FOOD SAFETY AND QUALITY ENGINEERING MSC THESIS

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EFFECT OF CARVACROL ON FOOD-BORNE BACTERIA ISOLATED FROM RAW PORK

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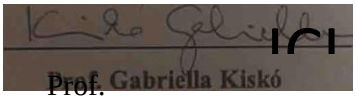

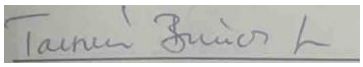

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1. INTRODUCTION

Public health is becoming concerned about foodborne illnesses caused by the consumption of food infected with pathogenic bacteria such as *Clostridium perfringes*, *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus (S. aureus)*. In the EU (European Union) in 2022, the European Food Safety Authority (EFSA) reported 5,763 -foodborne incidents, a 44% rise from 2021 (Report, 2023).

Numerous attempts have been made to use innovative preservation methods including vacuum packaging (VAP) and modified atmospheric packaging (MAP) to decrease foodborne pathogens and increase the shelf life of food. *Listeria monocytogenes* and other newly discovered foodborne pathogens are not eliminated by these techniques, either. Important dangerous bacteria in the food sector, such as *Listeria monocytogene*s, are resistant to cold temperatures, which is why the issue continues.

Scientists and researchers are searching for natural additives with a wide range of antibacterial activity due to growing public concern and awareness about the toxicity of artificial or chemical preservatives. Hence, this has sparked the interest in the use of some bioactive constituents of plants as an alternative. To decrease the presence and effects of foodborne microorganisms in food and prevent economic losses, the use of natural antimicrobial compounds is an effective way to extend the shelf life of food products.

Some plants are medicinal in nature. However, some parts of plants have benefits beyond their medicinal properties. Aromatic parts of plants have been used since ancient times to impart flavor and preserve food. Much interest has developed in the potential use of some aromatic compounds from plants with novel preservation methods like modified atmosphere packaging and vacuum packaging to inhibit the activity of pathogenic microorganisms in food. One group of emerging biocides is the use of Essential Oils (EOs) to inhibits foodborne pathogens. EOs are secondary plant metabolites which are odorous, volatile and are produced by plants mostly found in many leaves and stems, bark of plants, fruit and fruit peels which serve protective roles. They can also be found in some herbs and natural spices like thyme, cloves, oregano, nutmeg, anise seeds, tea tree, lavender, peppermint and cinnamon.

EOs are “Generally Regarded As Safe” (GRAS) by the US FDA and easily acceptable by consumers. They have long been used as flavoring agents, but due to their abundance in antimicrobial compounds, they can prevent food from microbial spoilage. EOs and its compounds contain monoterpenes, sesquiterpenes, and their oxygenated derivatives: alcohols, aldehydes, esters, ethers, ketones, phenols, and oxides and have antibacterial, antiviral, antiparasitic and antioxidant properties because of the phenolic functional group.

Gram-negative bacteria are more resistant to EOs than Gram-positive bacteria due to the complexity of the cell wall. The hydrophobicity of the cell wall of Gram-positive bacteria allow molecules to penetrate easily

into the cytoplasm. Most EOs are composed of phenolic compounds hence they can penetrate the cytoplasm of Gram-positive bacteria to exhibit its antimicrobial activity.

2. OBJECTIVES OF THE THESIS

The continuous improvement in the standard of living has gained recognition as consumers are currently becoming more concerned about the food they eat. The main causative agents of food spoilage and foodborne diseases are foodborne pathogens. This has made food safety gain much attention. Hence, several hands are needed on deck to fight foodborne related issues. Food additives or preservatives have been used extensively to inhibit the proliferation of foodborne pathogens and to extend the shelf like of food. Presently, chemical preservatives like sorbic acid and benzoic acid are used more. Further research has shown that carcinogenicity is associated with chemical preservatives.

The rise in consumer demand of a "more natural preservative" as compared to chemical preservatives has prompted researchers and scientists to develop alternative natural preservatives to inhibit the activity of important foodborne pathogens.

Meat and meat products safety has gained recognition after consecutive foodborne outbreaks and product recalls. In 2022 in the EU, the EFSA (Report, 2023) reported 34 cases of foodborne outbreak linked to pork. The most common foodborne diseases linked to this outbreak were campylobacteriosis, salmonellosis and yersiniosis. The causes of concern about meat safety are *Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC). Nevertheless, additional pathogenic bacteria like *Campylobacter jejuni, Salmonella* spp., *Clostridium perfringes* and *Listeria monocytogenes* are linked to foodborne illnesses connected to meat consumption.

Campylobacter is a Gram-negative bacteria found in the gastrointestinal tract of warm-blooded animals. Two species (*C. jejuni* and *C. coli*) are associated with meat products. *Clostridium* spp., however, is a gram-positive and spore-forming bacteria. *Clostridium perfringes* and *Clostridium botulinum* are well-established foodborne pathogens contrary to *Clostridium tetani*. *Clostridium perfringes* is found in meaty products like pig, lamb, beef and chicken products.

The goal of this work is to determine the effect of the essential oil carvacrol on microorganisms isolated from Raw Pork. To achieve this task, the following need to be carried out:

- To isolate and identify unknown bacteria strains from raw pork meat using morphological tests and MALDI-ToF MS (Matrix Assisted Laser Desorption Ionization- Time Of Flight).
- To determine the minimal inhibitory concentration (MIC) values of carvacrol regarding selected isolates by applying the Agar Disk Diffusion and the Kill-time method.

3. LITERATURE REVIEW

Carvacrol is one of the essential oil compounds, an isomer of thymol. It is a major compound found in essential oils of thyme and oregano. It belongs to the Labiatae family of plants (Antonia Nostro and Teresa Papalia, 2012). Carvacrol has been classified as GRAS and its usage in food has been approved. (Hyldgaard, Mygind and Meyer, 2012) reported that carvacrol has antimicrobial properties apart from its synergistic and antiparasitic effects. The antimicrobial action of carvacrol is stronger on Gram-positive bacteria than Gram-negative bacteria, and this relies solely on the bacterial membrane damage. This results in the dissipation of the proton motive force hence there is a reduction in ATP (Adenosine Triphosphate) synthesis affecting cellular processes like synthesis of enzymes and toxins that require ATP usage (Antonia Nostro and Teresa Papalia, 2012). In effect, studies have shown that carvacrol is able to inhibit Gram-negative and Gram-positive bacteria such as *Vibrio cholerae*, *V. vulnificus*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli (E. coli)* 0157:H7, *Salmonella* Typhimurium, *Pseudomonas fluorescens* (Hyldgaard, Mygind and Meyer, 2012).

According to the WHO (World Health Organization), the scope of issues related to food safety has expanded in recent years as consumers' awareness of packaged food has grown drastically. Also, the United Nations has forecasted that the human population is expected to increase to 10 billion by 2050 hence, several hands are needed on deck to produce safe and nutritious foods. This literature review aims to explore the effects of carvacrol derived from essential oils on foodborne pathogens isolated from pork.

3.1. Identification and Prevalence of Common Foodborne Pathogens in Pork

Pork is the most often consumed meat globally and can lead to foodborne illnesses. Understanding the types of significant pathogens impacting the pork industry, their frequency during various stages of swine production, and strategies to combat these pathogens in pork are essential for creating successful food safety interventions for the meat. Due to its widespread use in a wide range of products, pork is nonetheless important even if it is less linked to foodborne illness than other meat sources. More pork than any other meat is consumed by people in the EU (Devine, 2003).

The public's awareness of methicillin-resistant *S. aureus* (MRSA) infections in animals, particularly pigs, has created a need to understand this infection in relation to the production of pork. MRSA has recently surfaced. The selection of *Trichinella spiralis* and *Toxoplasma gondii* was based on their historical significance in the pork sector. Furthermore, 8% of hospitalizations for foodborne diseases are caused by *T. gondii*. Finally, *L. monocytogenes* was chosen because of its well-known lethality and its 24% contribution to foodborne illnesses that result in mortality (National Center for Emerging & Zoonotic Infectious Diseases, 2011).

It is evident that *Escherichia coli* was not included in this study. There is no denying the significance of *E. coli* O157:H7 to the food sector, although pigs rarely exhibit asymptomatic carriage of this disease, and pork is rarely the source of illness. In a UK research, 0.6 percent of pigs carried *E. coli* O157, a much lower percentage than *Salmonella* spp. and *Campylobacter* spp., which were found in 23 percent and 69 percent of pigs, respectively (Wijesundara *et al.*, 2021). Cattle-associated *E. coli* O157:H7 is more significant in foodborne infections originating from beef or leafy green vegetables. Excluded from this study is *Yersinia entercolitica*, another pathogen identified in swine but not a major source of foodborne illness, although being rather common in swine intestines. Factors contributing to the contamination of pork with foodborne pathogens include improper handling, processing and storage.

In transfer rate studies with inoculation pathogens, (Park *et al.*, 2020) studied the rates of pathogenic bacterial cross-contamination from gloves to meat and from meat to gloves during pork processing under meat-handling scenarios. The inoculated pork contained approximately 5–6 Log_{10}CFU/g pathogenic bacteria like *E. coli, Staphylococcus aureus (S. aureus), Listeria monocytogenes (L. monocytogenes*), and *Salmonella enterica* and its sub species (*Sal. enteritidis*). The cutting board, knife, and cotton gloves revealed 3.07–3.50, 3.29–3.92, and 4.48–4.86 Log_{10}CFU/g of bacteria after the meat was chopped. Fewer bacteria (3.12–3.75, 3.20–3.33, and 3.07–3.97 Log_{10}CFU/g respectively) were transmitted when polyethylene gloves were worn. Polyethylene gloves had a lower transition rate (cutting board 2.47–3.40, knife 2.01–3.98, and polyethylene glove 2.40–2.98 Log_{10}CFU/g) than cotton gloves when four pathogens (6 Log_{10}CFU/g) were put onto the gloves. These values were 3.46–3.96, 3.37–4.06, and 3.55–4.00 Log_{10}CFU/g for cotton gloves, in that order. On the other hand, after 10 hours of use, 3.09, 3.27, and 2.94 Log_{10}CFU/g of plate count bacteria were found on the knives, cutting board, and cotton gloves, while no bacterial count was found on the plastic gloves. They discovered that wearing polyethylene gloves resulted in optimum hand hygiene. Thus, during the preparation of meat, cross-contamination is decreased when polyethylene gloves are used instead of cotton ones.

3.2. Resistance of Foodborne Pathogens to Chemical Preservatives

Foodborne pathogens are pathogenic microorganisms, mainly bacteria which are introduced intentionally or unintentionally in food during processing. One major action that can lead to the introduction of foodborne pathogens in food includes cross-contamination. Some pathogenic bacteria like the *E. coli* and *Staphylococcus aureus* can produce toxins which enhance their growth and metabolic activities in food hence leading to food spoilage and deterioration. Emerging foodborne pathogens like *Listeria monocytogenes* are becoming more resistant to chemical preservatives and novel preservation technologies like VAP and MAP. This is due to two main factors. Firstly, the rise in consumer awareness and perception

of the use of chemical and synthetic preservatives as toxic has prompted the food industry to look for alternatives. Secondly, laws have limited the amount and usage of certain currently approved preservatives in various foods. The food industry is facing issues as some microbes are becoming less susceptible to most preservatives currently in use. There are two types of preservatives: Artificial and Natural. Currently, artificial or synthetic or chemical antimicrobials and additives are widely used. However, research on the use of natural preservatives is still limited.

3.3 Potential Use of Plant Volatile Oils in food.

There is a renewed focus on researching plant volatile oils' antibacterial properties, mechanism of action, and possible applications. In industrialized nations, conventional methods of preventing disease, pests, and spoilage of food and livestock appear to be making a comeback. As demonstrated by the wide variety of species that volatile oils have been tested against, this is particularly true regarding plant volatile oils and their antibacterial assessment. Plant volatile oils, also called plant essences, are mainly derived from herbs and spices. These compounds have different structural variations

(Gyawali and Ibrahim, 2014) provided a review illustrating these variations and how they affect the antibacterial properties of plant-derived components. Because of the way the hydroxyl (−OH) groups interact with the bacterial cell membrane, they break its structures and allow components to seep out, which is why the structural configuration of these compounds plays a major role in their antibacterial action. There is a growing need for volatile oils plant as opposed to synthetic preservatives. Plant volatile oils are extracts derived from spices, herbs and other parts of plants such as fruits, leaves, roots seeds, bark and pulp. Although these by-products have bioactive chemicals with antibacterial action and are attractive sources for their commercial exploitation, they are typically regarded as plant waste products (Gyawali and Ibrahim, 2014).

(Crista and Butnariu, 2023) investigated the potential use of plant volatile oils. From their findings, they stated that peppermint volatile oil has antiseptic, anti-inflammatory and anti-diarrheal properties and is used in the food industry as a flavoring agent. Volatile oils from nutmeg, cinnamon and black pepper improve blood circulation, reduces heart rate, reduces high blood pressure, corrects digestive disorders and reduces joint pain and stiffness.

Herbs and spices such as cinnamon, oregano, cloves, parsley, garlic, ginger, rosemary, thyme, nutmeg and coriander have antimicrobial and antioxidant properties which can inhibit pathogenic microorganisms when incorporated into food.

3.4 Essential Oils as Natural Antimicrobials

Antimicrobials and their compounds are used to inhibit microbial growth in food and to extend the shelf-life of packaged and pre-packaged foods. There seems to be a shift in industrialized cultures toward greener consumerism, with a desire for fewer artificial food additives and environmentally friendly items (Burt, 2004) (Sacchetti *et al.*, 2005). Natural compounds may be safer than synthetic ones, according to recent studies comparing synthetic antimicrobials with natural plant derivatives. Natural antimicrobials work by rupturing cell membranes, altering nucleic acid processes, causing the proton motive force to decay, and depleting adenosine triphosphate (ATP) (Antonia Nostro and Teresa Papalia, 2012). The negative effects of some synthetic preservatives on the health of the consumer are leading to more research on the exploitation of natural antimicrobials.

Antimicrobials produced from plants (polyphenols, essential oils), animals (lysozyme, lactoperoxidase and lactoferrin), microorganism metabolites, or algae extracts protect against foodborne pathogens via similar mechanisms of action.

Essential oils are plant secondary metabolites produced from some parts of the plants. Essential oils are the pungent, evaporative byproducts of the secondary metabolism of aromatic plants. They are typically produced in specific cells or cell clusters and present in various leaves and stems. They are often localized in one area, like the fruit, bark, or leaves, and when they are found in multiple parts of the same plant, their composition profiles are usually distinct. Even among oils derived from the same plant, there are frequently significant variations in the antibacterial activity reported.

Presently, EOs has been widely used in food as a food preservative. According to (Ju *et al.*, 2019), cinnamon and lilac EOs could significantly ($P < 0.05$) prolong the shelf life of the baked food. Also, (Zhang *et al.*, 2017) reported that cinnamon EOs could inhibit the accumulation of the total volatile basic nitrogen (TVB-N) and biogenic amines in carp fillets during storage, and compared with vacuum packaging, the shelf life of fish fillet was extended to 2 d. In earlier times, the related researchers explored the inhibitory effects of clove and cinnamon EOs on *Listeria monocytogenes* in ground beef, and the results showed that the two kinds of EOs can effectively control the growth and reproduction of *Listeria monocytogenes* in ground beef (Khaleque *et al.*, 2016). In addition, some researchers combined EOs with other preservatives applied to cheese (Artiga-Artigas, Acevedo-Fani and Martín-Belloso, 2017), sausage (Catarino *et al.*, 2017), fruits and vegetables (Munhuweyi *et al.*, 2018).

Meanwhile, (Tong, 2020) investigated the antimicrobial effect of 5 types of EOs on 5 bacteria and 4 strains of E. *coli* using the disk diffusion and MIC method. From their findings, they stated that the essential oils, particularly that from thyme and cinnamon showed great effectiveness on multi-antibiotic resistant Gram-positive bacteria rather than the Gram-negative bacteria hence indicating its use as a potential natural antimicrobial preservative agent in food storage.

Extensive research has revealed that the essential oils of oregano, thyme, sage, rosemary, cloves, coriander, garlic, and onion have antibacterial properties against bacteria, molds and germs. (Çetin, Çakmakçi and Çakmakçi, 2011) investigated the antimicrobial activity of thyme and oregano EOs using the disk diffusion and MIC methods. From their findings, the essential oils were tested against 43 microorganisms, including 26 bacteria, 14 fungi, and 3 yeasts species. The bacteria included *Bacillus cereus, Bacillus subtilis BC 3213, Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Pseudomonas fluorescens* and so on. The yeasts were *Candida albicans ATCC 1223, Saccharomyces boulardii BC 6128 and Saccharomyces cerevisiae BC 6541*. The fungi species included *Aspergillus* species and *Penicillium* species. The agar disk diffusion method was used to determine the MIC values of the bacteria strains and the microwell dilution assay was used to determine the MIC values of the yeast strains. About forty-seven components of their essential oils were identified using the Gas Chromatography (GC) and Gas Chromatography- Mass Spectrometry (GC-MS) system and the major components of the essential oil from thyme were carvacrol (47.5%), p-cymene (22.2%), borneol (3.4%), γ-terpinene (2.9%), β-caryophyllene (2.7%) and linalool (2.4%). On the other hand, essential oil from oregano contained mainly carvacrol (54.6%), p-cymene (12.5%), borneol (5.9%) and thymol (3.5%) together with linalool (1.8%) and terpinene-4-ol (1.5%). The MIC values and the mean inhibition zones of the bacterial strains varied from 7.8 and 500 mg/mL and 8-72 mm. The maximum inhibition zones and MIC values of the yeast and fungi species sensitive to the essential oils were 8-74 mm and 7.8-500 mg/mL, respectively. Their antimicrobial results showed that the essential oils of thyme and oregano had different antimicrobial activity. Owing to their increased phenolic component content, oregano and thyme have been extensively researched for their antibacterial action.

According to (Peñalver *et al.*, 2005), there is a direct correlation between the phenolic components carvacrol and thymol and the ability of the EOs of oregano and thyme to inhibit specific pathogenic bacterial strains, including *Salmonella* Enteritidis, *E. coli, Salmonella* Choleraesuis, and *Salmonella* Typhimurium. Carvacrol contains a phenolic hydroxyl group, which is responsible for its capacity to suppress infections such as *B. cereus* (Ultee, Bennik and Moezelaar, 2002). The findings of (Çetin, Çakmakçi and Çakmakçi, 2011) showed that the oils' antibacterial qualities varied widely, with inhibitory diameters ranging from 8 to 72 mm. Regarded as an economical source, their study could provide significant understanding into the use of thyme and oregano oils as natural antimicrobial food preservatives.

3.5 Comparative analysis of Carvacrol's antimicrobial efficacy against different foodborne pathogens

Mild preservation technologies are becoming more important in modern food industries. Consequently, spore-forming microorganisms are likely to proliferate and hence become a serious food safety risk. Mild processes are often combined to obtain safe products with improved organoleptic quality. A novel way to

reduce the proliferation of microorganisms is the use of EOs. Among the diverse group of chemical components in essential oils, carvacrol exerts a distinct antimicrobial action. It is a major natural constituent and is significantly present as an essential oil in aromatic plants and is well known for its numerous biological activities. However, not much is known about the mechanisms of action of this compound. A better knowledge of the mode of action is important regarding application in food system.

Because of its hydrophilic nature, carvacrol has antibacterial properties that may be related to interactions between the chemical and the microorganisms' cell membrane.

(Ultee, Kets and Smid, 1999) conducted research on the mechanism of action of carvacrol on the foodborne pathogen *Bacillus cereus*. Carvacrol and other hydrophobic substances may have an impact on biological membranes. According to their research, the two main roles of the bacterial cytoplasmic membrane are energy transduction and barrier function, which enable the membrane to create ion gradients that can be utilized to propel different processes and the creation of a matrix for proteins that are membrane embedded. Carvacrol prevented the cultivated *Bacillus cereus* from growing. Incubation for 30 min in the presence of 1 to 3 mM carvacrol reduced the viable cell numbers exponentially. Within seven minutes, carvacrol (2 mM) drastically reduced the intracellular ATP pool to zero. An increase in the extracellular ATP pool that was proportionate was not seen. A shift in the membrane potential was linked to the depletion of the internal ATP reserve. Lastly, it was found that the cytoplasmic membrane's permeability to potassium ions and protons had increased (at 0.25 and 1 mM carvacrol, respectively). Since carvacrol is a lipophilic molecule that preferentially partitions in this cell compartment, the bactericidal activity may be due to the loss of the membrane integrity. The membrane-associated energy-transducing mechanism is anticipated to be significantly impacted by disruption of the cytoplasmic membrane.

Their investigation concluded that carvacrol, a hydrophobic chemical, interacts with *Bacillus cereus* membranes by altering their permeability for cations such as H^+ and K^+. Ion gradient dissipation causes the cell's vital functions to malfunction, which ultimately results in cell death. The research demonstrated that carvacrol might be used as a flavoring agent in addition to an antibacterial agent.

3.6 Antimicrobial Properties of Carvacrol

Carvacrol is found in liquid form having molecular weight 150.22. Its boiling point is about 237−238 °C; density is 0.967 and melting point 0 °C. It is insoluble in water but soluble in 95% ethanol and acetone. Its content varies depending on the type of essential oil in which it is found.

The efficacy of the carvacrol depends on their phenolic group and hydroxyl group attached with the phenolic structure and increased membrane permeability by working on the microorganism at low concentration. The inhibitory activity of phenols may be clarified by associations with the cell membrane of microorganisms and is also associated with the hydrophobicity of the compounds. Most hydrophobic molecules are usually

harmful and the cytoplasmic membrane is often the main source of harmful activity. Besides, lipophilic products showed a strong affinity for cell membranes, and their insertions induced changes in the physicos-chemical properties of membranes and increased depletion of intercellular ATP (Ultee, Bennik and Moezelaar, 2002). The interactions between antimicrobial compounds and cell membranes are defined to affect both the lipid order and the stability of the bilayer, resulting in a decrease in membrane integrity and an increase in the passive flux of protons through the membrane. This effect is documented particularly for compounds with a log P greater than 3. By consensus, the most effective antimicrobial compound was carvacrol, which has a log P of 3.52 (Ben Arfa *et al.*, 2006). Carvacrol, by partitioning the phospholipid fatty acid chains, created ion channels across the membrane, eventually allowing ions to exit cytoplasm (Ultee, Bennik and Moezelaar, 2002).

3.7 In vitro studies assessing the antimicrobial activity and Minimum Inhibition Concentration of Carvacrol against different foodborne pathogenic strains.

Foodborne pathogens associated with raw pork include *E. coli, Salmonella, Listeria monocytogenes, Staphylococcus aureus, Yersinia enterocolitica, Streptococcus pyogenes, Klebsiella pneumoniae and Enterococcus faecalis.* These pathogens are also found in undercooked pork meat.

An in vitro study focusing on the chemical constituent, minimal inhibitory concentration, and antimicrobial efficiency of essential oil from *Oreganum vulgare* against *Enterococcus faecalis* was conducted by (Janani et al 2020). The Agar diffusion method was used to evaluate the antimicrobial efficiency of different concentrations of oil. GC analysis showed the major constituent of the oregano essential oil was carvacrol, containing 42% of the total constituent. The MIC value recorded was 25 μg/ml.

MIC determines the susceptibility of microorganisms to antimicrobials. In this, the microorganisms are tested for their ability to produce visible growth in microtitration plate wells of broth containing serial dilutions of antimicrobial agents. MIC is defined as the minimum concentration of antimicrobial agents that caused inhibition in the growth of test microorganisms. Stock solution of the oregano essential oil was made and different lower dilutions were prepared from it. The MIC value was determined by the broth microdilution method. From this study, oregano essential oil was reported to be an effective antimicrobial agent against *Enterococcus faecalis.*

(de Almeida de Souza *et al.*, 2021) conducted a study to determine the antimicrobial activity of carvacrol against multidrug-resistant *Klebsiella pneumoniae*. From this research, the antimicrobial activity of carvacrol was evaluated in vitro using broth microdilution and time-kill methods. The in vitro results showed that carvacrol had antimicrobial activity against all isolates evaluated. The survival curves showed that carvacrol eradicated all the bacterial cells within 4h. The antimicrobial action of carvacrol and its time-kill curves provided evidence of its rapid action. The inhibitory effects of carvacrol could be attributed to

the interactions between the structural and functional properties of the cytoplasmatic membrane, where carvacrol interacts with the lipid bilayer and aligns itself between fatty acid chains, leading to the expansion and destabilization of the cytoplasmic membrane (Antonia Nostro and Teresa Papalia, 2012) (Nazzaro *et al.*, 2013).

The antimicrobial and antioxidant properties of carvacrol and thymol were evaluated by (Rúa *et al.*, 2019) against 19 strains of *Staphylococcus aureus* derived from clinical samples, meat, and milk. The minimum bactericidal concentration (MBC) and (MIC) of the two phenolics, both separately and together, were ascertained using the microdilution test assay. Based on this investigation, it was possible to determine that the individual MIC values *Staphylococcus aureus* strains ranged from 200 to 400 lg/mL for carvacrol and from 300 to 600 lg/mL for thymol. Depending on the origin of the *S. aureus* strains (clinical, dairy, and meat), the combination of carvacrol and thymol demonstrated both an antagonistic and a noninteraction effect. Consequently, when using thymol or carvacrol as food preservatives, strain variation must be considered. The antioxidant action of the combination is additive at low concentrations of both components and antagonistic at high concentrations of at least one of the components. As a result, they concluded that low concentrations of carvacrol and thymol (between 50 and 200 lg/mL) may be advantageous for both human health and the preservation of food's organoleptic qualities.

3.8 Investigation of the influence of environmental factors (pH, temperature, and food matrix) on the antimicrobial efficacy of carvacrol

A study was conducted by (Rattanachaikunsopon and Phumkhachorn, 2010) on the assessment of factors affecting the antimicrobial efficacy of carvacrol and cymene on *Vibrio cholerae* strains (ATCC 14033, VC1 AND VC9) in carrot juice. Several factors that may influence the effect, including bacterial strain, temperature, bacterial cell number and food substrate were also evaluated.

The food samples used in this study were carrot juice, vegetable soup and fish broth. All these food samples were inoculated with 7.5 ppm carvacrol, cymene 7.5 ppm and *Vibrio cholerae* ATCC 14033 (10^5 CFU/ml). The control samples were the ones without the addition of carvacrol and cymene. The results obtained from this study indicated that the fat content and the complexity of the food influenced the antimicrobial activity of carvacrol and cymene against *V. cholerae* ATCC 14033. Higher concentrations of the natural compounds were needed to inhibit bacterial growth in fish broth than vegetable soup, which had a higher fat content than did carrot juice. This could be due to the hydrophobicity of carvacrol and cymene, which may migrate to the fatty compounds of the foods, leaving the aqueous fraction, where the bacterium develops, free of antimicrobials. Generally antimicrobial activity increases with increasing concentration of the agent.

It was observed from this study that the highest concentrations of carvacrol and cymene reduced drastically, the viable count of *V. cholerae* ATCC 14033 in the fish broth than in the vegetable soup although both

foods are high in fat. Generally, more complex foods are less affected by natural antimicrobials as compared to less complex foods. Food ingredients might serve as barriers to protect bacterial cells from inhibitory substances.

In this study, more emphasis was placed on carrot juice. Different storage temperature was applied at various combinations of the compounds. Concentrations of 7.5, 10, 12.5 and 15 ppm of carvacrol and cymene were used for this experiment. The carrot juice, which was duplicated was treated with the different concentrations of carvacrol and cymene and stored at 25°C, 4°C and 15°C. This study showed that temperature has a greater influence on the antimicrobial activity of carvacrol. At 15°C and 25°C, there was a complete reduction in the cell number of the bacteria strains. Hence, the sensitivity to the antimicrobials of the bacterial strain decreased as the temperature reduced.

Treatments with increasing concentrations of carvacrol added separately to carrot juice inoculated with *V. cholerae* (ATCC 14033, VC and VC7) showed increasing antimicrobial effect on the three strains tested. The effect of bacterial cell number on the antimicrobial activity of carvacrol and cymene against *V. cholerae* ATCC 14033 was studied at 25 °C in carrot juice containing different bacterial concentrations, 10^3, 10^5, and 10^7 CFU/ml. By using 7.5 ppm of carvacrol together with 7.5 ppm of cymene, bacterial cells could not be detected in the juice containing 10^3 and 10^5 CFU/ml of *V. cholerae* ATCC 14033. With the same doses of carvacrol and cymene, bacterial cells were detected in the juice containing 10^7 CFU/ml. The concentrations of the compounds needed to be increased to obtain zero detectable viable count of *Vibrio cholerae ATCC 14033* as the number of the bacterial cells in carrot juice was increased.

The strains of bacteria could also be a factor influencing the antimicrobial activity of carvacrol and cymene. Among all the tested bacteria strains, *V. cholerae VC7* was the most sensitive strain to carvacrol and cymene, whereas *V. cholerae ATCC 14033* was the least sensitive one.

On the other hand, cymene when used alone did not show any activity against the *V. cholerae* strains. Treatments with increasing concentrations of carvacrol and cymene added simultaneously to carrot juice inoculated with *Vibrio cholerae (ATCC 14033, VC and VC7)* showed increasing sensitivity of the bacteria, which sensitivity was higher than that observed when carvacrol was added individually. It should be noted that cymene is a biological precursor of carvacrol, hence when these two phenolic compounds are combined, cymene can enhance the inhibitory activity of carvacrol.

This study presented the potential use of carvacrol and cymene as preservatives against *V. cholerae*. It also presented several factors influencing the antimicrobial activity including strains of bacteria, temperature, bacterial cell number, and type of food.

3.9 Toxicity, Safety and Regulatory Considerations of carvacrol for use in food preservation

Essential oils are being used to preserve food increasingly since they were granted GRAS classification. Encapsulation and edible films/coatings are two forms of their application. According to research, EOs can be promising means to combat food safety concerns. Although EOs work well, there are a few limitations to keep in mind. These include their organoleptic qualities and the insufficiency of utilizing only one EO component. To achieve maximum efficiency, a single component could not provide enough potency. Additionally, low concentrations need to be considered because of their aromatic profile, which may affect consumer perception and potentially change how effective their antibacterial properties are.

The low bioavailability of carvacrol, high volatility, unpleasant and pungent taste at higher doses, sensitivity to processing conditions (heat, other ingredients), and the acidic environment of the digestive tract present challenges to its widespread use in food and feed. Some studies have tried to address this problem through specific carriers or modified release carvacrol formulations, which may improve the uptake of the active compound and its residence time in various organs or matrices. The application of colloidal systems, such as microencapsulation and nanotechnology, which were thoroughly covered in the review of Wang and Wu, appears to be the answer to the issues mentioned (Wang and Wu, 2021).

Regarding the wider use of carvacrol as an antimicrobial agent, its bioavailability is a major limitation. To this end, (Mauriello, Ferrari and Donsì, 2021) encapsulated carvacrol using various stabilizers, such as whey protein isolate and surfactant Tween 80, and measured its antimicrobial activity against *Saccharomyces cerevisiae*. The obtained results indicated antifungal activity at a carvacrol concentration of <500 mg/L. Pure carvacrol had a stronger fungicidal effect than the Tween 80 emulsion containing 25% and 50% carvacrol. When whey protein isolate was used, the antimicrobial activity was proportional to the dissolution of carvacrol, suggesting that the emulsion droplets act as micrometric reservoirs for the compound, which is gradually released in the aqueous phase. Researchers postulate that the use of encapsulation may also contribute to extending its durability. In turn, (Mechmechani *et al.*, 2022) investigated the effect of microcapsules of carvacrol on the biofilm-forming capacity of *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Carvacrol reduced the *Pseudomonas aeruginosa* biofilm to below the detection limit after only 15 min.

After a vigorous review of the previous studies on inhibition of foodborne pathogens by essential oils, carvacrol is a naturally occurring compound that has achieved great importance, mainly due to its well-documented antimicrobial activity against many strains of bacteria, yeasts, and molds. The combination of the hydroxyl group and the delocalized electron of the aromatic ring is essential for its activity. Importantly, the position of the hydroxy group is of less importance, as carvacrol often has comparable activity to its isomer, thymol. Carvacrol easily penetrates the cell membrane and can bind ATP or monovalent cations

(e.g. K^+), changing the cell membrane potential and influencing homeostasis. Changes in the respiratory chain and, consequently, decreases in ATP synthesis are also observed. More research is needed on its safety and toxicity, as there seems to be limited information on the safety, toxicity and regulatory considerations of carvacrol for food preservation.

The literature demonstrates the significant potential of carvacrol derived from essential oils as an effective antimicrobial agent against foodborne pathogens isolated from pork. However, further research is needed to address safety concerns, optimize application methods, and overcome practical challenges for widespread adoption in the food industry.

4. MATERIALS AND METHODOLOGY

4.1. Materials

4.1.1. Diluent

1 g of peptone powder and 8.5 g Sodium chloride (NaCl) was measured into a glass bottle to which 1000 ml of distilled water was added.

To carry out the decimal dilutions, 9 ml diluent containing test tubes were prepared by filling test tubes with 9 ml of peptone water using a Jencons pipette dispenser. The diluents were then autoclaved at 121℃ for 15 minutes. To prepare stock solution of food sample diluent in 500 ml volume was also autoclaved at 121℃ for 15 minutes.

4.1.2. Culture

The cultures were maintained in 1 ml TSB by the addition of 0.5 ml glycerin and refrigerated at -81ºC to preserve the strains as stock cultures for the subsequent experiments.

Fresh cultures were prepared from the stock cultures by inoculation of sterile Tryptic Soy Agar (TSA) agar slants which were incubated at 30 °C for 24 hours.

4.1.3. Meat sample

Pork sample was purchased from a local supermarket and delivered to the laboratory of the Department of Food Microbiology, Hygiene and safety for microbiological investigation.

4.2. Methods

4.2.1. Microbiological analysis

8.5 g of raw pork and 76.5 ml of sterile diluent was homogenized for 5 mins in a stomacher bag. to prepare a stock solution. A serial dilution was prepared from the stock solution. Total plate count was determined by pour plating the appropriate members of the serial dilutions with Tryptone Glucose Extract (TGE) Agar. The number of lactic acid bacteria was determined on De-Man -Rogosa-Sharpe (MRS) agar by spread plating instead of the usual pour plating to make the isolation step easier. The plates were incubated in anaerobic jars. Spread plating was used to determine the number of pseudomonads on Cetrimide Agar (CET) and the number of coliforms and E. coli on Chromocult Agar. The plates of TGE, MRS and CET were incubated at 30 °C and that of Chromocult at 37 °C.

4.2.2. Isolation of microorganisms

After counting the colonies, they were checked for micromorphology. Colonies were clustered based on their similar morphology and well separated colonies were isolated from MRS agar to MRS agar plates while from other media to TSA plates. The isolates from the Chromocult plates were labelled as P1 and P2, isolates from the MRS plate were labelled as P2 and P3. Those from the Cetrimide plate were labelled as P5 and P6.

4.2.3. Preparation of colonies for Phenotypic Identification

4.2.3.1. Microscopic Examination

4.2.3.1.1 Preparation of specimen for staining

i. A clean glass slide was pulled over a flame to remove dirt.
ii. Microbial suspension was made from all six isolates.
iii. A drop of distilled water was placed on the slide and a loopful of microbial culture was taken and mixed with the distilled water. This was repeated for all the six isolates.
iv. The content of the slide was left to dry by passing over the flame at 0.5 m for 1 minute and then to the flame to fix it (Figure 1).
v. The specimen was then stained using crystal violet.
vi. The stained specimen was rinsed with distilled water immediately after staining.
vii. It was left to dry over a flame.
viii. After, it was viewed under the microscope with ×100 power objective.

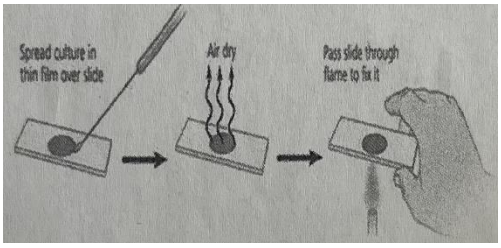


Figure 1: Specimen preparation prior to staining for microscopic examination

4.2.3.2 Catalase Test

i) A colony from all the six isolates were taken with a plastic loop onto 6 glass slides and a catalase reagent was added.

ii) The appearance of bubbles immediately indicated a catalase positive test and if no bubbles were present, it signified a catalase negative test.

4.2.3.3 Oxidase Test

i) Six drops of the oxidase reagent were placed on 6 pieces of paper on 6 glass slides. A colony from all the six isolates were taken with a plastic loop onto the pieces of paper and rubbed.

ii) The appearance of violet coloration indicated an oxidase positive test.

4.2.3.4 Potassium Hydroxide (KOH) Test

i) A drop of KOH was taken with the pipette and placed on a glass slide.

ii) Using a plastic loop, a microbial culture was taken and rubbed in the drop of KOH solution on the glass slide.

iii) The appearance of a slimy string indicated a Gram-negative microorganism.

4.2.3.5 MALDI-TOF (Mass Spectrometry) sample preparation

i) Pure cultures were prepared from all the six isolates and incubated at 30 °C at 24 hours prior to MALDI-TOF analysis.

ii) Colonies of all six isolates were taken with toothpicks and placed in wells on the MALDI-TOF test kit.

iii) Drops of formic acid were added to each well with a pipette and distilled water was added.

iv) About 1µl of a matrix solution was added and left to evaporate. Analysis was started with the MALDI-TOF Mass Spectrometer.

4.2.3.6 Antimicrobial Agent Activity Test

i) The pure cultures of five isolates out of six were inoculated on TSA plates prior to antimicrobial agent testing. As this thesis work is a part of a project, in the frame of the thesis work, 17 microbial isolates from pepper and 8 microbial isolates from raw milk were also used.

ii) The optical density of the isolated cultures was set to OD=0.5 (10^8 CFU/ml) with the help of a Density meter and it was diluted to reach an initial cell count of 10^6 CFU/ml.

iii) To make lawns, 1ml of the microbe suspension was pipetted onto prepared TSA plates and swirled. The agar plate was tilted slightly sideways. The unabsorbed suspension was pipetted out. This was repeated for all 30 test tubes. The plates could dry.

iv) Three agar discs were put on each of the 30 TSA plates using sterile forceps.

v) 10µl of 15 mg/ml of carvacrol solution (carvacrol was dissolved in ethanol) was pipetted onto each agar disc on all 30 TSA plates, allowed to dry and incubated at 30 °C to check for inhibition. Diameters of inhibition zones were measured using a ruler and recorded.

vi) A control test was carried out to check the inhibitory effect of ethanol using thirty small TSA plates but instead of carvacrol, 10 µl of 50% alcohol (considering the highest ethanol concentration in the carvacrol solution in the antimicrobial test) was pipetted onto each agar disk and incubated at 30 °C.

4.2.3.7 *Agar Disk Diffusion Test for MIC determination*

i) The six isolates that were very sensitive to different antimicrobials (antibiotic, disinfectant and carvacrol) were used for this test. Isolates P16 and P28 were very sensitive to Oxacillin, isolates RM4 and RM12 were sensitive to Flora sept and isolates PM5 and PM1 were sensitive to carvacrol. It should be noted that the P isolates were isolated from pepper and the RM isolates were isolated from raw milk.

ii) About 1-1ml microbial suspension of each isolate with approximately 10^6 cell/ml concentration was prepared as described in the previous test to make lawns on six TSA plates.

iii) After, four paper disks were put on each of the six TSA plates.

iv) Dilution series of carvacrol were prepared as follows: A 60 mg/ml of carvacrol stock solution was prepared. Concentrations of 30 mg/ml, 15 mg/ml, 7.5 mg/ml and 3.75 mg/ml were prepared from the stock solution with a serial half dilution. They were prepared in Eppendorf tubes.

v) 10µl of the appropriate members of the dilution series with the concentrations of 30 mg/ml, 15 mg/ml, 7.5 mg/ml and 3.75 mg/ml of carvacrol was transferred separately onto the paper disc labelled as 1, 2, 3 and 4. The 30 mg/ml concentration was transferred to the paper disc labelled 1 and so on.

vi) The TSA plates were incubated at 30ºC.

4.2.3.8 Optical Density measurement

i) Overnight cultures of all six isolates that were very sensitive to the antimicrobials were prepared.

ii) The same steps for the Agar Disk Diffusion test were repeated just that for the dilution series, all the five concentrations were used including the stock solution.

iii) A double concentrated TSB was prepared.

iv) The microbial suspensions, serial dilutions of carvacrol, distilled water and double concentrated TSB were filled into the microtiter wells based on the volumes in Table 1 and 2.

v) About 300 µl of distilled water was pipetted into empty wells (Figure 2).

vi) The measurement was done in such a way that two out of six isolates were used daily until all six isolates were used and results recorded.

Figure 2: Microtiter plate ready for measurement

Table 1: Arrangement of microbial suspension, TSB, Carvacrol dilution series and distilled water in wells on microtiter plate

	1	2	3	4	5	6	7	8	9
a	PM1+C60	PM1+C60	PM1+C60	P16+C60	P16+C60	P16+C60	B C(60)	PM1	P16
b	PM1+C30	PM1+C30	PM1+C30	P16+C30	P16+C30	P16+C30	B C(30)	PM1	P16
c	PM1+C15	PM1+C15	PM1+C15	P16+C15	P16+C15	P16+C15	B C(15)	PM1	P16
d	PM1+C7,5	PM1+C7,5	PM1+C7,5	P16+C7,5	P16+C7,5	P16+C7,5	B C(7,5)		
e	PM1+C3,75	PM1+C3,75	PM1+C3,75	P16+C3,75	P16+C3,75	P16+C3,75	B C(3,75)		TSB

Table 2: Volumes of microbial suspension, TSB, Carvacrol and distilled water used in the wells on the microtiter plate

	2xcc TSB (µl)	Antimicrobial (in proper conc.) (µl)		Dest water (µl)	Microbe suspension (µl)
Grey marked wells:	135	135		-	30
Yellow marked wells	135	135		30	-
Green marked wells	135	-		135	30
Blue marked well	135	-		165	-

5. RESULTS AND DISCUSSION

This chapter presents the study findings on the effects of carvacrol on microorganisms from Raw Pork, Pepper and Raw Milk.

5.1 Inoculation of Microorganisms from Pork

The culture media used for the isolation of bacteria from pork were isolated from Pork were TGE Agar, CET, Chromocult and MRS Agar.

TGE Agar is used for the enumeration of microorganisms in water, milk and dairy products. It is composed of tryptone (an essential amino acid), Meat extract, Glucose and Agar. The tryptone and the meat extract provide nitrogenous compounds and Vitamin B complex for the microorganisms to utilize for growth. The Glucose serves as a source of energy. Typical microorganisms that can be enumerated using the TGE Agar include *Bacillus subtilis, Escherichia coli, Lactobacillus casei, Staphylococcus aureus* and *Pseudomonas aeruginosa.* From the results of this study, the plate counts of colonies were above 300cfu/ml on all the three TGE plates of the first, second and third serial dilutions.

Figure 3: Colonies on Chromocult plate

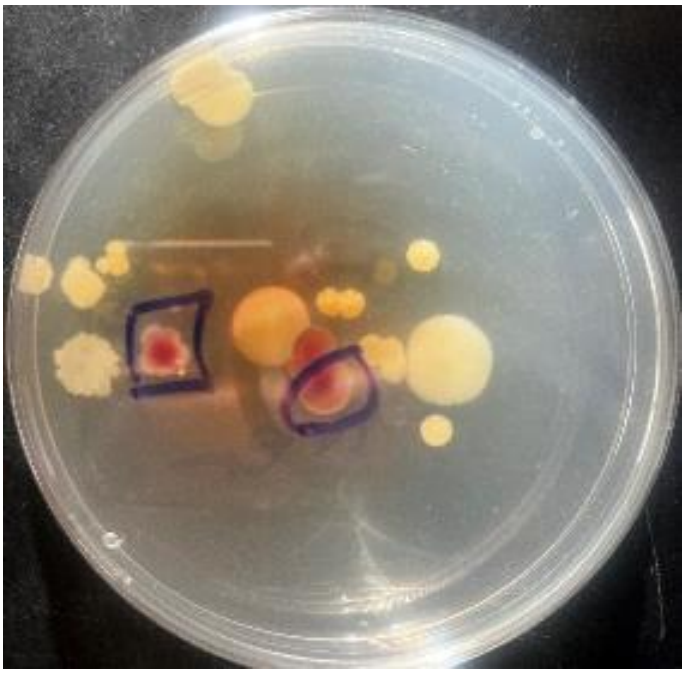


Figure 4: Colonies on TGE, Centrimide and MRS plates

Lactobacillus species are enumerated using the MRS Agar. Peptone, Glucose, sodium acetate, Dipotassium hydrogen phosphate, Triammonium citrate, Yeast extract, Beef extract, and Magnesium sulfate are among its contents. The microorganisms' robust nutritional foundation is provided by the inclusion of polysorbate, acetate, magnesium, and manganese. Typically, *Lactobacillus acidophilus*, *Lactobacillus fermentum* and *Lactobacillus bifidum* are microorganisms which can grow on MRS Agar plates. After incubation on MRS agar plates, two types of colony morphology could be distinguished: big and small yellow colonies. The results from *Figure 4* showed the growth of yellow big and small colonies as well as pink colonies after incubation on the Chromocult plate. Interestingly, the colonies were spherical in shape.

Chromocult Coliform Agar is both a selective and differential Medium used for the enumeration of microorganisms in water samples. It is able to detect the presence of *E.coli* and *coliform* bacteria in water. The presence of coliform bacteria on the Chromocult plate is possible due to the presence of D-Galactosidase, an enzyme which is characteristic of coliform bacteria. This culture media contains casein, yeast extract, sorbitol and sodium pyruvate as sources of carbon, nitrogen, fermentable carbohydrate and other essential growth nutrients for the microorganisms' growth. Disodium hydrogen phosphate and sodium dihydrogen phosphate are the buffering agents. Sodium chloride maintains the osmotic equilibrium in the medium. As seen in *figure 3*, there was the appearance of yellow big and small colonies and interestingly, Big colonies which were pink in color were also seen.

Centrimide Agar on the other hand, is a selective medium for the enumeration of *Pseudomonas aeruginosa*. The use of cetrimide, also known as N-Cetyl-N,N,N-trimethyl ammonium bromide, reduces interference with *P. aeruginosa* growth by primarily inhibiting the growth of the surrounding microbial flora.

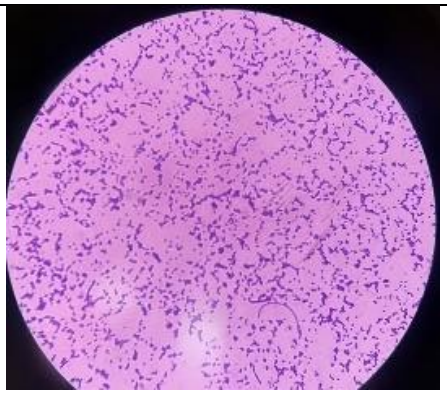
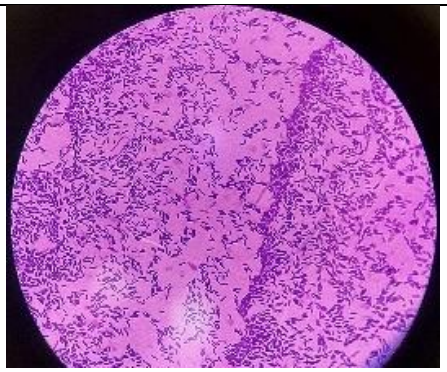
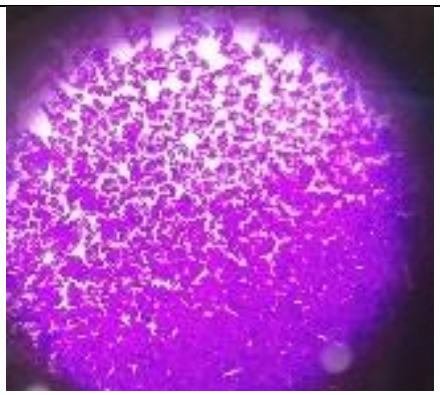
Pseudomonas aeruginosa does not produce less pigment in this media. This medium contains gelatine peptone, Magnesium chloride, Potassium sulfate, Cetrimide and Agar. The appearance of a cluster of colonies, big and small were seen on the Centrimide plates after incubation.

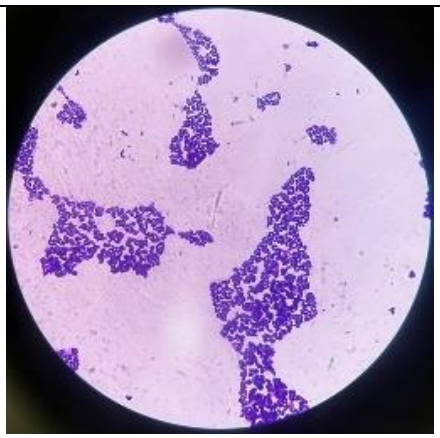
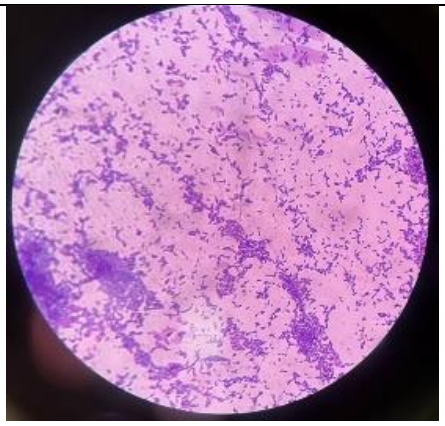
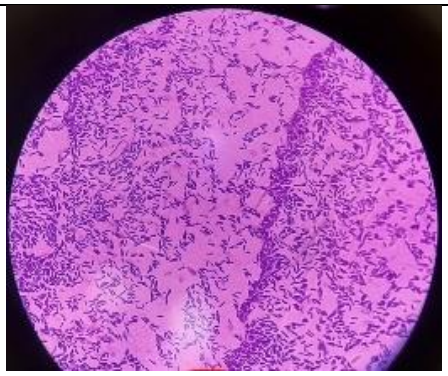
5.3 Isolation of bacteria on TSA

After the inoculation, the two pinkish colonies from the Chromocult plates were isolated. One of the pinkish colonies was zigzag in shape and the other, circular. The colony with the zigzag shape was labelled as P1 on the TSA plate and the one which was circular in shape was labelled P2. Also, both the small and the big spherical colonies from the MRS plate were isolated on the TSA plates and labelled as P3 and P4 respectively. Additionally, the one of small and big colonies from the Centrimide plates were isolated on the TSA plates and labelled P5 and P6 respectively.

The TSA is a general culturing or plating medium for most bacteria, yeasts and molds. It is composed of casein and soy peptones supplying organic nitrogen, particularly amino acids, and longer-chained peptides. Sodium chloride maintains the osmotic equilibrium, and the natural sugars from soy peptone are the energy source. Agar is the solidifying agent. It was used for isolation because of its versatility and the ability of gram-positive and gram-negative bacteria to grow.

Table 3: Macro and micromorphology of isolates

Serial number	Isolated colony characteristic	Colony morphology	Micromorphology	Labeling of the isolate
1	pinkish colonies from Chromocult agar	zigzag colony	 Purple colored, tiny spherical shaped colonies arranged in chains.	P1
2	pinkish colonies from Chromocult agar	circular colony	 Purple colored colonies arranged in clusters.	P2
3	Small colonies from MRS plate	Circular colonies	 Purple colored colonies packed together.	P3

4	Big colonies from MRS plate	Curvy rod-shaped colonies	 Purple colored rod-shaped colonies were observed.	P4
5	Small colonies from Centrimide plate.	Rod-shaped colonies	 Purple colored colonies with few arranged in small rods	P5
6	Big colonies from Centrimide plate	Rod-shaped colonies	 Purple colored colonies with few arranged in rods.	P6

5.5 Catalase Test

Catalase test is another way of identifying, characterizing and classifying bacterial species. Catalase is an enzyme produced by most bacteria. This enzyme acts as a protective mechanism for the bacteria from hydrogen peroxide. Hydrogen peroxide is very toxic and can damage the cellular DNA, lipids and proteins. The enzyme, catalase converts hydrogen peroxide to liquid water and oxygen. Aerobic bacteria produce hydrogen peroxide from their metabolism. To know if bacteria is catalase positive, a drop of hydrogen peroxide is added to the bacteria in an agar slant or a slide. The presence of bubbles indicate that the bacteria is catalase positive which means the bacteria can break down hydrogen peroxide into water and oxygen gas. The absence of bubbles indicates an organism is catalase negative.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

From the results below, it was observed that the isolates P1, P2, P5 and P6 were catalase-positive due to the presence of bubbles. The isolates P3 and P4 were catalase negative due to the absence of bubbles.

Table 4: Catalase test results for all six isolates

Specimen	**Observation**
P1	Presence of bubbles
P2	Presence of bubbles
P3	No bubble was present
P4	No bubble was present
P5	Presence of bubble
P6	Presence of bubble

5.6 Oxidase Test

Oxidase test is used for differentiation and microbial identification, particularly of Gram-negative bacteria, based on the presence of enzyme cytochrome oxidase. Oxidase-positive bacteria produces the enzyme cytochrome oxidase (indophenol oxidase) that catalyzes the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). Tetramethyl-p-phenylenediamine dihydrochloride contained in Oxidase Test Stick acts as an artificial electron donor and is oxidized by oxidase-positive bacteria forming the colored compound indophenol blue (Forbes et al., 1998).

The oxidase test is based on bacterial production of an intracellular oxidase enzyme and some organisms may produce more than one type of oxidase enzyme. These enzymes participate in the cellular respiration process and catalyze removal of hydrogen from a substrate using oxygen as a hydrogen acceptor. The active substrate in oxidase reagent, N, N, N, N-tetramethyl-p-phenylenediamine dihydrochloride, acts as an artificial electron acceptor for the enzyme oxidase and is oxidized to form a violet colored compound. A positive reaction is a purple color within 30 seconds and a negative reaction is a colorless change after 1 minute (Forbes et al., 1998).

Table 5: Oxidase test results for all six isolates

Specimen	**Observation**
P1	Oxidase-positive
P2	Oxidase-positive
P3	Oxidase-negative
P4	Oxidase-negative
P5	Oxidase-positive
P6	Oxidase-positive

From the results, it was observed that P1, P2, P5 and P6 were oxidase positive whereas P3 and P4 were oxidase-negative hence there was no formation of any purple colored compound.

5.7 Potassium Hydroxide (KOH) Results

The Potassium Hydroxide Test is used to differentiate gram-negative bacteria from gram-positive bacteria. A drop of potassium hydroxide is smeared or added to a loopful of bacteria colony on a microscopic slide. The formation of a slimy string between the loop and the slide indicates that the microbe is gram-negative. Potassium Hydroxide scan break down the peptidoglycan present in the cell walls of gram-negative bacteria but not in the case of gram-positive. The breakdown of the cell wall of gram-negative bacteria exposes the contents of the cells including the DNA. The bacterial solution becomes viscous due to the presence of DNA in the solution and the solution will stick to the plastic loop when touched. Gram-positive bacteria have a thick peptidoglycan layer in the cell wall hence, will not be affected by KOH. Therefore, the cell wall will not break down, its contents like the DNA will not be exposed hence no viscous solution would be observed.

From the Table 5, the positive result indicated the formation of a slimy string hence the microbe is gram-negative. A negative result indicated the absence of the formation of a slimy string hence the microbe is gram-positive. P1, P2, P5 and P6 could be gram-negative bacteria whereas P3 and P4 could be gram-positive bacteria.

Table 6: Potassium hydroxide test results

Specimen	Observation
P1	Positive results (Gram-negative)
P2	Positive results (Gram-negative)
P3	Negative results (Gram-positive)
P4	Negative results (Gram-positive)
P5	Positive results (Gram-negative)
P6	Positive results (Gram-negative)

5.8 MALDI-ToF Identification

MALDI-ToF mass spectrometry is used to identify microorganisms down to the genus level and in most cases the specie and the strain level (Singhal *et al.*, 2015). It is done by comparing the Peptide Mass Fingerprint (PMF) of an unknown microorganism to a known microorganism in the PMF database. The microorganism or the sample that is to be identified is mixed with an organic compound called the matrix solution. Examples of matrix solutions like α-cyano-4-hydroxycinnamic acid and 2,5-di-hydroxy benzoic acid. The composition of the matrix solution is made up of water and a mixture of organic solvents that contains ethanol, methanol or even acetonitrile which can dissolve the matrix. The matrix solution crystallizes upon drying and the microorganism gets trapped to the matrix solution and crystallizes. The organic solvents contained in the matrix solution can penetrate the cell wall of the microorganism and extract the intracellular proteins. The proteins are precipitated or dried as well as other cellular compounds by the ethanol or alcohol, resuspended in 70% formic acid and analyzed by the MALDI-ToF MS. For this study, MALDI-ToF MS was used to identify all the six isolates from the raw pork.

Table 7: MALDI-ToF results

Sample	Identified Strain
P1	Buttiauxella gaviniae
P2	Aeromonas eucrenophilia
P3	Kocuria salsicia
P5	Pseudomonas lundensis
P6	Pseudomonas fluorescens

Antimicrobial Activity Test results using Carvacrol and Alcohol

The antimicrobial activity of carvacrol was tested on the five isolates from Pork, nine isolates from raw milk (Diana Lazalde's work) and 17 isolates from pepper (Yomalli Mera Cruz's work). Also, 50% alcohol was used in place of the carvacrol for all 29 TSA plates for the isolates as a control experiment. It should be noted that the carvacrol was dissolved in 96% alcohol (concentrated alcohol) and the stock dissolved with water before usage hence, the main aim of the control experiment was to find out if the alcohol in the carvacrol would have effect on the inhibition of the bacteria.

Figure 5: Clear zones of inhibition around papaer disk on TSA plate for Microbacterium arborescens

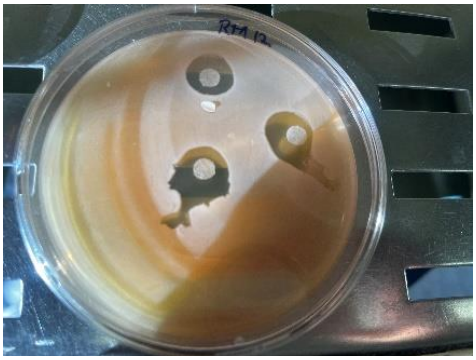


Figure 6: Zones of inhibition for Macrococcus caseolyticus

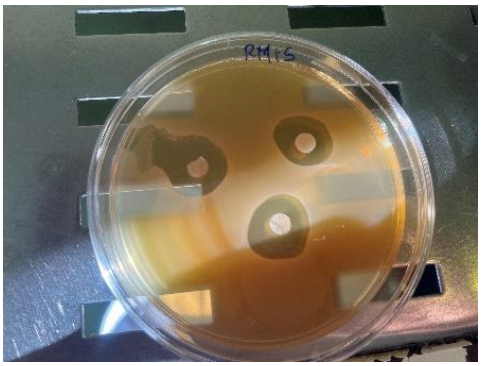


Figure 7: Zones of inhibition for Buttiauxella noackiaes

The effects of carvacrol were seen on all the isolates except *Bacillus cereus* and *Hafnia alvei*. Generally, the presence of phenolic compounds in essential oils show antimicrobial activity against gram-positive bacteria but this wasn't the case in *Bacillus cereus*. This could be attributed to the fact that contamination occurred during the addition of the carvacrol or the right concentration of carvacrol was not used. Gram-negative bacteria are more resistant to EOs than Gram-positive bacteria due to the complexity of the cell wall (Nazzaro, Fratianni and Martino, 2013). *Hafnia alvei* is a gram-negative facultative anaerobic bacillus which is rarely pathogenic, probably the reason there was no inhibition. The pictures above were some selected isolates whose inhibition zones were very clear. The Antimicrobial testing was repeated because on the first occasion, 0.3mg/ml of carvacrol was used and there were no inhibition zones around the agar disks. On the second occasion, the concentration of the carvacrol was increased to 15mg/ml before inhibition zones were detected. Generally, the efficiency the carvacrol increases as the concentration increases. This also could depend on the food matrix from which the microorganisms were isolated. As already stated in the literature review from the study of (Rattanachaikunsopon and Phumkhachorn, 2010),

the matrix of the food could be a barrier to the efficacy of the antimicrobial agent. Pork meat is high in fat hence high concentration of carvacrol was needed for it to exhibit its activity. Table 8 is the results of the diameters of the inhibition zones of all the 29 isolates tested. These are the results of the 23 isolates because the MALDI-ToF MS was not able to identify the rest of the isolates.

Table 8: Antimicrobial Agent Test results and diameter of inhibition measurement of isolates

Isolate	Identification result	Average of inhibition zone diamater (mm)
PM1	*Buttiauxella gaviniae*	16.67
PM2	*Aeromonas sp*	11.67
PM3	*Kocuria salsicia*	15
PM5	*Pseudomonas lundensis*	16.67
PM6	*Pseudomonas fluorescens*	11.67
RM20	*Hafnia alvei*	0
RM19	*Stenotrophomonas maltophilla*	10
RM12	*Macrococcus caseolyticus*	16.67
RM6	*Staphilococcus chromogenes*	16.67
RM4	*Kocuria salsicia*	16.67
RM15	*Buttiauxella noackiae*	18.33
RM21	*Pantoea agglomerans*	16.67
RM13	*Acitenobacter johnsonii*	20
P21	*Pseudomonas flavescens*	15
P26	*Microbacterium arborescens*	17.5
P28	*Pseudonomas antarctica*	16.67
P29	*Pseudomonas fulva*	11.67
P32	*Bacillus cereus*	0
P33	*Microbacterium arborescens*	18.33
P19	*Pluralibacter pyrinus*	10
P30	*Pseudonomas oryzihabitans*	11.33
P9	*Bacillus cereus*	15
P16	*Micrococcus luteus*	20

As already stated, most of the bacteria responded positively to the antimicrobial test agent with varying diameters. The results showed that carvacrol could inhibit positively both pathogenic and non-pathogenic bacteria.

5.9. Agar Disk Diffusion Test

The Agar Disk Diffusion Test was used to test for antimicrobial activity of serial dilutions of carvacrol. Bacterial suspension of six isolates that were susceptible to the antimicrobial testing were used for the Agar Disk Diffusion Test. The six isolates were PM5 (*Pseudomonas lundensis)*, PM1 (*Buttiauxella gaviniae)*, RM12 (*Macrococcus caseolyticus)*, P16 (*Micrococcus luteus)*, P28 (*Pseudonomas antarctica)* and RM4 (*Kocuria salsicia)*. The bacterial suspension was used to prepare lawns on TSA plates with an O.D 0.5, in three parallels. Four serial dilutions of carvacrol was prepared with concentrations 60mg/ml, 30mg/ml, 15mg/ml and 7.5mg/ml. Four Agar Disks were put on each six TSA plates labelled 1, 2, 3 and 4. 10µ of the dilution series of carvacrol was transferred to each agar disk paper labelled 1, 2, 3 and 4. The four concentration of carvacrol corresponded to the labelled agar disks on the TSA plate. So, the 60mg/ml concentration of carvacrol was transferred to the first agar disk labelled 1 and so on. Table 8 shows the results of the six isolates that were more susceptible to carvacrol. The pictures below (Figures 8-9) were some selected isolates out of the six. It was observed that the clear zones around the agar disk labelled 1 and 2 were the first and second dilutions of carvacrol, meaning there was an inhibition. There was little to no clear zone around the agar disks labelled 3 and 4 which were from the third and fourth dilutions of carvacrol. This means that the higher the concentration of carvacrol, the higher its efficacy of inhibition.

Figure 8: Zones of inhibition for some selected strains

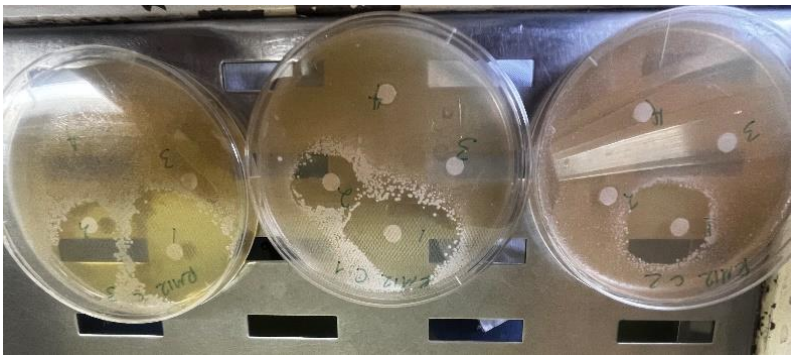


Figure 9: Inhibition zones of some selected isolates

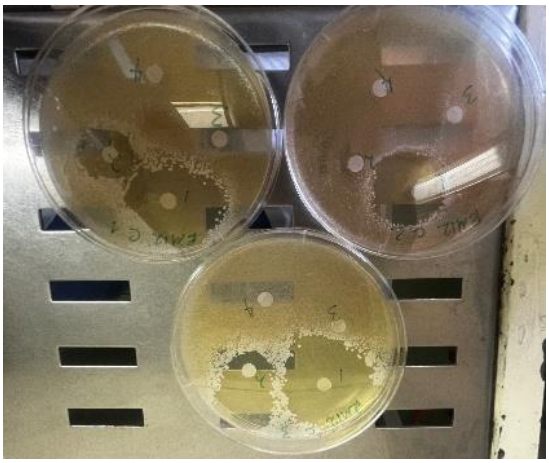


Table 9: Agar Disk Diffusion Test results

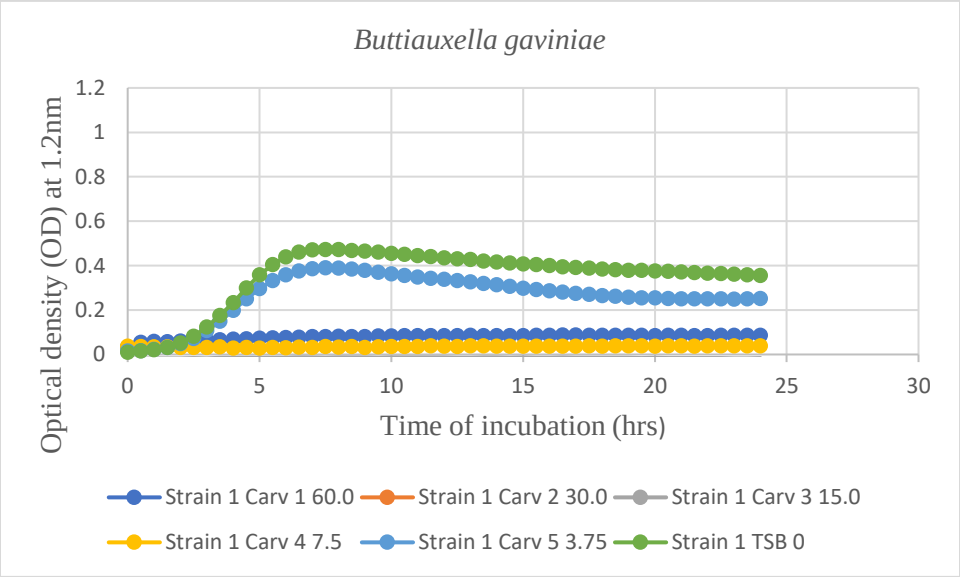
Isolates	Diameter (mm)			
	1st dilution	2nd dilution	3rd dilution	4th dilution
PM5 C1	5.00	5.00	5.00	5.00
PM5 C2	30.00	0.00	0.00	0.00
PM5 C3	15.00	10.00	0.00	0.00
PM1 C1	15.00	10.00	10.00	5.00
PM1 C2	20.00	0.00	0.00	0.00
PM1 C3	35.00	23.00	0.00	0.00
RM12 C1	30.00	0.00	0.00	0.00
RM12 C2	40.00	25.00	0.00	0.00
RM12 C3	45.00	40.00	0.00	0.00
P16 C1	15.00	10.00	0.00	0.00
P16 C2	0.00	0.00	0.00	0.00
P16 C3	15.00	10.00	5.00	0.00
P28 C1	10.00	10.00	0.00	0.00
P28 C2	10.00	0.00	0.00	0.00
P28 C3	15.00	0.00	0.00	0.00
RM4 C1	5.00	10.00	0.00	0.00
RM4 C2	10.00	0.00	0.00	0.00
RM4 C3	30.00	15.00	0.00	0.00

5.10. Optical Density Measurement for MIC determination

The optical density measurement was done to check the growth of the microorganisms in the presence of carvacrol and to determine the MIC values. The MIC values were determined by measuring the optical density. In addition, positive (no carvacrol) and negative (no bacteria) controls were included for each strain. The six microorganisms that were more susceptible to carvacrol were used for this measurement. The six include *Pseudomonas lundensis*, *Buttiauxella gavinia*, *Macrococcus caseolyticus*, *Micrococcus luteus*, *Pseudonomas antarctica* and *Kocuria salsicia.* Triplicates of each microbial suspension were put in six wells on the microtiter plate.

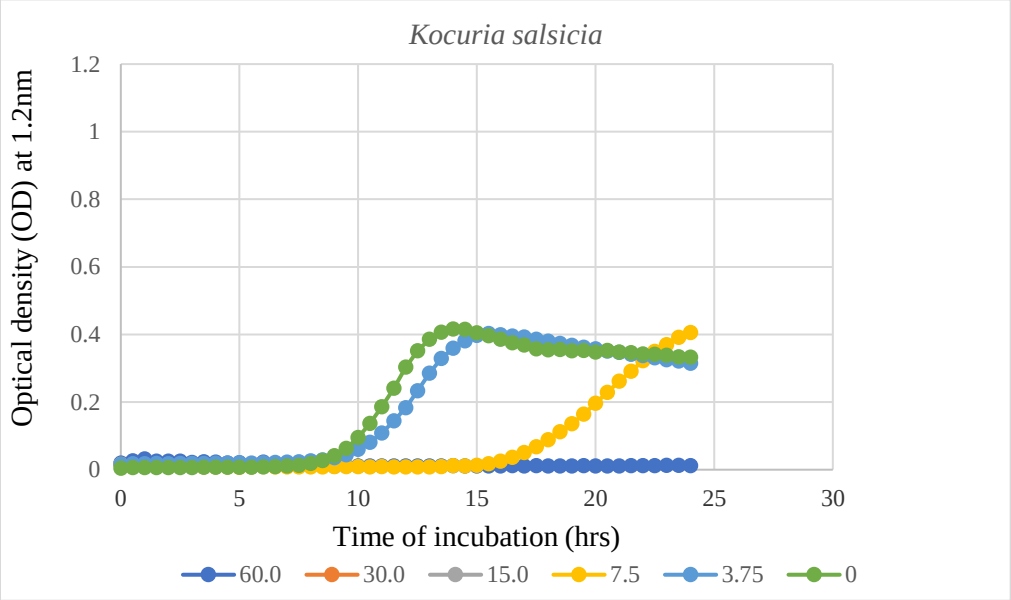
The microtiter plates were incubated at 30°C for 24 hours in Mulstiskan Accent equipment. The MIC value was determined visually by figures, considered as the lowest concentration of carvacrol able to inhibit the visible growth of the microorganism at a standard incubation time.

Figure 10: Turbidimetric curve of Buttiauxella gaviniae after 24 hours of incubation



The MIC value is the lowest concentration of carvacrol needed to inhibit the growth of bacteria completely. From Figure 11, the MIC value of *Buttiauxella gaviniae* was 3.75 mg/ml. It has been reported that the MIC values found for carvacrol ranges between 250 to 2500 µg/ml depending on the microorganism and the conditions of the experiment (Ben Arfa *et al.*, 2006). The MIC value reported here is 3750 µg/ml which shows that carvacrol can effectively inhibit foodborne microorganisms. The green curve is for the control (without the carvacrol) so growth of *Buttiauxella gaviniae* was expected to occur because there was no addition of carvacrol. It should be noted that *Buttiauxella gaviniae* is a Gram-negative bacteria and generally, Gram-negative bacteria are more resistant to essential oils.

Figure 11: Turbidimetric curve of Kocuria salsicia after 24 hours of incubation.



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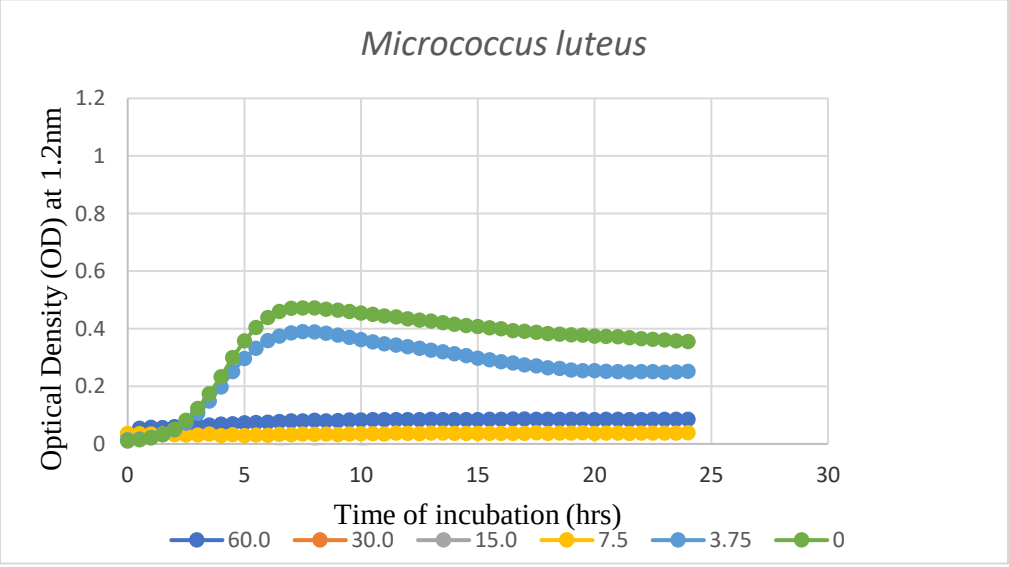
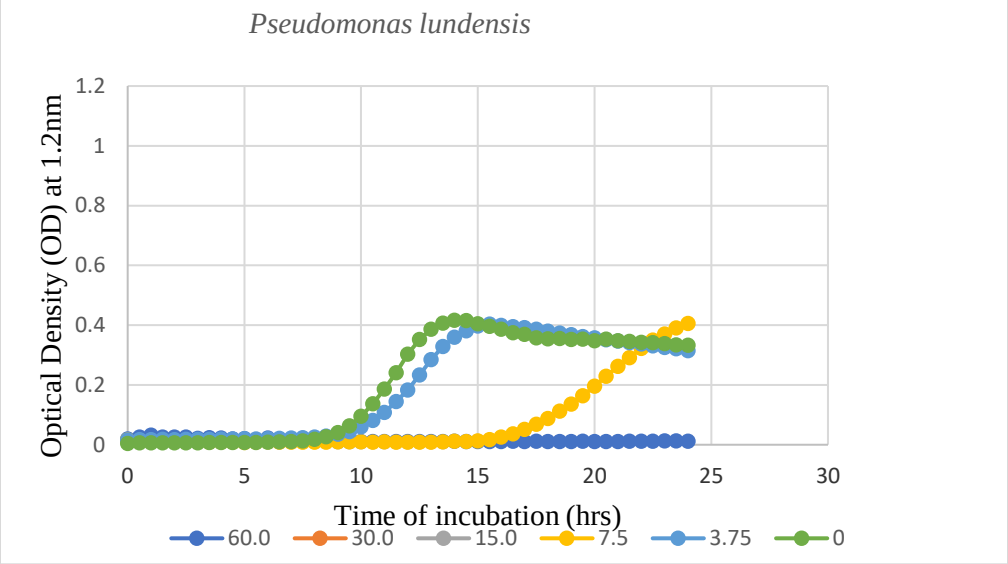
Kocuria salsicia is a Gram-positive bacteria. The MIC value reported was 7.5 mg/ml.

Figure 12: Turbidimetric curve of Micrococcus luteus after 24 hours of incubation



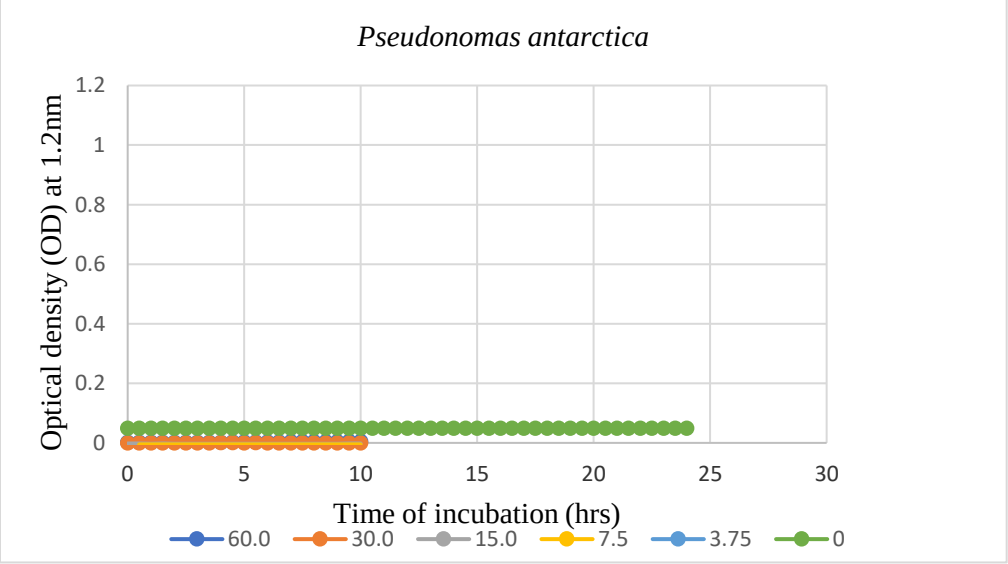
Micrococcus luteus is a Gram-positive bacteria. The MIC value reported here was 30.00 mg/ml.

Figure 13: Turbidimetric curve of Pseudomonas lundensis after 24 hours of incubation



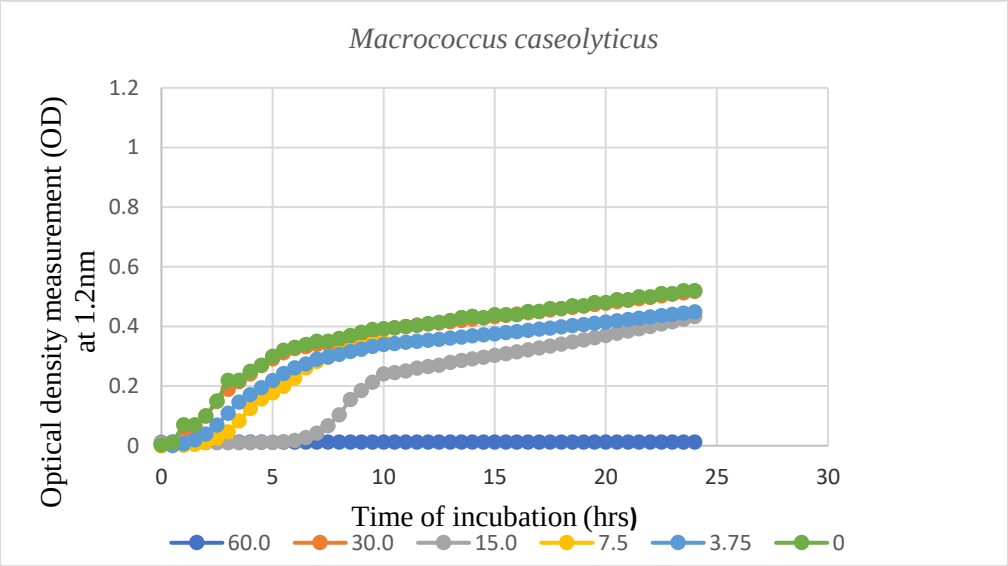
Pseudomonas lundensis is a Gram-negative bacterium. The MIC value recorded here was 7.5 mg/ml. According to (Hussein *et al.*, 2020) who worked on the antimicrobial activity of carvacrol against *Pseudomonas lundensis*, the reported value of the MIC was 15.3 µl/ml. However, after another 24 hrs of incubation, the MIC was 3.9 µL/mL. It was observed from their study that the lowest concentration of MIC values for *P. lundensis* were observed for carvacrol.

Figure 14: curve of Pseudomonas antartica after 24 hours of incubation



It was observed that the *Pseudonomas antarctica* was not able to growth at the provided circumstances in the broth that was used for the experiments, therefore its MIC value could not be determined. Further experiments should be done by changing the growth medium and incubation temperature.

Figure 15: Turbidimetric curve of Macrococcus caseolyticus after 24 hours of incubation



Macrococcus caseolyticus is a Gram-positive opportunistic pathogen. Its MIC value for carvacrol was found to be 30 mg/ml.

6. CONCLUSION AND PROPOSAL

The MIC values recorded for all the six strains is an indication that carvacrol can effectively inhibit the growth of foodborne microorganisms. Generally, the efficacy of natural antimicrobials in food is lower than in model solutions or solid media because food components affect their activity. To validate and affirm the activity and safety of carvacrol for use in food systems, additional research is required.

7. SUMMARY

Essential oils can inhibit pathogenic and spoilage bacteria in food. The effect of carvacrol on foodborne microorganisms isolated from pork is a study aimed to find the potential use of carvacrol as an alternative to artificial preservatives. Carvacrol is an essential oil compound found in spices such as oregano and thyme. Essential oils have long been used as flavoring agents in the ancient times but there hasn't been comprehensive research on its use as natural antimicrobials in foods.

The phenotypic characterization of the six microorganisms isolated from TGE, Centrimide, MRS and Chromocult was carried out by catalase, oxidase and KOH tests as well as microscopic investigations. The MALDI-Tof mass spectrometry was used to identify the isolated strains. It should be noted that in the frame of this work, the Agar Disk Difussion test and the Optical Density measurement was done on 29 strains (9 strains isolated from raw milk by Diana Lazalde) and (15 strains isolated from pepper by Yomalli Mera Cruz). The Agar Disk Difussion test was done to know the susceptibility of the strains to carvacrol. It was observed that there was inhibition zones around the paper disks at the first and second concentrations of carvacrol (30 mg/ml and 15 mg/ml), meaning that a high concentration of carvacrol is needed for inhibition. All the strains tested positive to carvacrol except *Bacillus cereus* and *Hafnia alvei.* A control experiment with only alcohol was used basically to find out if the alcohol present in the carvacrol contributed to the inhibition since the carvacrol was dissolved in concentrated ethanol and diluted with water.

Based on the results of the agar disk diffusion method, 6 strains were selected for their MIC value determination which included *Pseudomonas lundensis, Buttiauxella gaviniae, Macrococcus caseolyticus, Micrococcus luteus, Pseudonomas antarctica* and *Kocuria salsicia* in the broth microdilution method as well in liquid phase. Optical Density measurement was conducted to generate the turbidimetric curves of all the six strains. The MIC values are the lowest concentration of carvacrol that can completely inhibit the growth of the microorganisms, yeasts and moulds. The MIC values of the six strains of tested microorganisms were visualized using figures.

The MIC values indicated that carvacrol can be used as an alternative natural antimicrobial to inhibit foodborne microorganisms in food.

8. REFERENCES

- de Almeida de Souza, G.H. *et al.* (2021) 'In vitro and in vivo antibacterial activity assays of carvacrol: A candidate for development of innovative treatments against KPC-producing Klebsiella pneumoniae', *PLoS ONE*, 16(2 February), pp. 1–13. Available at: https://doi.org/10.1371/journal.pone.0246003.
- Antonia Nostro and Teresa Papalia (2012) 'Antimicrobial Activity of Carvacrol: Current Progress and Future Prospectives', *Recent Patents on Anti-Infective Drug Discovery*, 7(1), pp. 28–35. Available at: https://doi.org/10.2174/157489112799829684.
- Ben Arfa, A. *et al.* (2006) 'Antimicrobial activity of carvacrol related to its chemical structure', *Letters in Applied Microbiology*, 43(2), pp. 149–154. Available at: https://doi.org/10.1111/j.1472-765X.2006.01938.x.
- Arfa, A. Ben *et al.* (2006) 'Antimicrobial activity of carvacrol related to its chemical structure', 43, pp. 149–154. Available at: https://doi.org/10.1111/j.1472-765X.2006.01938.x.
- Artiga-Artigas, M., Acevedo-Fani, A. and Martín-Belloso, O. (2017) 'Improving the shelf life of low-fat cut cheese using nanoemulsion-based edible coatings containing oregano essential oil and mandarin fiber', *Food Control*, 76, pp. 1–12. Available at: https://doi.org/10.1016/j.foodcont.2017.01.001.
- Burt, S. (2004) 'Essential oils: Their antibacterial properties and potential applications in foods - A review', *International Journal of Food Microbiology*, 94(3), pp. 223–253. Available at: https://doi.org/10.1016/j.ijfoodmicro.2004.03.022.
- Catarino, M.D. *et al.* (2017) 'Development and performance of whey protein active coatings with Origanum virens essential oils in the quality and shelf life improvement of processed meat products', *Food Control*, 80, pp. 273–280. Available at: https://doi.org/10.1016/j.foodcont.2017.03.054.
- Çetin, B., Çakmakçi, S. and Çakmakçi, R. (2011) 'The investigation of antimicrobial activity of thyme and oregano essential oils', *Turkish Journal of Agriculture and Forestry*, 35(2), pp. 145–154. Available at: https://doi.org/10.3906/tar-0906-162.
- Crista, M.-E. and Butnariu, M. (2023) 'Citation for this article: Madalina-Elena CRISTA and Monica BUTNARIU. Plant Products Containing Volatile Oils and their Uses', *Nutrition And Food Science Journal*, 6(1), p. 141. Available at: https://www.researchgate.net/publication/369912560.
- Devine, R. (2003) 'La consommation des produits carnés', *Productions Animales*, 16(5), pp. 325–327.
- Gyawali, R. and Ibrahim, S.A. (2014) 'Natural products as antimicrobial agents', *Food Control*,

46, pp. 412–429. Available at: https://doi.org/10.1016/j.foodcont.2014.05.047.

- Hussein, K.N. *et al.* (2020) 'In vitro antimicrobial activity of plant active components against Pseudomonas lundensis and Listeria monocytogenes', *Progress in Agricultural Engineering Sciences*, 16, pp. 163–172. Available at: https://doi.org/10.1556/446.2020.20018.
- Hyldgaard, M., Mygind, T. and Meyer, R.L. (2012) 'Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components', *Frontiers in Microbiology*, 3(JAN), pp. 1–24. Available at: https://doi.org/10.3389/fmicb.2012.00012.
- Ju, J. *et al.* (2019) 'The inhibitory effect of plant essential oils on foodborne pathogenic bacteria in food', *Critical Reviews in Food Science and Nutrition*, 59(20), pp. 3281–3292. Available at: https://doi.org/10.1080/10408398.2018.1488159.
- Khaleque, M.A. *et al.* (2016) 'Use of cloves and cinnamon essential oil to inactivate Listeria monocytogenes in ground beef at freezing and refrigeration temperatures', *Lwt*, 74, pp. 219–223. Available at: https://doi.org/10.1016/j.lwt.2016.07.042.
- Mauriello, E., Ferrari, G. and Donsì, F. (2021) 'Effect of formulation on properties, stability, carvacrol release and antimicrobial activity of carvacrol emulsions', *Colloids and Surfaces B: Biointerfaces*, 197(July 2020), p. 111424. Available at: https://doi.org/10.1016/j.colsurfb.2020.111424.
- Mechmechani, S. *et al.* (2022) 'Microencapsulation of carvacrol as an efficient tool to fight Pseudomonas aeruginosa and Enterococcus faecalis biofilms', *PLoS ONE*, 17(7 July), pp. 1–20. Available at: https://doi.org/10.1371/journal.pone.0270200.
- Munhuweyi, K. *et al.* (2018) 'Physical and antifungal properties of β-cyclodextrin microcapsules and nanofibre films containing cinnamon and oregano essential oils', *Lwt*, 87, pp. 413–422. Available at: https://doi.org/10.1016/j.lwt.2017.09.012.
- National Center for Emerging & Zoonotic Infectious Diseases (2011) 'Pathogens causing the most illnesses, hospitalizations, and deaths each year', pp. 3–4. Available at: www.cdc.gov/foodborneburden.
- Nazzaro, F. *et al.* (2013) 'Effect of essential oils on pathogenic bacteria', *Pharmaceuticals*, 6(12), pp. 1451–1474. Available at: https://doi.org/10.3390/ph6121451.
- Nazzaro, F., Fratianni, F. and Martino, L. De (2013) 'Effect of Essential Oils on Pathogenic Bacteria', pp. 1451–1474. Available at: https://doi.org/10.3390/ph6121451.
- Park, J.M. *et al.* (2020) 'Transfer rates of pathogenic bacteria during pork processing', *Journal of Animal Science and Technology*, 62(6), pp. 912–921. Available at: https://doi.org/10.5187/JAST.2020.62.6.912.

- Peñalver, P. *et al.* (2005) 'Antimicrobial activity of five essential oils against origin strains of the Enterobacteriaceae family', *Apmis*, 113(1), pp. 1–6. Available at: https://doi.org/10.1111/j.1600-0463.2005.apm1130101.x.
- Rattanachaikunsopon, P. and Phumkhachorn, P. (2010) 'Assessment of factors influencing antimicrobial activity of carvacrol and cymene against Vibrio cholerae in food', *Journal of Bioscience and Bioengineering*, 110(5), pp. 614–619. Available at: https://doi.org/10.1016/j.jbiosc.2010.06.010.
- Report, S. (2023) 'The European Union One Health 2022 Zoonoses Report', *EFSA Journal*, 21(12). Available at: https://doi.org/10.2903/j.efsa.2023.8442.
- Rúa, J. *et al.* (2019) 'Combination of Carvacrol and Thymol: Antimicrobial Activity Against Staphylococcus aureus and Antioxidant Activity', *Foodborne Pathogens and Disease*, 16(9), pp. 622–629. Available at: https://doi.org/10.1089/fpd.2018.2594.
- Sacchetti, G. *et al.* (2005) 'Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods', *Food Chemistry*, 91(4), pp. 621–632. Available at: https://doi.org/10.1016/j.foodchem.2004.06.031.
- Singhal, N. *et al.* (2015) 'MALDI-TOF mass spectrometry: An emerging technology for microbial identification and diagnosis', *Frontiers in Microbiology*, 6(AUG), pp. 1–16. Available at: https://doi.org/10.3389/fmicb.2015.00791.
- Tong, N.T.A. (2020) 'Antimicrobial activity of essential oils on food pathogens', *The Journal of Agriculture and Development*, 19(4), pp. 64–72. Available at: https://doi.org/10.52997/jad.8.04.2020.
- Ultee, A., Bennik, M.H.J. and Moezelaar, R. (2002) 'The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus', *Applied and Environmental Microbiology*, 68(4), pp. 1561–1568. Available at: https://doi.org/10.1128/AEM.68.4.1561-1568.2002.
- Ultee, A., Kets, E.P.W. and Smid, E.J. (1999) 'Mechanisms of action of carvacrol on the food-borne pathogen', *Applied and Environmental Microbiology*, 65(10), pp. 4606–4610. Available at: https://doi.org/10.1128/aem.65.10.4606-4610.1999.
- Wang, P. and Wu, Y. (2021) 'A review on colloidal delivery vehicles using carvacrol as a model bioactive compound', *Food Hydrocolloids*, 120(July 2020), p. 106922. Available at: https://doi.org/10.1016/j.foodhyd.2021.106922.
- Wijesundara, N.M. *et al.* (2021) 'Carvacrol exhibits rapid bactericidal activity against Streptococcus pyogenes through cell membrane damage', *Scientific Reports*, 11(1), pp. 1–14. Available at: https://doi.org/10.1038/s41598-020-79713-0.

- Zhang, Y. *et al.* (2017) ‘Effect of cinnamon essential oil on bacterial diversity and shelf-life in vacuum-packaged common carp (Cyprinus carpio) during refrigerated storage’, *International Journal of Food Microbiology*, 249, pp. 1–8. Available at: https://doi.org/10.1016/j.ijfoodmicro.2016.10.008.

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