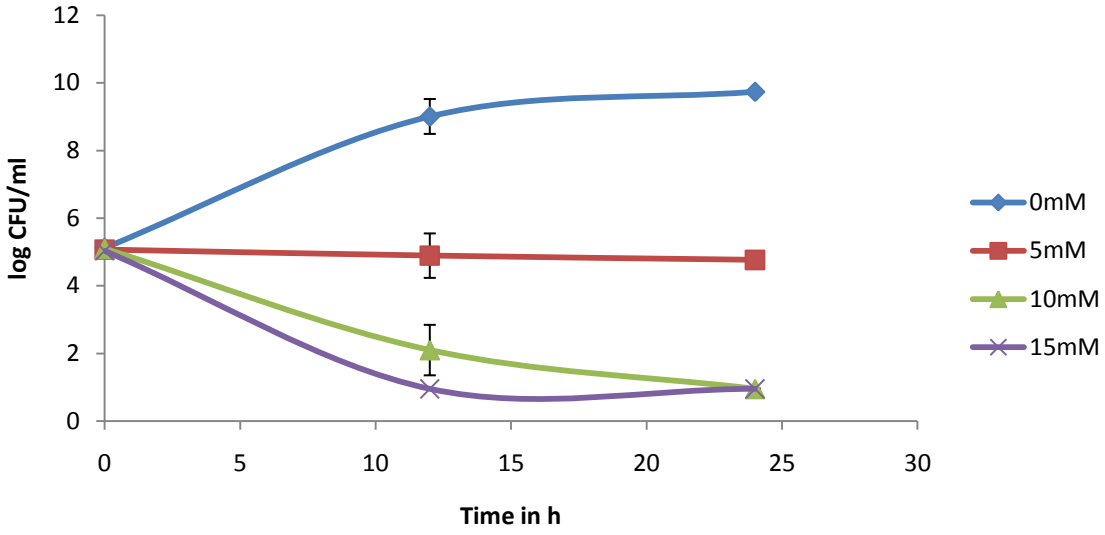

Fig 17b.

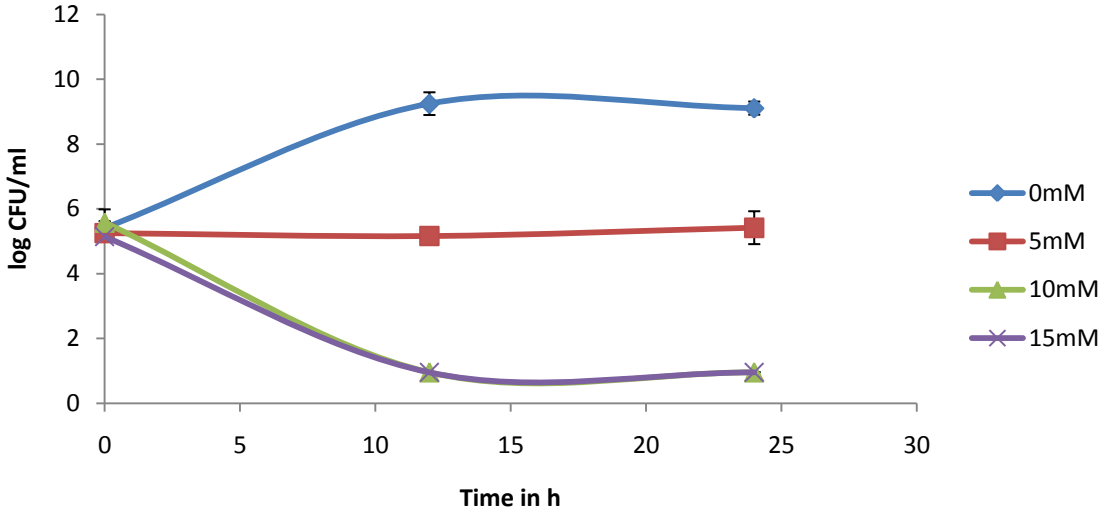

Figure 17. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of cinnamic aldehyde subjected to 60°C for 4 h.

Fig 18a.

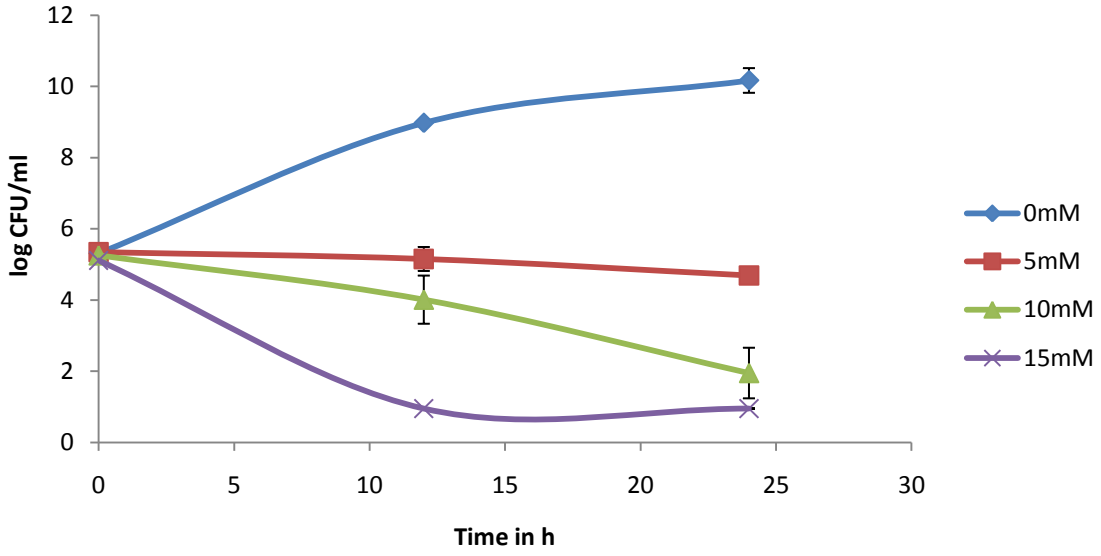

Fig 18b.

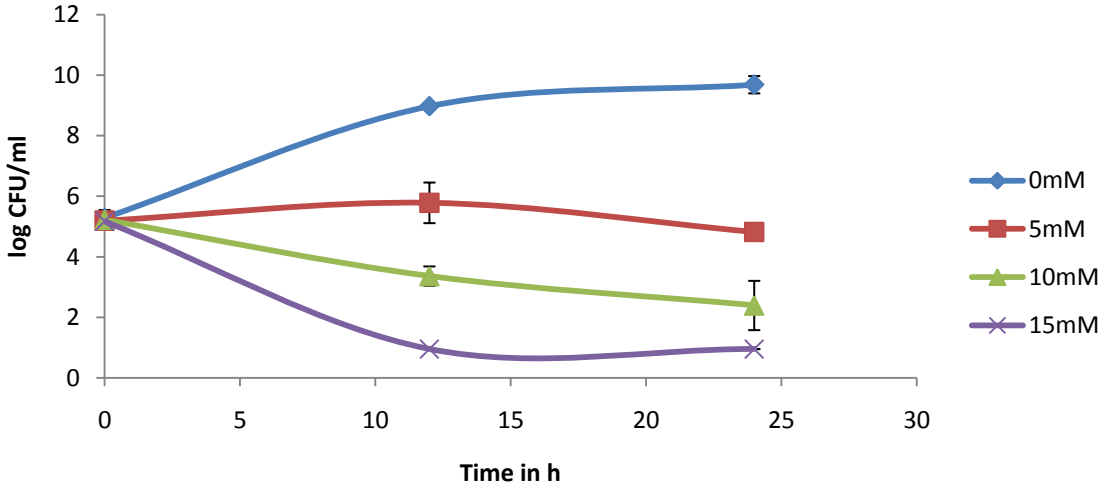

Figure 18. The population of a) *E. coli* O157:H7 and b) *Salmonella* incubated in UHT whole milk for 24 h at 37°C in the presence of cinnamic aldehyde subjected to 70°C for 4 h.