Characterization of mānuka and rosemary oils as antimicrobial and antioxidant agents for meat applications

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Abstract

The usage of chemical preservatives in meat products has been associated with adverse health effects, which is driving consumers' preferences towards natural preservatives. Raw and processed meats have been linked to cancers due to the presence of nitrate/nitrite as a chemical preservative. In recent years, extensive innovative and promising approaches have been exploited to entirely or partially replace synthetic preservatives. Plant-based natural preservatives possessing antioxidant and antimicrobial characteristics can be ideal for food applications. Essential oils are aromatic liquids extracted from different plant parts, such as leaves, bark, roots, and seeds, and are rich sources of bioactive compounds like monoterpenes, sesquiterpenes, and oxygenated sesquiterpenes and phenolic compounds. As per the literature, essential oils possessing antioxidant and antimicrobial characteristics have been reported to decline the rate of oxidative reactions and microbial growth. This study aims to harness the potential of essential oil obtained from the indigenous plant of New Zealand, i.e., mānuka, as a natural antioxidant and antimicrobial agent for meat preservation than chemical preservatives like nitrates/nitrites. As per the available literature, β-triketones are responsible for the antimicrobial characteristics of mānuka oil. We hypothesise that using antioxidants and antimicrobial bioactive compounds of *Leptospermum scoparium* (mānuka) will improve the shelf life and stability of the meat products.

The first research objective characterised and compared the antioxidant and antimicrobial potential of mānuka oil with different triketone contents (5, 25, and 40 %) and kānuka oil with a commonly used natural preservative, i.e., rosemary oil. In chemical composition, kānuka oil possessed higher levels of α-pinene, while rosemary oil exhibited higher amounts of 1,8 cineole and α-pinene as primary compounds. In mānuka oils, the concentration of other compounds decreased as triketone content increased from 5 to 40 %. A comparison of the antioxidant

characteristics of these oils was also made with chemical antioxidants, i.e., butylated hydroxytoluene (BHT). It was observed that mānuka oils possess higher antioxidant properties than rosemary and BHT (at both the lowest tested concentrations of 0.1 % and 1 %). In the antimicrobial efficacies assay results, all mānuka oils showed more effectiveness against *Listeria monocytogenes* and *Staphylococcus aureus* than *Salmonella* and *Escherichia coli*. However, the inhibition effect of rosemary oil was greater against *Salmonella* and *Escherichia coli* than mānuka oil **(Chapter 3)**. The minimum inhibitory concentration of all mānuka oils required to inhibit *Listeria monocytogenes* and *Staphylococcus aureus* was below 0.04 %, while kānuka and rosemary oil inhibited these microbes at 0.63 and 2.5 %, respectively. On the other hand, a minimum 2.5 % concentration of all oils was needed to inhibit *Salmonella* and *Escherichia coli.* These results indicated that mānuka oil can be used as an antimicrobial agent, particularly against tested Gram-positive microbes (at a very concentration of 0.04 %) in meat products, while rosemary oil can be used against all tested microbes at 2.5 %. However, meat constituents such as fats have a significant effect on the efficacies of added bioactive compounds, therefore, it is essential to have insights into the lipophilicity of added essential oils and their bioactive compounds.

In the next research experiment, to confirm the lipophilic behaviour of chemical compounds present in mānuka oil, the octanol-water partition coefficient of beta-triketones (leptospermone, isoleptospermone, and flavesone), α-pinene and γ-terpinene were elucidated using shake flask method (Gas chromatography and mass spectrometry) and predicted using EPI software (**Chapter 4**). High values of the octanol-water partition coefficient of these compounds indicate their more affinity towards the fat than the water. Further, when the concentration of the compounds separated in 3 and 12 % beef-fat and water systems was determined, all compounds showed higher concentrations in water of the low-fat system than in the high-fat. The findings pointed out that essential oils may exert an antioxidant effect in

the high-fat system to prevent lipid oxidation; however, their antimicrobial effect may be reduced due to the presence of fat, and higher concentrations of these oils may be needed to achieve an antimicrobial effect against selected microbes.

In the third research experiment, selected mānuka and rosemary oils were used as natural antioxidants and antimicrobial agents in low and high-fat meat pastes prepared from commercial-breed and wagyu beef tenderloins, respectively **(Chapter 5)**. These effects were compared against the chemical preservative sodium nitrate and butylated hydroxytoluene during refrigerated storage of meat pastes at 4 °C. In commercial and wagyu beef pastes, a lower number of *Listeria monocytogenes* and *Staphylococcus aureus* were observed in mānuka and rosemary oil treatments than in the sodium nitrate and control samples (without added preservative). Rosemary oil also delayed the growth of *Salmonella* and *Escherichia coli* more than mānuka oil added and control samples. In terms of oxidative stability, mānuka oil added wagyu beef pastes were more stable and showed the lowest lipid oxidation values than all treatments. In commercial beef samples, no significant difference between essential oils added samples, either mānuka or rosemary oil and control samples was observed. There was a significant change in pH values of all wagyu and commercial beef samples, whilst these changes were greater in untreated samples (controls) than in the essential oils-treated samples. Despite the promising antioxidant and antimicrobial characteristics of essential oils, these are rarely utilised in food products owing to their easy degradation, low water solubility, low stability, and unwanted odour and flavour. The application of essential oils in encapsulated form is an effective and innovative approach to overcome these limitations by covering the core materials (oil droplets) in carrier materials. In addition, it improves stability and provides controlled release and targeted delivery of essential oils in foods.

In the next research objective, mānuka and rosemary oils-containing nonentities (nanoemulsions and nanocapsules) made of sodium alginate and whey protein were fabricated and compared for their thermal stability and release characteristics **(Chapter 6)**. The particle size and zeta potential of prepared nanoentities were between 100 -600 nm and -10 to -40 mV, confirming that the obtained nanoemulsions and nanocapsules were stable and in the nano range. The obtained nanoentities were observed to be more thermostable, sustained release profile than the free form of oils while showing a lower *in vitro* antioxidant effect. The release mechanism of the essential oil from nanoemulsions and nanocapsules was also studied using different mathematical models. The release mechanism of essential oil from nanoemulsions and nanocapsules followed Higuchi's law, which indicates that the solvent first penetrates the encapsulated matrix and then dissolves the embedded oil droplets through the diffusion process. The delayed or sustained release from encapsulated oil might influence the antioxidant and antimicrobial activity of essential oils in meat pastes. However, a food matrix made up of different constituents can affect the partitioning and release of essential oils from the carrier material, and consequently, their preservative effect may vary according to the meat paste.

An improvement in the antioxidant activity of oils after emulsification was observed as nanoemulsions of both oils had the lowest TABRS values in crossbred and wagyu pastes **(Chapter 7)**. Mānuka oil and its nanoentities had more antioxidant effects than rosemary oil. In wagyu pastes, there was a significant difference in nanoemulsions added pastes than the other treatments, while in crossbred pastes, no significant differences were noted between free oils and nanoentities containing beef pastes. Despite the antioxidant efficacies, the antimicrobial activity of free, nanoemuslfisied and nanoencapsulated oils was also determined in the wagyu and crossbred beef pastes during refrigerated storage (4 °C) of two weeks. These antimicrobial effects were compared against controls (without added preservatives) and sodium nitrite-added paste samples. There was a significant increase in microbial counts of all

inoculated-paste samples, whilst this increase was lower in preservatives added samples than in the controls. In wagyu and crossbred beef pastes, mānuka oil and its nanoentities delayed the growth of *Listeria monocytogenes* and *Staphylococcus aureus*, and mānuka-nanoemulsions exhibited the lowest number of these microbes than all other treatments. However, rosemary oil and its nanoforms effectively inhibited *Salmonella* and *Escherichia coli* during refrigerated storage at 4 °C. To better understand the mechanism for the antimicrobial activity of essential oils against selected pathogens, cell viability membrane integrity and the release of intracellular compounds and proteins through fluorescence-based assays were determined. In all these assay results, mānuka and rosemary oils treatment of *Listeria monocytogenes*, *Staphylococcus aureus, Salmonella* and *Escherichia coli* exhibited a decline in cell viability, disrupted cell-wall permeability and enhanced release of intracellular compounds and proteins from cells than the untreated cells. Scanning electron micrographs also confirmed that these mechanisms were responsible for the antibacterial efficacy of mānuka and rosemary oil. To correlate the effect of fat content on varied antimicrobial characteristics of essential oils in meat pastes, the partitioning of essential oils in different phases, such as octanol, beef and water, was determined.

Overall, the work showed that mānuka oil has the potential to be used in meat pastes as an antimicrobial agent, especially against tested Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*). In addition, this oil can be used to completely replace synthetic antioxidants like butylated hydroxytoluene to inhibit lipid oxidation in high-fat meat systems. Due to the lipophilic nature of oils, the fat content of meat systems significantly affects the partitioning of these oils in water and fat phases, which in turn affect their antimicrobial efficacies.

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List of abbreviations

ABTS	2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
b*	Yellowness
BHT	Butylated Hydroxytoluene
cfu	Colony forming units
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediamine tetra-acetic acid
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FRAP	Ferric Radical Absorbing Power
FTIR	Fourier Transform-Infrared Spectroscopy
FU	Fluorescence Units
g	Grams
IR	Infra-red
K_{ao}	Octanol-air partition coefficient
K_{aw}	Air-water partition coefficient
kg	Kilograms
KO	Kānuka oil
$K_{ow\ or}$ *log P*	Octanol-water partition coefficient
L*	Lightness
log	Logarithmic
M	Molar

MBC	Minimum Bactericidal Concentration
MC	Mānuka nanocapsules
MDA	Malondialdehyde
ME	Mānuka nanoemulsion
mg	Milligrams
MIC	Minimum Inhibitory Concentration
mL	Millilitres
MO	Mānuka oil
MW	Molecular weight
N	Normality
PCA	Principal Component Analysis
RC	Rosemary nanocapsules
RE	Rosemary nanoemulsion
RO	Rosemary oil
SEM	Scanning electron microscopy
TBARS	Thiobarbituric acid
TCA	Trichloroacetic acid
USFDA	United States Food and Drug Administration
V	Volume
W	Weight
α	Alpha
β	Beta
γ	Gamma
δ	Delta

° C	Degree celsius
µg	Micrograms
µL	Microlitres
a*	Redness
$CaCl_2$	Calcium chloride

List of Publications

1. Kaur, R., Gupta, T. B., Bronlund, J. and Kaur L. (2021). The potential of rosemary as a functional ingredient for meat products- A Review. *Food Reviews International*. https://doi.org/10.1080/87559129.2021.1950173
2. Kaur, R. and Kaur L. (2021). Encapsulated natural antimicrobials: A promising way to reduce microbial growth in different food systems. *Food Control*. https://doi.org/10.1016/j.foodcont.2020.107678
3. Kaur, R., Kaur L., Gupta, T. B., Singh, J. and Bronlund, J. (2022). Multi-target preservation technologies for chemical-free sustainable meat processing. *Journal of Food Science*. https://doi.org/10.1111/1750-3841.16329
4. Kaur, R., Kaur L., Gupta, T. B. and Bronlund, J. (2023). Effectiveness of mānuka and rosemary oils as natural and green antioxidants in wagyu and normal beef pastes. *International Journal of Food Science and Technology*. https://doi.org/10.1111/ijfs.16390
5. Kaur, R., Kaur L., Gupta, T. B. and Bronlund, J. (2023). Mānuka oil vs rosemary oil: Antimicrobial efficacies in wagyu and commercial beef against selected pathogenic microbes. *Foods*. https://doi.org/10.3390/foods12061333
6. Kaur, R., Gupta, T. B., Bronlund, J. and Kaur L., (2023). Synthesis and characterisation of mānuka and rosemary oils-based nano-entities: Application in wagyu and crossbred beef pastes preservation. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2023.137600
7. Kaur, R., Gupta, T. B., Bronlund, J. and Kaur L., (2023). Effectiveness of mānuka and rosemary oils- containing nanoemulsions and nanocapsules as a natural antimicrobial agent in wagyu and crossbreed beef (*Food Research International*, to be submitted).

8. Kaur, R., Bronlund, J., Gupta, T. B. and Kaur L. (2023). Partitioning of essential oil compounds in beef fat and water: using an octanol-water partition coefficient approach (To be submitted).

Chapter 1 Introduction

Consumers' demand for natural, healthy, safe, and stable food products is rising. Foods like meat, seafood, fruits, and vegetables are highly perishable, so these are extensively prone to chemical, microbial, and enzymatic spoilage reactions. All these deteriorative mechanisms lead to the loss of nutritional values of food products through the breakdown of macronutrients (carbohydrates, protein, and fats) and the development of undesirable odours and flavours (Dave & Ghaly, 2011). As a result, these food products become objectionable to consumers and pose significant economic and nutritional losses (Dave & Ghaly, 2011; Iulietto et al., 2015). Thereby, food researchers and processers constantly strive to improve the shelf life, safety, and quality of food formulations through a wide range of conventional and modern techniques. Various preservative treatments have been optimised and utilised to enhance the shelf life of food products. However, food preservation is not as straightforward as in historic times, it has evolved from an art level to an interdisciplinary science (Rahman, 2020). These days, food manufacturers and producers prefer mild preservation methods to meet consumer demands for the natural appearance and nutritional characteristics of foods than robust techniques (Smid & Gorris, 2020).

Artificial preservatives have long been employed to control the abovementioned spoilage mechanisms; and thereby improve the shelf life, safety and quality of food products. However, some of these preservatives, e.g., nitrates/ nitrites in meat and meat products, have been suspected as carcinogenic (Rather et al., 2016; dos Reis et al., 2017). Although the exact mechanism and evidence are not clear and precise, but it is suspected that cancer precursors may be excess fat, protein, iron, and curing agents (salt, nitrates, nitrites), and compounds produced during smoking (heterocyclic amines, polyaromatic hydrocarbons) (Botez et al., 2017). Raw meats are intrinsically low in endogenic antimicrobial and antioxidant compounds,

while their nutrient-rich environment makes them prone to various spoilage reactions. In addition, processing operations facilitate the reactions by loss of muscle structure integrity, exposure to oxygen/air/prooxidants, increase in surface area reduction in endogenous enzymes and nonenzymatic components and increase in surface area (Iulietto et al., 2015; Bekhit et al., 2021). Thus, the preservation of meat and meat-based products through different approaches is of utmost importance. The adverse health effects of artificial preservatives and a recent rise in consumer popularity and awareness of plant-based preservatives have driven the meat industry to consider alternatives to synthetic preservatives (Jayasena & Jo, 2013).

Plant-based preservatives are gaining importance over chemical preservatives as a good source of antioxidant and antimicrobial compounds (Vital et al., 2016). The utilisation of essential oils as natural preservatives has been reported to alleviate the spoilage oxidative reactions and microbial growth, thus enhancing the shelf life of meat and meat products (Karre et al., 2013). Various essential oils such as clove, thyme, oregano, tea tree, lemon, rosemary, lavender, eucalyptus, and peppermint oil have been widely studied and have been documented for their antioxidant and antimicrobial effectiveness through *in vitro* and *in vivo* studies (Moarefian et al., 2013; Bakhtiary et al., 2018). Plant essential oils effectively inhibited the growth of pathogenic microbes such as *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, *Clostridium* spp., *Campylobacter jejuni*, and *Aeromonas hydrophila* found in meat and meat products (Moarefian et al., 2012; Moarefian et al., 2013; Bakhtiary et al., 2018; Salvaneschi et al., 2020). Among these, *Rosemarinus officinalis*, recognised as rosemary, is a widely studied and promising aromatic herb belonging to the *Lamiaceae* family. The potential antioxidant, antimicrobial, anticancer, and anti-inflammatory characteristics of rosemary oil/extract have been reported in the literature (Jonatas et al., 2017; Choi et al., 2019; Ahmed & Babakir-Mina, 2020; Moczkowska et al., 2020). Rosemary oil and extracts have been shown to reduce the rate of spoilage oxidative reactions and the growth of food pathogenic and spoilage microbes in a

different range of food products (Gouveia et al., 2016; Pires et al., 2017; Schilling et al., 2018; Kaur et al., 2021).

Leptospermum scoparium and *Kunzea ericoides*, recognised as "mānuka" and “kānuka”, are small trees or shrubs indigenous to New Zealand. The mānuka plant is considered a *Taonga* by the Māori and has profound cultural importance. In the past, mānuka parts were used by the traditional Māori healers to treat wounds, burns, muscle inflammation, and fevers (Perry et al., 1997; Porter & Wilkins, 1999). The leaves of this plant were also used in tea preparations. The literature confirms that essential oils of both "mānuka" and “kānuka” have great potential in pharmaceuticals, antibiotics, food supplements and cosmetology agents (Chen et al., 2016). The mānuka oil contains monoterpenes, sesquiterpenes and triketone and is utilised as an antimicrobial agent. However, New Zealand kānuka oil possesses elevated amounts of monoterpenes, especially α-pinene, followed by other monoterpenes, sesquiterpenes and trace amounts of leptospermone (Perry et al., 1997; Fuller et al., 2022). Studies elucidating both essential oils' antioxidant and antimicrobial potential are available in the literature, but their application in food products still needs to be explored.

Nevertheless, these are seldom utilised due to some limitations associated with using essential oils in food products, such as interactions with food constituents, volatility, instability (under light and temperature conditions), lower-water solubility, and strong odour (Wu et al., 2022; Wang et al., 2023). The food matrix constituents, mainly fats, are a key factor influencing the retention of free essential oils and partitioning/releasing bioactive compounds. Owing to the lipophilic nature of essential oils, they exhibit a high affinity towards meat fat, which provides diluting effect to these oils (Hyldgaard et al., 2012; Wang et al., 2020). Consequently, their influence may differ according to the fat content of food (Wang et al., 2020; Kaur et al., 2023). For instance, meat products produced from wagyu and other beef breeds are different in fat

content and composition, owing to the presence of higher intramuscular fat content and high amounts of unsaturated fatty acids in the latter beef (Boylston et al., 1996; Bermingham et al., 2021). Understanding the effect of essential oil, either in free or nanoencapsulated form, in meat products prepared from different breeds having different compositions will allow the usage of these natural preservatives in various food formulations.

Nanoencapsulation is one of the pioneering and efficient solutions to overcome the abovementioned limitations and to preserve the beneficial properties of natural preservatives by isolating them from harsh conditions (Hussein et al., 2017; Abandansarie et al., 2019). It is an innovative and practical branch of nanotechnology that deals with the encapsulation of particles on a nanometer scale (Jafari, 2017) and is used in different areas, including materials engineering, pharmaceuticals and food industries (Liu et al., 2017; Kong et al., 2019; Wu et al., 2020). In this process, carrier material provides protection to the active material from unfavourable processing or storage conditions, such as high temperature, moisture and oxygen, certain pH levels and light, as well as helping to hide sensory characteristics which are usually unliked by the food consumers (Castro-Rosas et al., 2017). In addition, an increase in the surface area of nano-size particles increases the practical application of bioactive compounds (Rashidi & Khosravi-Darani, 2011). Contemplating food safety, the Food and Drug Administration (FDA) has confirmed the methods associated with nanoencapsulation-based food constituents for mass production (Chau et al., 2007). It has been reported that biodegradable natural materials used for nanoencapsulation are believed as low-risk materials than polymeric synthesised nanocapsules (Katouzian & Jafari, 2016). The promising applications of nanoencapsulation are in the field of consumer products regulated by the United States Food and Drug Administration (USFDA), such as cosmetics, medical devices, new and over-the-counter drugs, and food and food packaging. Despite the economic and social potential of nanoencapsulation, FDA faces several issues in the regulation of these products

(Sandoval, 2009). Recently, USFDA issued a draft guidance for products containing nanomaterials, describing the definition and quality attributes of products containing nanomaterials (Emily et al., 2018). To date, some uncertainties regarding the consumption of nanoencapsulated food materials still exist, despite their influence on human health and the environment require to be investigated (Dowling, 2004; Amenta et al., 2015).

Several researchers have reported the development of innovative and promising nanoentities like nanocapsules, nanospheres, nanoemulsions, and nanoparticles using different carrier materials and methods (Abbasi et al., 2019). For instance, nanoemulsions are devised because their composition can be optimised and altered to achieve bioactive compounds' required solubility and transformation. While nanocapsules can efficiently provide protection against harsh storage and processing conditions (light, pH, temperature, and oxygen), targeted delivery and controlled release characteristics to the bioactive compounds (Ghaderi-Ghahfarokhi et al., 2016).

For the preparation of nanoemulsions and nanocapsules, different natural polymers, including alginate, chitosan, gelatin and albumin, while in colloid stabilisers, Tween® 20 or Tween ®80 dextran, poly (vinyl alcohol), and copolymers are the most widely used (Vauthier & Bouchemal, 2009). Alginate is one of the easily available, reduced-cost, non-toxic polymeric materials used as a carrier for bioactive compounds in emulsion, capsule and film forms. Several studies have fabricated alginate nanoparticles, nanocapsules, nanoemulsions and films to encapsulate essential oil or their bioactive compounds (Salvia-Trujillo et al., 2013; Zhang et al., 2022). Similarly, whey protein-based nanoengineered approaches have been fabricated to encapsulate food systems. Due to their high nutritional value, GRAS (Generally recognised as safe) status, and good gelling ability, the designed nanostructures can be easily optimised, prepared, and controlled (Abbasi et al., 2014; Ramos et al., 2019). Proteins like whey isolates/

concentrates can also conjugate various food ingredients, including vitamins, minerals, flavours, odours, and antioxidants. This behaviour can facilitate the targeted delivery and controlled release of bioactives because of the swelling of the gel in the presence of external stimuli like pH, temperature, enzymes and ionic strength, and ameliorate the stability and bioavailability of bioactives (Ramos et al., 2019).

This research aims to investigate and compare the antioxidant and antimicrobial potential of mānuka, kānuka and rosemary oils as natural preservatives for food applications against chemical preservatives like sodium nitrates/nitrites. The influence of selected mānuka and rosemary oils to inhibit changes in lipid oxidation, pH, colour and microbial growth was investigated in low and high-fat meat pastes. Further, the efficacies of emulsified, encapsulated, and free oils were compared and characterised against sodium nitrite in low and high-fat beef pastes. Overall, this thesis presents and discusses comprehensive studies focusing on the mechanisms of understanding the antioxidant and antimicrobial characteristics of mānuka oils and their interactions with meat fat.

1.1. Research hypotheses

The antimicrobial and antioxidant properties of the mānuka and kānuka oils have been reported in the literature. Therefore, this research hypothesises that these essential oils can be used in meat products to improve their oxidative and antimicrobial stability, prolonging the shelf life of meat. In order to mask the undesirable odour and flavour and improve the stability, controlled release and targeted delivery of essential oils, previous studies have suggested the application of nanotechnology to overcome these challenges. This research, therefore, also hypothesises that nanoemulsions and nanocapsules of mānuka and rosemary oils may show better thermal stability and enhanced antioxidant and antimicrobial activities to extend the shelf life of meat products.

1.2. Overall goal, questions, and objectives of the research

This research project aims to understand the antioxidant and antimicrobial potential of mānuka oil as a natural preservative for meat products and make a comparison with commonly used natural, i.e., rosemary oil, and chemical preservatives, i.e., sodium nitrates/nitrites. This project also aimed to understand the influence of nanoencapsulation and nanoemulsification on mānuka oil and rosemary oil characteristics, including antioxidant, antimicrobial, stability, and release, was studied.

To achieve the abovementioned goal, the key objectives and questions of this research are discussed as follows:

RQ 1 Do mānuka and kānuka oils possess antioxidant and antimicrobial characteristics?

Objective 1

Characterisation and comparison of the antioxidant and antimicrobial potential (against selected pathogenic microbes) of mānuka and kānuka oils with commonly used rosemary oil and synthetic antioxidant-butylated hydroxytoluene (BHT).

RQ 2 How do mānuka oil and its bioactive compounds get partitioned into different phases?

Objective 2

Investigation of octanol-water coefficient of major compounds of mānuka oil and their partition in beef fat and water.

RQ 3 Whether mānuka oil possess the same antioxidant and antimicrobial characteristics as natural preservatives, i.e., rosemary oil and chemical preservatives-sodium nitrate, when added to meat systems?

RQ 4 How do the mānuka and rosemary oils influence the physiochemical characteristics of low and high-fat meat pastes in comparison to rosemary oil and a commonly used chemical preservative, sodium nitrate?

Objective 3

Determination of the influence of selected mānuka and rosemary oils to inhibit changes in pH, colour, lipid oxidation, and microbial growth in low and high-fat meat pastes against sodium nitrate and BHT.

RQ 5 How do nanoencapsulation and nanoemulsification affect the thermal stability and kinetics of the release of mānuka and rosemary oils?

Objective 4

Fabrication and characterisation of nanocapsules and nanoemulsions containing mānuka and rosemary oils using food-grade carrier materials and investigation of their antioxidant activity, stability and release properties compared with the free oils.

RQ 6 How do nanoencapsulation and nanoemulsification affect the antioxidant and antimicrobial activity of mānuka and rosemary oils in low and high-fat beef pastes?

Objective 5

Comparison of the antioxidant and antimicrobial potential of mānuka and rosemary oils and their nanoforms in low and high-fat beef pastes with sodium nitrite and understanding of mechanisms responsible for the antimicrobial potential of mānuka and rosemary oils against selected pathogenic microbes.

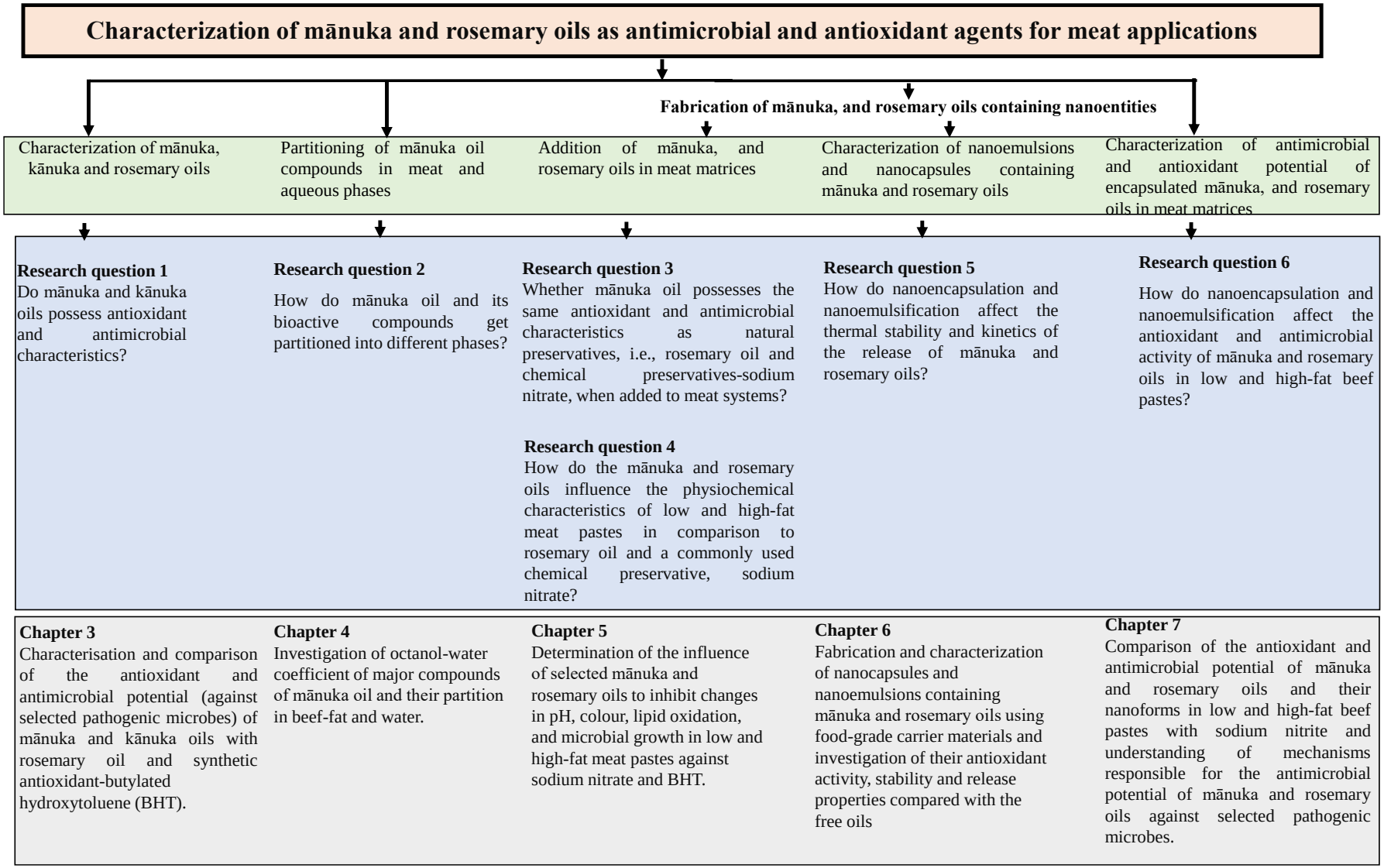


Figure 1.1. A schematic presentation of how the chapters are related to the research question of this project.

Chapter 2 Literature Review

2.1. Overview of food preservation

Food preservation comprises of application of science-based knowledge through a wide range of available technologies and procedures to prolong the shelf life and ensure the microbiological safety of food products (Rahman, 2007). The development of food preservation methods has been driven by various reasons. Firstly, with the significant developments in food production and trade, a demand also arises to accommodate food items from one region to another (Bopp, 2019). Thereby, food is transported across variable distances to reach consumers. However, due to technical, economic and other barriers, food is mainly lost before reaching consumers in developing countries (Nellemann, 2009; FAO, 2011). Nellemann (2009) reported that approximately 30 to 40 % of food in developed and developing nations is wasted, even though the reasons are very different. To ensure that food reaches its destination in good condition, special requirements such as proper packaging, environmental conditions and food preservation are needed (Cheng et al., 2010; Lerner & Lerner, 2011; Becerril et al., 2013; Carocho et al., 2014). Additionally, it has been predicted that the global population will reach around 8 billion by 2025. Feeding the growing population is widely acknowledged as one of the biggest challenges for the whole planet, especially from an environmental viewpoint (Carocho et al., 2014). There is a growing demand for fresh and suitable foods that should be available throughout the year; however, their limited and short shelf-life negatively impacts consumer health, economic loss and the environment (Rozenblit et al., 2018). Food preservation is imperative in addressing world hunger and reducing food wastage in this critical situation. As fresh food spoilage is due to microbial, chemical and enzymatic reactions, food preservation processes can inactivate bacteria, mould, yeast, and enzymes and avoid food contamination (Food & Division, 1997; Aste et al., 2017).

Hence, the concept of food preservation materializes as a technology to improve food quality, safety and shelf life (Li et al., 2020; Li et al., 2021). Rahman (2007) defined preservation as the action or method used to maintain foods at desirable characteristics or nature as long as possible and for maximal benefits. Various factors influence the characteristics of preserved food, which determines the nature and method used for preservation. For instance, foods with low water activity/perishability (dry foods) are easy to preserve, while it is challenging to preserve highly perishable foods like meat, seafood, fresh fruits and vegetables (Leistner, 2007). Based on hurdles/parameters (high/low temperature, water activity (a_w), pH, redox potential (E_h), competitive flora and added preservatives), commonly used preservation methods are chilling, heating, freezing, freeze-drying, curing, drying, salting, acidification, fermentation, smoking etc. For some food preservation methods, these hurdles are of prime importance, while these may act as secondary hurdles in other methods (Leistner et al., 1978; Leistner, 1985; Leistner & Gorris, 1995).

In the literature, thermal (sterilization, canning, drying, pasteurization, freezing and cooling) and non-thermal treatments (high-pressure, irradiation, and others) and their combinations have been reported to inhibit/ inactivate microbes and produce safe food products with prolonged shelf-life (Deak, 2014). However, depending on processing conditions, thermal treatments at high temperatures can adversely affect the heat-labile nutrients (vitamins, minerals), colour, texture and flavour (Roobab et al., 2018; Chiozzi et al., 2022). Several limitations, such as high instalment cost, less consumer awareness, formation of rules and regulations, and processing parameters and design, also pose a challenge to using non-thermal technologies in the food industry (Chacha et al., 2021).

Among the different food preservation methods, food additives are always chosen over the above-discussed methods as the least expensive method (Saltmarsh & Saltmarsh, 2013). As

per the Codex Alimentarius food additive is "any substance, which is not normally consumed as a food itself, not utilized as a typical food ingredient, whether or not it has a nutritive value, the intentional reason for its addition to a food is a technological purpose may in preparation, processing, manufacturing, packing, holding or transport of such food results in or may be reasonably expected to result (either directly or indirectly) in it or its byproducts becoming a component of or otherwise influencing the properties of such foods". Under this term, food contaminants or substances incorporated into food for maintaining or ameliorating nutritional qualities are not included (Motarjemi et al., 2013; Codex Alimentarius, 2017). Even though the term “food additives” has been utilized frequently in recent times, its use has been practised since ancient times and most likely dating back to much earlier than the Palaeolithic age (hunter-gatherer era) (Desiree & Geethi, 2017). Evidence has reported that sulphur dioxide was used by Egyptians over 3000 years ago, and Greeks are renowned for using salt and sodium nitrate combinations to preserve meat in the times of Homer. Likewise, several compounds, including salt and spices preservatives, have been used since immemorial times (Saltmarsh & Saltmarsh, 2013). Historically, the use of additives has changed drastically due to the commencement of industrialization (Carocho et al., 2014; Carocho et al., 2015). These additives have been used to improve the quality of food products for centuries; however, today, these additives are used for preserving the nutritional values, improving the quality, organoleptic characteristics and consumer acceptability, reducing food wastage, and facilitating food processing items and readily available food (Gilsenan, 2011; Amit et al., 2017). These can be added during the processing, packaging, and storage of food products to achieve desirable changes in food properties (Amit et al., 2017).

Depending on their function in food products, food additives can be categorized into six groups: preservatives, colouring agents, flavouring agents, texturizing agents, nutritional agents, and miscellaneous agents, as shown in Figure 2.1 (Carocho et al., 2014; Carocho et al., 2015). Food

colouring agents, also known as dyes, are used to change or provide colours to food to improve their attractiveness to consumers. Flavouring agents are the food additives employed to change the taste of food by increasing its sweetness or completely replacing the final taste of the food product. These agents are broadly divided into flavour enhancers, sweeteners, and natural and artificial flavours. Nutritional agents are natural compounds like amino acids, vitamins, fibres, fatty acids, polyphenols, and others used as nutritional enrichments. Texturizing agents are a class of food additives used in foods to ameliorate their overall texture or mouthfeel, and they are classified into two groups stabilizers and emulsifiers (Branen et al., 2001). As per the European Union, all food additives, either approved for food use or not, are designated with the letter "E" and a special number to easily identify the food additives across the globe (Council Regulation (EC) 1333/2008; Council Regulation (EC) 1129/2011). Under this list, food preservatives are listed from E200 to E299.

2.2. Different types of preservatives used for food products

Preservatives are the specific food additives used to ensure safety and avoid loss of food quality by preventing physical, chemical, and enzymatic spoilage. Although preservatives may serve more than one function in foods, this group of additives consists of antimicrobial, antioxidant and anti-enzymatic agents, each having a particular mode of action. Antimicrobial agents are employed in foods to control natural spoilage of food and microbial contamination for food safety concerns (Tajkarimi et al., 2010). In other words, antimicrobials are natural or chemically derived substances capable of reducing or arresting the growth of microbes and deteriorative reactions resulting from their presence. However, antioxidants are used to prevent the oxidation of molecules through the donation of electron or hydrogen atoms, converting themselves to the reduced form, in radical form. These radical antioxidants are stable and do not proceed reactions to take place compared with other radicals, thereby preventing spoilage

oxidative reactions (Carocho & Ferreira, 2013). Lipid peroxidation/rancidification is one of the most common oxidations occurring in foodstuff during storage. Antioxidants are used to prevent these reactions, extend shelf life and impede decay without affecting the taste, odours and appearance of the food (Nanditha & Prabhasankar, 2008).

Food preservatives can be classified as natural or synthetic/chemical depending on their origin. Natural preservatives are found in natural sources (produced naturally), while synthetic preservatives are produced synthetically or chemically (Msagati, 2013).

2.2.1. Chemical preservatives

The introduction of chemical preservatives has a significant influence on the transformation of food production patterns and eating habits than any other food additive. In 1992 Parke and Lewis stated the benefits of chemical preservatives as "Gone are the fears of eating meat that was not cooked fresh, one developed food poisoning and botulinum, and problems related with the rancidity of food with fat, from butter and meat to ice cream and biscuits, appear to have gone forever" (Parke & Lewis, 1992).

Chemical preservatives can be categorized as organic and inorganic, depending on their action and chemical nature. The organic preservative category includes formic acid, benzoates, propionic acid, an ester of p-hydroxybenzoic acid and their calcium and sodium salts. However, borates, sulphites, hypochlorite, peroxide, nitrites/nitrates, sulphurous acid, peroxide, and hypochlorite come under inorganic preservatives (Surekha & Reddy, 2014). Weak organic acids (e.g., sorbic and benzoic acid) have promising antimicrobial action because they do not ionize entirely and contain both dissociated (charged) and undissociated fractions. As the pH under microbial cells (cytoplasm has neutral pH) is higher than the pH of acid, the undissociated fraction of weak acid diffuses through the microbe's cell membrane to the cytoplasm, triggering the dissociation of acid into protons and corresponding ions (Pilatus &

Techel, 1991; Legiša & Grdadolnik, 2002; Jernejc & Legiša, 2004; Plumridge et al., 2004). Due to the lipid insolubility of charged species, these will accumulate in the cell membrane (cytoplasm), resulting in a pH drop of the cytosol, which is detrimental to the microbial cell and hinders all metabolic activities (Krebs et al., 1983; Msagati, 2013). Benzoates are effective against yeast and mould, while minimally against bacteria, and are predominantly used in high-acid foods (juices, jams, ketchup, jellies, soft drinks and salad dressings) (Marshall et al., 2016). Organic acids such as citric, lactic, malic, butyric, acetic, benzoic, ascorbic, propionic, formic, sorbic, succinic tartaric acids and some of their salt have been authorized as Generally recognized as safe (GRAS) for their food use by United States Food and Drug Administration (US FDA) (Gurtler & Mai, 2014). Several factors, such as acid type, function, food type and desired function, determine the quantities of organic acids to be added to the food products. For instance, preservatives (antioxidants) and flavourings are added sparingly (for example, 100-500 ppm), while acidulants are incorporated in higher quantities (several parts/100) (Gurtler & Mai, 2014).

The antimicrobial action of nitrites and sulphites is due to their complex formation with food components, which is toxic to microbes (but not mammals). For instance, all sulphates (sulphites, bisulfites, sulphur dioxide, and metabisulfite) produce sulphurous acid in aqueous environments, which acts as an antimicrobial agent. In some fruits and vegetables, sulphites are used as antibrowning agents to inhibit enzymatic and non-enzymatic browning (Marshall et al., 2016). Sulphites are used in dried fruits, the wine industry (to disinfect equipment), and some fruit juices.

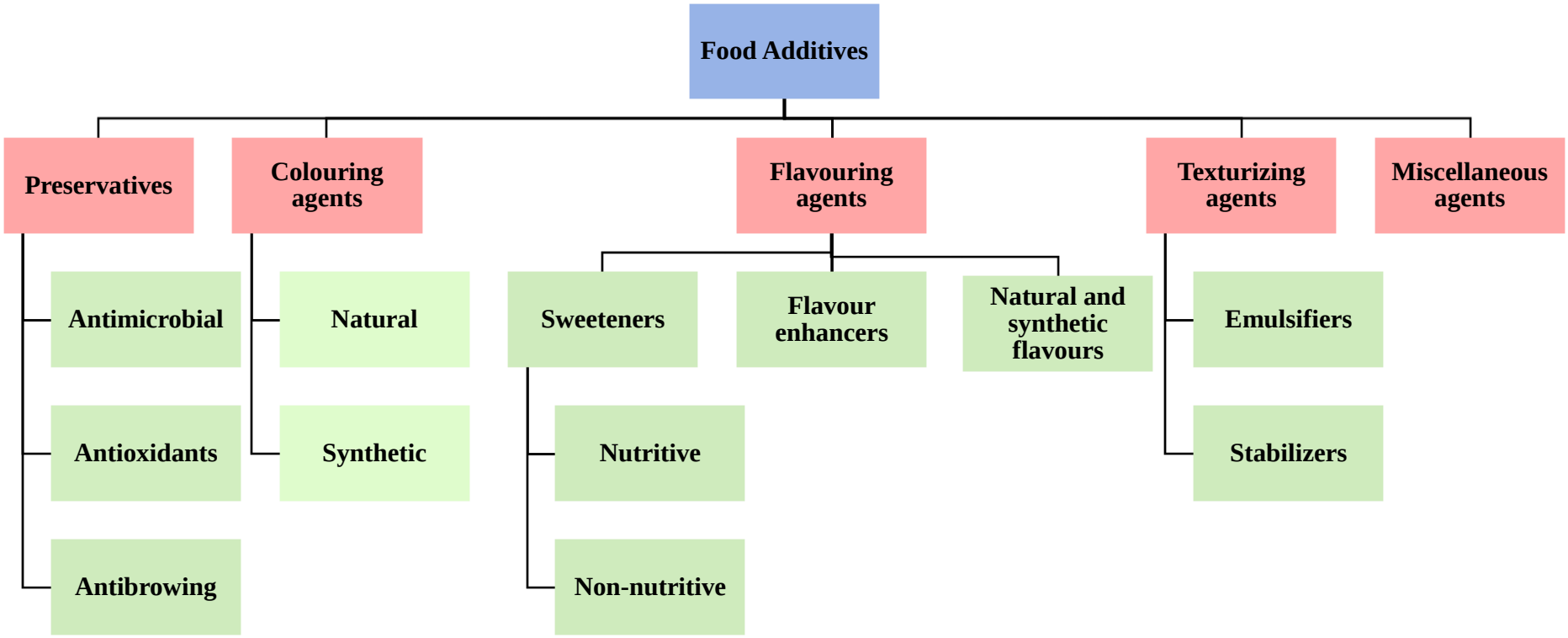


Figure 2.1. An overview of the classification of food additives, depending on their functions.

This figure was adapted from Carocho et al. (2014) with copyright permissions.

2.2.1.1. Sodium nitrites/nitrates

Nitrates and nitrites are added to meats such as ham, bacon, and sausages during the curing process. In the earliest times, adding nitrate impurities in salt during the drying of meat was utilized as a meat preservation system (curing process) to provide a pink colour, reduce microbial spoilage and increase food safety during food storage (Pegg & Shahidi, 2008; Pegg & Honikel, 2014). However, nowadays, the biochemical reactions involving the conversion of nitrate to nitrite, further nitrite to nitric oxide, and resulting nitroso heme pigments to provide cured colour to meat products have been widely documented in the literature (Oostindjer et al., 2014). The reduction reactions demonstrate that nitrate is only effective after being converted to nitrite (Pegg & Shahidi, 2008; Oostindjer et al., 2014). So, it is well understood that nitrite is the curing agent, which is an effective antimicrobial agent to retard the growth of bacteria such as C*lostridium botulinum* and some pathogenic microbes and improve the colour and flavour of processed meats (Pegg & Shahidi, 2008; Pegg & Honikel, 2014; Crowe et al., 2019). The meat industry has hugely benefitted from the use of sodium nitrite in the curing process by producing ameliorated, safe and enhanced shelf life meat products with excellent storage stability (Pegg & Shahidi, 2008). Nitrite, a multifunctional curing agent, can act as a bacteriostatic and bactericidal agent against *Clostridium botulinum* and *Staphylococcus aureus*, thereby providing a preservative effect (Sindelar & Milkowski, 2011).

Moreover, it functions as an antioxidant and effectively prevents or delays lipid oxidation in meat products (Thomas et al., 2014). Besides the preservative effect, meat curing with nitrites also helps to develop a unique colour and flavour to the cured meat products, as shown in Figure 2.2. Throughout the curing process, nitrates are added, which convert to nitrites and further react with the myoglobin to produce nitroso-myoglobin, which is responsible for the characteristics of cured meat colour in the meat products (Honikel, 2008). The allowed

concentration of nitrates or nitrites in meat products is generally 100-200 mg/kg (IARC, 2018). However, besides desirable effects, the consumption of nitrites can form endogenous N-nitrosamine by reacting with amines and amino acids under some conditions (Pegg & Shahidi, 2008). Some of these N-nitrosamines have been suspected as carcinogenic, thus driving the need to use natural preservatives in food products.

2.2.2. Natural preservatives

In recent years, controversies on the association of chemical preservatives with adverse health implications have driven the need to use natural preservatives in food products. Living organisms (plants, animals and microorganisms) possess several antimicrobial compounds, which act as a host defence and have potential applications in the food industry as preservatives. For instance, lysozyme in egg white, lactoperoxidase in milk, bacteriocins from lactic acid bacteria, bioactive compounds from plants, saponins and flavonoids obtained from herbs and spices, and chitosan in shrimp shells. Plant extracts or oils, which possess antimicrobial and antioxidant bioactive compounds, have great potential to be used in food products to prevent oxidative, microbial and discolouration reactions (Pateiro et al., 2021). Using plant-based natural preservatives has been shown to reduce the rate of oxidative reactions and microbial growth, thereby enhancing the shelf life of food products (Karre et al., 2013).

2.2.3. Essential oils as natural preservatives

The ever-growing consumer interest towards natural sources has forced the food industries to use natural, plant-based, or herbal-origin preservatives rather than chemical preservatives to produce safe and extended shelf-life food products (Al-Maqtari et al., 2022). Among all, essential oils are one of the most studied natural products. In 2020, the worldwide demand for essential oils was 247 kilotons, and the market size was 18.6 USD billion.

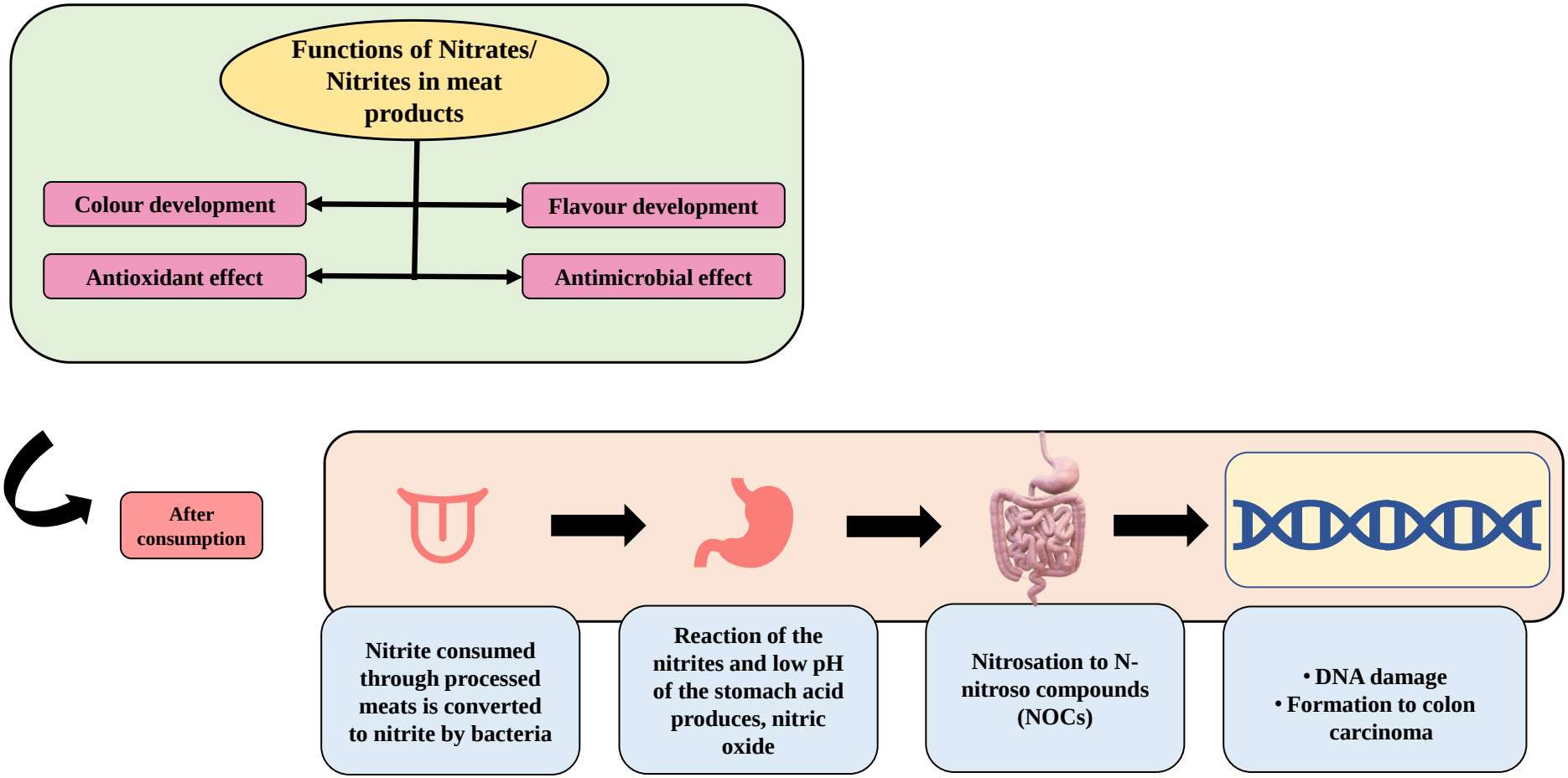


Figure 2.2. Functions of nitrites in meat products and their harmful health effect after consumption.

This figure was adapted from Crowe et al. (2019), an open-access article.

Table 2.1. Examples of some commonly used essential oils in different food products.

Family/	Essential oil	Major Active compound	Properties	Tested in food products	References
Lamiaceae	Thyme oil	Thymol, Borneol, Carvacrol, (Bhavaniramya et al., 2019)	Antimicrobial, Antioxidant	Vegetables, Meat, par-baked wheat, sourdough bread, tomato paste, hamburger, sausages, ready-to-eat-meat,	Iseppi et al. (2019), Liu and Liu (2020), Debonne et al. (2018), Omidbeygi et al. (2007), Radünz et al. (2020), Sharma et al. (2020), Quesada et al. (2016), Viuda-Martos et al. (2010)
	Rosemary oil	Eucalyptol, camphor, α-pinene (Micić et al., 2021)	Antimicrobial, Antioxidant	Vegetables, Chicken, Fresh dough, beef patties, mortadella	Iseppi et al. (2019), Harmankaya and Vatansever (2017), Teodoro et al. (2014), Mohamed and Mansour (2012), Viuda-Martos et al. (2010)
	Mint oil	Carvone (Bhavaniramya et al., 2019)	Antimicrobial, Antioxidant	Juices, Meat, cheese, Iranian and white brined cheese	Guedes et al. (2016), Djenane et al. (2012), Moosavy et al. (2013), Tehrani and Sadeghi (2015)
	Oregano	Terpin-4-ol and α-terpineol (Bhavaniramya et al., 2019)	Antimicrobial, Antioxidant	Maize, Fruits, fresh pork meat, beef muscle, processed meat product	Munhuweyi et al. (2017), Hernández-Hernández et al. (2017), Catarino et al. (2017)
	Peppermint oil/ mint oil	Menthol (Bhavaniramya et al., 2019)	Antimicrobial, Antioxidant	Juices, Meats, Minced beef	Guedes et al. (2016), Almeida et al. (2019), Smaoui et al. (2016)

Myrtaceae	Clove oil	Eugenol (Bhavaniramya et al., 2019)	Antimicrobial, Antioxidant	Chicken, Baked foods, Tomato Paste, Sausages, Ground beef, cooked pork sausages	Harmankaya and Vatansever (2017), Ju et al. (2018), Omidbeygi et al. (2007), Sharma et al. (2020), Khaleque et al. (2016), Lekjing (2016), Zengin and Baysal (2015)
	Tea tree oil	Terpin-4-ol, γ-terpinene, α-terpineol, p-cymene, α—pinene (Groot & Schmidt, 2016)		Ground beef	Silva et al. (2019)
Rutaceae	Lemon oil	Linalool Bhavaniramya et al. (2019)	Antimicrobial, Antioxidant	Fish	Yazgan et al. (2019)
Lauraceae	Cinnamon oil	Cinnamaldehyde, cinnamate, cinnamic acid (Rao & Gan, 2014)	Antimicrobial, Antioxidant	Fruits, Baked foods, Fruits, Ground beef, cooked sausage, Fresh Italian sausages, pork slices	Mousavian et al. (2018), Bhavaniramya et al. (2019), Ju et al. (2018), Khaleque et al. (2016), Aminzare et al. (2018), Zhang et al. (2019), He et al. (2015)
Apiaceae	Caraway oil	Carvone (Bhavaniramya et al., 2019)	Antimicrobial, Antioxidant	Baby carrots	Gniewosz et al. (2013)
Rosaceae	Rose oil	Farnesol (Bhavaniramya et al., 2019)	Antimicrobial, Antioxidant	Probiotic fermented whey	Dinçoğlu and Rugji (2021)

Zingiberaceae	Ginger	Citral, thujene, zingiberene, pinene, camphene (Kalhoro et al., 2022)	Antimicrobial, Antioxidant	Chicken meat, Maize, pork meat, beef patties	Bhavaniramya et al. (2019), Noori et al. (2018), Nerilo et al. (2020), Wang et al. (2017), Dzudie et al. (2004)

This market is anticipated to rise at an annual rate of 7.5 % from 2021 to 2028, driving through the trend of consumption of natural products (Grand View Research, 2020; Bruno et al., 2021). Essential oils are highly concentrated lipophilic/hydrophobic liquids extracted from plants and are a complex mixture of several metabolites like terpenes, phenolics, ketones, terpenoids, phenylpropenes, aldehydes, alcohols, ketones, acids and esters, and ethers in chemical composition (Burt, 2004; Prakash et al., 2018; Falleh et al., 2020; Al-Maqtari et al., 2022). Besides the aromatic profile, the antimicrobial characteristics of essential oils against a broad range of microflora have provided convincing evidence of their suitability as a candidate to be utilized as a natural preservative for food applications (Table 2.1) (Falleh et al., 2020).

2.2.3.1. Rosemary oil

Rosemary is one of the most promising, versatile, and widely explored natural preservatives that have been documented to reduce the rate of oxidations and microbial growth rate in meat products, thus extending their shelf life. Rosemary, *Rosmarinus officinalis*, is an aromatic plant that belongs to the family *Lamiaceae* and is indigenous to the *Mediterranean region.* Rosemary leaves have been utilized as a food additive or ingredient for flavouring purposes (Ribeiro-Santos et al., 2015; Kaur et al., 2021). Rosemary extract or rosemary oil has been confirmed for its bioactive properties, such as antioxidant, anti-inflammatory, and anti-cancer (Jonatas et al., 2017; Choi et al., 2019; Jonatas et al., 2019; Ahmed & Babakir-Mina, 2020; Moczkowska et al., 2020). Moreover, various studies have emphasized its potential role as antifungal, antibacterial, insecticide, and hepaprotective (Nieto et al., 2018). These characteristics of rosemary are due to its chemical constituents: rosmanol, carnosol, carnosic acid, ursolic acid, rosmariquinone, caffeic acid, and rosmaridiphenol (Manhani et al., 2018; Choi et al., 2019; Ielciu et al., 2021). It has been reported that 90 % of the antioxidant of rosemary extract is associated with carnosic acid and carnosol constituents (Erkan et al., 2008). However, many

other bioactive constituents, such as flavonoids, diterpenes, steroids, and triterpenes, are also found. The promising biological and functional properties of rosemary are due to the presence of bioactive compounds like phenolic diterpenes, flavonoids, and triterpenes. In the food industry, the standardized extracts of dried rosemary leaves containing a definite percentage of carnosol and carnosic acid were introduced in the early 1990s but have recently turned into a leading natural antioxidant in the market (Erkan et al., 2008).

Looking at the excellent safety profile of rosemary extracts, containing a definite percentage of carnosol and carnosic acid with stipulated amounts of volatile oils, they even have been permitted by both the European Union (E392) and the United States as a natural antioxidant for food use (EFSA, 2018; Phipps et al., 2021). European Food Safety Authority (EFSA) has reviewed the safety levels of rosemary extract and concluded that the high-intake estimates vary from 0.09 (elderly) to 0.81 mg/kg (children) per day of carnosol and carnosic acid. Moreover, in the European Union, rosemary extracts have been incorporated in food and beverages at levels up to 400 mg/kg (Carnosol+ Carnosic acid) (EFSA, 2008a).

Several studies have reported that rosemary extract could be a natural antioxidant for partial replacement or substitution for synthetic antioxidants in meat products (Table 2.2). Al-Hijazeen and Al-Rawashdeh (2019a) compared the effects of rosemary extract (RE) (300 ppm and 350 ppm), L-ascorbic acid (300 ppm), sodium nitrite (200 ppm) and BHA (14 ppm) on stability and quality of cooked chicken meat. It was reported that both RE (350 ppm) and sodium nitrite (200 ppm) exhibited the highest effect on maintaining the low carbonyl values during the storage period (4 °C for 7 days). However, no significant difference was observed between all other treatments and control samples (prepared without adding preservatives) (Al-Hijazeen & Al-Rawashdeh, 2019).

Table 2.2. Studies on the application of rosemary to prevent lipid oxidation in different meats and meat products.

Functional component	Applied Concentration	Method of application	Meat product	Observations	Reference
Rosemary oil and basil oil	5.0 and 2.5 mg/mL	Soaking of meat sample in a solution containing rosemary oil	Chicken breasts	• A short contact time (15 min) of rosemary oil showed the best effect on the reduction of mixed microflora • Rosemary treatment reduced the number of salmonella cells in meat samples than the control • Highest antioxidant effect compared with other treatment	Stojanović-Radić et al. (2018)
Rosemary essential oil and modified atmospheric packaging	0.2 %	-	Poultry fillets	• The addition of rosemary essential oil in combination with modified atmospheric packaging declined lipid oxidation in meat samples • 0.2 % rosemary oil showed no significant reduction of *Salmonella Typhimurium* and *Listeria monocytogenes*	Kahraman et al. (2015)
Rosemary essential oil and modified atmospheric packaging	-	Spray of rosemary oil on the surface of packaging material	Beef	• Combined use of rosemary oil with packaging positively influenced the colour characteristics of beef, especially redness (*a**) • Microbial counts were lower in active packaging containing rosemary oil than the non-active packaging	Sirocchi et al. (2017)
Rosemary and sage essential oils	0.1 %	Antioxidants were dissolved in 10 mL ethanol before being	Liver Pâté	• In the case of liver pâté, the incorporation of the oils showed better antioxidant activity and lipid oxidative stability than BHT	Estévez et al. (2005)

		added to the raw batter and minced further			
Rosemary oil and sodium alginate active packaging containing rosemary, cinnamon, nisin	5 mg/mL	Added in sodium solution during the preparation of active packaging	Chicken fillets	• Alginate active packaging containing both rosemary and cinnamon oil showed a higher antimicrobial effect than the individual use of preservatives and control	Raeisi et al. (2016)
Rosemary and ginger oils in whey protein films	1 %	Mixing in the whey protein solution	Lamb meat	• Treatment of lamb meat with films containing essential oils reduced the lower lipid oxidation values and significant delay in microbial spoilage • No significant difference in the results of rosemary and ginger oil was observed	Tsironi et al. (2022)
Lyophilized rosemary extract	0.02 %	Added with ingredients	Chicken Burgers	• After 21 storage days, lyophilized rosemary extract added to chicken burgers prevented 48.29 % of lipid oxidation in contrast with the control • Lower production of malonaldehyde	Pereira et al. (2017)
Rosemary Extract	18.6 mg/kg, 480 mg/kg	With other ingredients	Chicken Burgers	• After 120 days, chicken burgers prepared with 480 mg rosemary exhibited a similar TBARS index to 20 mg BHA-added samples	Pires et al. (2017)

Rosemary extract and sodium ascorbate	0, 125, 250, 375, and 500 ppm	Mixing with ingredients	Liver Pâté	• Rosemary extract was found to be an effective antioxidant against lipid oxidation and lowered TBARS values	Haile (2015)
Thyme, Green Tea, and Rosemary Extract	0.02 %	Blending of ingredients	Minced Pork Meatballs	• Rosemary exhibited the highest ability to alleviate the nutritional value losses of the proteins	Hęś and Gramza-Michałowska (2017)

Similarly, the combined usage of rosemary and citrus extracts in cured meat products like Spanish chorizo as an alternative to produce clean, natural-label meat products free of artificial additives has been reported by Martínez et al. (2019). The usage of rosemary as an effective antimicrobial agent in meat and meat-based products has been shown abundantly in the literature (Ahn et al., 2002; Ntzimani et al., 2010; Mohamed & Mansour, 2012; Azizkhani & Tooryan, 2015; Gouveia et al., 2016; Schilling et al., 2018). The addition of 0.3 % rosemary extract exhibited a powerful antimicrobial effect against *Listeria monocytogenes* in ready-to-eat (RTE) pork liver sausages (Pandit & Shelef, 1994). Similarly, during chilled storage, rosemary extract-containing ground pork patties were documented with lower rates of bacterial population, lipid, and protein oxidation than control patties prepared without any extract. Moreover, patties blended with 0.2 g/kg and 0.3 g/kg of rosemary extract had higher cooking yields and lower pH throughout the chilled storage period (Yin et al., 2016). The physical interactions of an antimicrobial extract with the food matrix components, such as fat, proteins, etc., may decrease or increase their effectiveness. In one research, as per the results of culture-based assays, the rosemary extract was effective against lactic acid bacteria and *Listeria* but not active against *Bacillus thermosphacta*. While in the case of meatballs, it only reduced the lactic acid bacteria slightly (Fernandez-Lopez et al., 2005).

Many studies revealed the antimicrobial perspective of rosemary in combination with various other antimicrobial compounds. The effects of various preparations (vitamin C, vitamin C+vitamin E, taurine+vitamin C, rosemary extract+vitamin C) on the extension of quality characteristics of beef steaks for 29 days period of storage (1±1 ℃) was determined by Djenane et al. (2002). The use of both rosemary extract and vitamin C in fresh beef steaks led to reduced numbers of psychotropic aerobic microbes than other treatments during the whole period of storage (30 days at 1±1 ℃), and the difference was only significant from day 22 of storage onwards (Djenane et al., 2002). However, the combined use of rosemary and marjoram

essential oil at a 200 mg/kg concentration could not significantly reduce the psychotropic bacterial numbers. The possible reason could be the fat and/or protein content responsible for reducing the antibacterial efficacy of the essential oils in the food (Mohamed & Mansour, 2012). While, in the case of the beef sausages stored at 4 °C, incorporation of the rosemary extract alone or in combination with tocopherols (toc) or *Mentha logifolia* (ME) reduced the total viable, lactic acid bacteria, yeasts, and moulds counts (Azizkhani & Tooryan, 2015).

2.2.3.2. Mānuka oil

Leptospermum scoparium, also known as mānuka, is the most widely distributed and environmentally tolerant flowering plant native to New Zealand; it usually grows as a shrub or small tree. The early settlers of New Zealand used the leaves of mānuka plants to make tea, which is why these plants are generally known as "tea trees", "red mānuka", and kahikatoa. It is different from its cousin plant, i.e., kānuka in leaf size, flower and wood types, which is also known as white or tree mānuka (Maddocks-Jennings et al., 2005; Maddocks, 2021). These plants are often confused with "*Melaleuca alternifolia*" or "Australian tea tree oil", which is endemic to Australia and different in chemical composition but belong to the same family (*Myrtaceae*). In recent times, Mānuka honey, derived from the *Leptospermum* spp. has acquired widespread attention, due to the non-peroxide antimicrobial efficacy of methylglyoxal, also known as Unique Mānuka factor. This honey is produced by the honeybees foraging on the nectar of mānuka shrubs (Bonifacio et al., 2018; Nolan et al., 2020). In the past, mānuka plant parts were used by the traditional Māori healers to treat wounds, burns, muscle inflammation, fevers, and a host of other problems associated with the eyes, mouth and skin (Porter & Wilkins, 1999). The leaves were utilised to make an infusion, which acted as an effective tea supplement to cure numerous internal complaints such as back stiffness, breast inflammation, and eye-related problems (Chen et al., 2016). As per Crop and Food Broadsheet

(2000), this plant's leaves were also used to scent the vapour baths and toilet oils. An essential oil obtained from the selected mānuka plant line is still being developed as an antimicrobial product (Brooker et al., 1987; Riley, 1994; Perry et al., 1997), particularly against antibiotic-resistant strains (Douglas et al., 2004). Several studies reported the antimicrobial properties of mānuka and kānuka oils against a diverse range of microbes, as shown in Table 2.3. The existing research documented that the powerful antimicrobial characteristics of the mānuka oil are due to the presence of triketones.

As presented in Table 2.3, Rhee et al. (1997) reported the antimicrobial effect of mānuka oil towards ten kinds of microbes, including the inhibition of Gram-positive bacteria *Staphylococcus aureus* and *Micrococcus luteus* at a minimum inhibitory concentration (MIC) value of 3.05 µg/mL. For *Aspergillus niger*, a MIC value of 24 mg/mL was reported, but for other fungi like *Candida albicans* and *Tricophyton mentagrophytes*, MIC values were greater than 1000 µg/mL. However, this oil remained ineffective against Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris or Klebsiella pneumonia*). Williams et al. (1998) documented the powerful antimicrobial effects of New Zealand mānuka oil against *Staphylococcus aureus.* Porter and Wilkins (1999) stated the antimicrobial efficacy of this oil against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Pseudomonas aeruginosa*.

Table 2.3. Chemical composition of the mānuka and kānuka oils obtained from different regions.

Oil Type	Region	Composition	Reference
Mānuka	Far North of New Zealand	High α-pinene	Perry et al. (1997)
	East Cape of New Zealand	High β-triketone content	Perry et al. (1997), Douglas et al. (2004)
	Australia	High total monoterpenes (about 51 %) and 1,8-cineole (20 %)	
Kānuka		High α-pinene levels (about 40 %)	
Mānuka	Southern New Zealand	Presence of sesquiterpenes (about 71 %), esters of sesquiterpenes alcohol (14.5 %), citronellyl cinnamate (6 %), and terpene (1 %)	Gardner (1925)
Mānuka	Unknown	Sesquiterpenes hydrocarbon (48.2 %) and oxygenated sesquiterpenes (36.6 %) were present in higher amounts, followed by cis-calamenene (22.7 %) and leptospermone (19.2 %)	Fratini et al. (2019)
Mānuka	East Cape of New Zealand	sesquiterpenes (≥ 60 %) was the major component, followed by oxygenated sesquiterpenes and triketones (≤ 30 %)	Porter and Wilkins (1999)
Kānuka	East Cape of New Zealand	High α-pinene levels (≥ 50 %) and lower levels of viridiflorol and virdiflorene (≤ 10 %)	

From a chemical composition viewpoint, sesquiterpenes (≥ 60 %) were the major component in the tested mānuka oil, followed by oxygenated sesquiterpenes and triketones (≤ 30 %) as remaining components (Porter & Wilkins, 1999) (Table 2.3). In comparison with the tea tree oil, mānuka oil remained more active towards Gram-positive bacteria with minimum bactericidal concentration (MBC) and MIC values of 0.12-0.5 % and 0.06-0.25 %, respectively (Harkenthal et al., 1999). When three different oils, i.e., mānuka, kānuka and Australian tea tree oil (*Melaleuca alternifolia),* were compared for their antimicrobial and antioxidant actions, noticeable differences were observed. The antifungal activity of kānuka remained inversely proportional to its powerful antibacterial activity, whereas mānuka showed more powerful antifungal activity but was not as potent as Australian tea tree oil. However, mānuka showed more consistent antioxidant activity than kānuka, whereas Australian tea tree oil displayed no antioxidant activity (Lis-Balchin et al., 2000).

Similarly, in another study, *in vitro* antimicrobial potential of the Australian tree, mānuka, kānuka, cajuput and niaouli oils were compared with a β-triketone complex of mānuka oil. Findings documented that high effectiveness displayed by mānuka oil against Gram-positive bacteria at MIC from 0005-0.15 % and dermatophytes at 0.30-0.40 %. However, this oil remained ineffective towards the *Candida albicans.* The Australian tea tree oil exhibited the overall best antimicrobial activity, but mānuka oil differed from other tea tree oils with its inhibition effect on Gram-positive bacteria (due to its β-triketone content) (Christoph et al., 2000). Vuuren et al. (2014) observed a negligible effect of monthly variation on the composition of mānuka and kānuka oils from different species (*Leptospermum scoparium, Leptospermum petersonii,* and *Kunzea ericiodes*). In the same study, *Leptospermum petersonii* oils exhibited considerable antibacterial properties, especially towards the *Brevibacterium* genus (*Brevibacterium agri* at lowest MIC of 0.06 mg/mL). Kānuka oil alone did not show wide-spectrum inhibition against tested 16 microbes but combined with the *Leptospermum*

petersonii, and it represented in an additive manner (Vuuren et al., 2014). In one study, mānuka essential oil showed inhibition towards Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium phlei* and *Bacillus subtilis* and moderate inhibition towards Gram-negative bacteria like *Escherichia coli* and *Serratia marcescens*. It was observed that treatment of mānuka oil on MRSA cells resulted in morphological changes, including damaged cells, cellular lysis and free cellular contents. Mānuka oil treated *Escherichia coli* cells had shortened and showed distorted shapes, and few cells had lost their integrity (Alnaimat et al., 2015). The effects of mānuka and kānuka essential oils on inhibition of disease and inflammation-causing microorganisms (*Trichosporon mucoides, Candida albicans, Malassezia furfur, Candida tropicalis, Streptococcus sobrinus, Escherichia coli* and *Streptococcus mutans*), were studied by (Chen et al., 2016). The oils showed promising fungicidal characteristics ranging from 0.78 % to 3.13 % and excellent antibacterial properties by 100 % inhibition of the tested bacteria. Moreover, both oils reduced the tumour necrosis factor-α released after lipopolysaccharide stimulation in a human acute monocytic leukaemia cell line. Consequently, this study recommended that these oils have antimicrobial and anti-inflammatory properties without negatively influencing the immune system (Chen et al., 2016).

Table 2.4. Antimicrobial properties of mānuka and kānuka essential oils.

Oil type	Tested microbe	Method used	Results	References
Mānuka oil	Ten microbes (including different bacteria and fungi)	Two-fold serial dilution method and agar plate two-fold dilution method	• Oil showed an antimicrobial effect towards *Staphylococcus aureus* and *Micrococcus luteus* at a MIC value of 3.05 µg/mL for both bacteria • MIC value against *Aspergillus niger* was 24 mg/ mL • For other fungi like *Candida albicans* and *Trichophyton mentagrophytes*, MIC values were >1000 µg/mL • Oil remained ineffective against Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Pseudomonas vulgaris* or *Klebsiella pneumonia)*	Rhee et al., (1997)
Mānuka oil	*Bacillus subtilis* and *Trichophyton mentagrophytes*	Disc diffusion	• East Cape chemotype oil from New Zealand exhibited the strongest antimicrobial properties towards both microbes	Perry et al. (1997)
Mānuka oil	*Staphylococcus aureus, Escherichia coli, Candida albicans* and *Pseudomonas aeruginosa*	Two-fold serial dilution	• Oil remained capable of killing the bacteria at MBC values (w/v) of *Staphylococcus aureus* (0.039 %), *Escherichia coli* (1.25 %), MRSA (0.0195 %), *Candida albicans* (0.31 %), *Pseudomonas aeruginosa* (1.25 %)	Porter and Wilkins (1999)
Mānuka oil	*Enterobacter aerogenes, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Salmonella choleraesuis, Staphylococcus flexneri, Bacillus subtilis, Staphylococcus xylosus and Staphylococcus saprophyticus.*	Broth dilution	• Mānuka oil remained more active towards Gram-positive bacteria with MBC and MIC values of 0.12-0.5 % and 0.06-0.25 %, respectively, than tea tree oil	Harkenthal et al. (1999)

Mānuka oil	*Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans*	Microdilution chequerboard method	• mānuka oils exhibited significant antibacterial properties towards the *Brevibacterium* genus (*Brevibacterium agri* at lowest MIC of 0.06 mg/mL) • Kānuka oil alone did not show wide-spectrum inhibition against tested 16 microbes • The combination of mānuka and kānuka represented an additive manner against microbe inhibition	Vuuren et al. (2014)
Mānuka oil	Methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium phlei*, *Bacillus subtilis*, *Escherichia coli* and *Serratia marcescens.*	Disk diffusion assay and MIC and MBC	• The inhibitory activity of mānuka essential oil towards Gram-positive bacteria, including moderate inhibition towards Gram-negative bacteria, was observed	Alnaimat et al. (2015)
Cinnamon, mānuka, and winter savoury oil	*Listeria monocytogenes*	Two-fold serial dilution and microdilution chequerboard	• Synergistic inhibition and antibacterial effect against *Listeria monocytogenes* were observed	Fratini et al. (2019)

Three different essential oils, such as cinnamon, mānuka, and winter savoury oil (mixed in binary and ternary combinations), demonstrated their synergistic inhibition against the *Listeria monocytogenes* as alternatives to antibiotics used in human and veterinary medicines. From the chemical analysis of oils, it was observed that sesquiterpenes hydrocarbon (48.2 %) and oxygenated sesquiterpenes (36.6 %) were the predominant compounds present in mānuka oil, followed by cis-calamenene (22.7 %) and leptospermone (19.2 %) (Fratini et al., 2019). A study by Perry et al. (1997) reported that mānuka essential oil composition varies throughout New Zealand's habitats. They found different chemotypes of mānuka in different parts of New Zealand, as shown in Table 2.3, of which high triketones containing chemotypes were found in East Cape and Marlborough Sounds. Because different chemotypes of New Zealand mānuka have different biological activities, standardization of oil composition is important before the development of this oil for medicinal purposes. The oil obtained from the East Cape chemotype reportedly exhibited the strongest antimicrobial properties towards *Bacillus subtilis* and *Trichophyton mentagrophytes*, both in terms of minimum inhibitory concentration and inhibition zones (Perry et al., 1997).

The existing research documented that the antimicrobial characteristics of the mānuka oil are due to the presence of triketones. Additionally, geographical variation has an imperative effect on the composition of these oils (Maddocks-Jennings et al., 2005).

2.2.3.3. Kānuka oil

Like the mānuka, kānuka is a native plant of New Zealand, also known as the tea tree, due to its historical association that Captain Cook used both these plant leaves as equivalents to make tea (Lis-Balchin & Hart, 1998). Kānuka belongs to the genus *Kunzea* and shows close association with *Leptospermum*, *Callistemon*, and *Melaleuca* while differentiated by the stamen part (Thomas et al., 2010). In recent years, an extensive interest in *Myrtaceous* species

oils such as mānuka (*Leptospermum scoparium*), kānuka (*Kunzea ericoides*), and Australian tea tree oil (*Melaleuca alternifolia*) has been noticed. Steam-distilled oil from the leaves of the kānuka plant is commercially sold on a small scale in undiluted form and is used in pharmaceutical preparations.

Due to the folk-medicine use of this plant, various studies have reported the chemical composition and antimicrobial characterization of kānuka and mānuka oils (Hood, 1998; Armstrong, 2004; Thomas et al., 2010). The chemistry of kānuka is less studied than mānuka, which is a close relative of this plant and exhibits distinct regional foliage chemotypes (Fuller et al., 2022). Nevertheless, mānuka oil is different in chemical composition due to high amounts of β-triketones, including leptospermone, isoleptospermone and flavanone.

In chemical composition, excessive amounts of volatile compound α-pinene followed by traces of monoterpenes, sesquiterpenes and leptospermone have been found in kānuka oil, as discussed in the literature. Kānuka oil obtained from different parts of New Zealand is considerably different in chemical composition. The steam-distilled kānuka oil from Northern New Zealand contains high amounts of α-pinene (around 77 %) and lower amounts of citral, terpineol, aromadendrene, and β-triketone (leptospermone). However, kānuka oil obtained from Southern New Zealand had 52 % of α-pinene and lower amounts of 1,8 cineole, β-pinene, β-terpinene, linalool, α-terpineol, aromadendrene (Perry et al., 1997). A recent study by Fuller et al. (2022) confirmed the presence of two distinguishable flavanones in kānuka oil, which have been reported to possess activity against *Phytophthora*.

2.2.3.4. Mechanism of action of essential oils

Essential oils (EOs) are well recognized for harbouring antimicrobial activity against a broad spectrum of microbes (Burt, 2004; Falleh et al., 2020). The antimicrobial activity of essential oils can be either bacteriostatic (inhibit/stop bacterial growth without killing them) or

bactericidal (killing bacterial cells). The mechanism of action of antibacterial activity of essential oils has still not been formally established, possibly due to the variability in their chemical compounds (Hyldgaard et al., 2012) (Figure 2.3). As per the available literature, the antimicrobial efficacy of essential oils could be attributed to their ability to permeate through bacterial membranes to the inside of the cell and show an inhibitory effect on the functional and lipophilic properties of the microbial cells (Guinoiseau et al., 2010; Bajpai et al., 2012). The lipophilicity/hydrophobicity of essential oils permits them to permeate the cytoplasmic cell membranes and permeabilizes the different microbial layers made up of phospholipids, polysaccharides, and fatty acids (Burt, 2004). The disintegration effect of essential oils on the bacterial cell wall and cytoplasmic membrane structures through permeabilization of different layers results in a reduction of important ions, membrane potential, ATP pool, and leakage and collapse of cell contents and proton pump, respectively. Including the fragilization of cell membranes and loss of vital molecules, every one of the negative mechanisms is the main reason for the damage to vital cell processes and cell lysis (Gutiérrez-del-Río et al., 2018).

Rodriguez-Garcia et al. (2016) reported that the exterior membrane of Gram-negative bacteria cell wall restricts the flow rate of lipophilic essential oils through the lipopolysaccharides layer, whereas lipophilic ends of lipoteichoic acid in Gram-positive allows essential oils to diffuse through the membranes. It could be the probable reason for more sensitivity of Gram-positive microbes to essential oils than Gram-negative bacteria (Dhifi et al., 2016; Falleh et al., 2020). Besides this, the cell structure of Gram-positive bacteria is less complex than Gram-negative, consisting of a thin layer of peptidoglycan covered by an exterior membrane made up of lipopolysaccharides having a hydrophilic character that serves as a selectively permeable barrier (Behbahani et al., 2019; Bruno et al., 2021). This outer membrane constrains the diffusion of lipophilic compounds of essential oils, thereby preventing bioactive compound accumulation in the cell membrane.

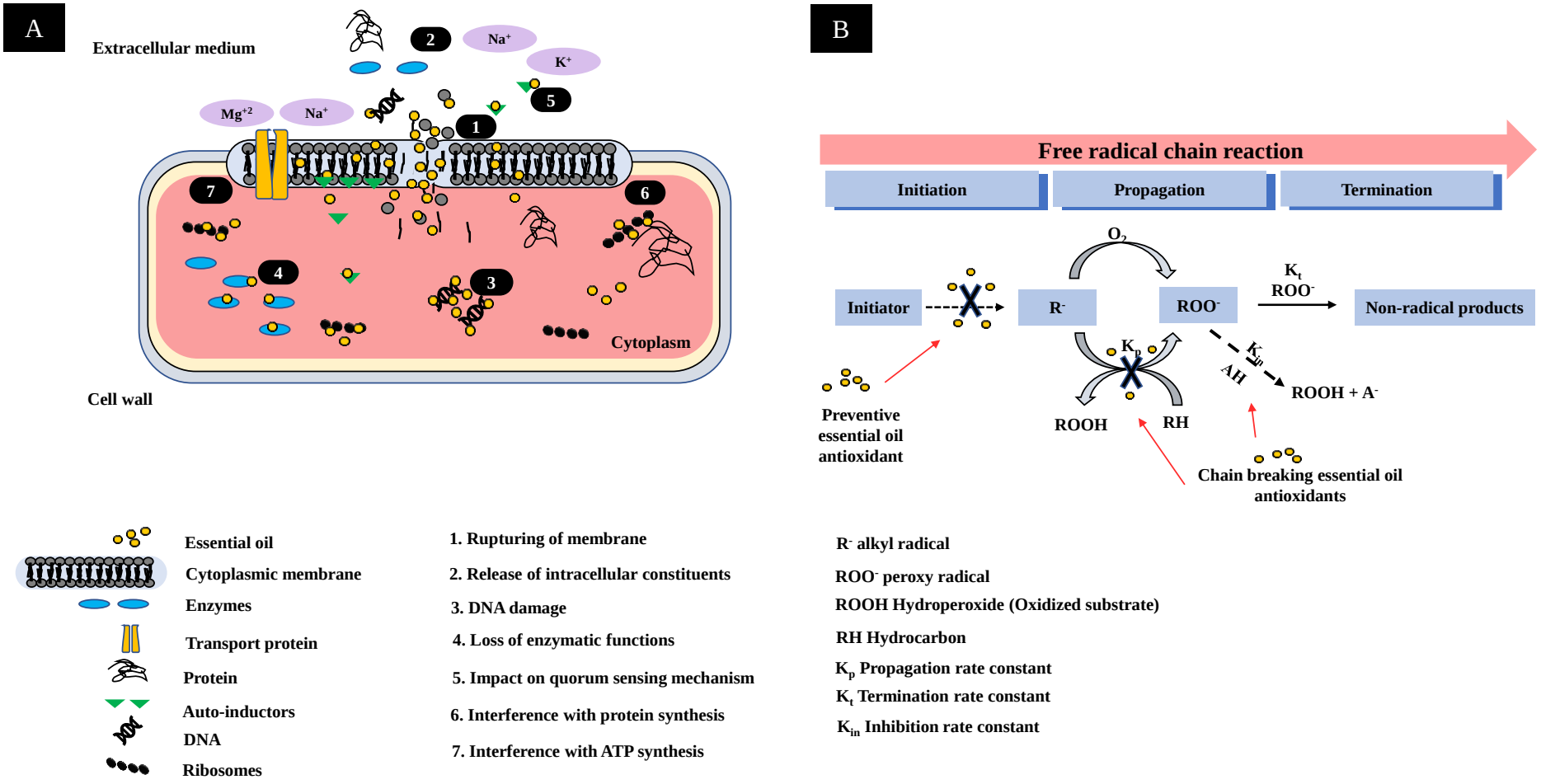


Figure 2.3. Proposed mechanism of antimicrobial (A) and antioxidant (B) activity of essential oils.

This figure was adapted from Silva et al. (2022) with copyright permissions and Basavegowda and Baek (2021) (an open-access article).

However, Gram-positive bacteria cell wall is composed of about 90-95 % peptidoglycan, which permits compounds to diffuse and act on the cytoplasmic membrane, thereby increasing antimicrobial activity (Nazzaro et al., 2013; Bruno et al., 2021). In addition, essential oils can cause coagulation of cytoplasm and inhibition of various enzyme systems, which are responsible for the production and energy regulation of structural components (Burt, 2004; Falleh et al., 2020). However, some of these inhibition mechanisms are not completely understood. For instance, the Filamenting temperature-sensitive strain Z (FtsZ) protein is a promising target due to its important role in bacterial division. The sesquiterpene germacrene could interreact with this binding pocket and serve as a natural preservative (Šarac et al., 2014).

According to the available literature, essential oils possessing high amounts of phenolic compounds exhibit higher antimicrobial activity towards food-borne pathogens (Jemaa et al., 2018; Gutiérrez-del-Río et al., 2018). These terpene phenols, such as carvacrol, eugenol, and thymol, alter the bacterial membrane's permeability by targeting the protein amine and hydroxylamine, resulting in cell death (Hyldgaard et al., 2012; Adelakun et al., 2016; Gutiérrez-del-Río et al., 2018). Nevertheless, it is imperative to mention the antimicrobial activity of essential oils due to the synergistic action of major and minor compounds present in these oils. The synergistic antimicrobial action between carvacrol and *p*-cymene has promising potential to be used as a natural preservative. Rattanachaikunsopon and Phumkhachorn (2010) reported that microbial growth inhibition was significantly weaker when *p*-cymene was added to the medium separately while combining both compounds in the same medium (at the same time) inhibited microbial growth. A possible explanation could be that *p*-cymene serves as a substitutional impurity in the bacterial membrane and may moderately disturb the membrane potential of intact cells (Rattanachaikunsopon & Phumkhachorn, 2010). This combination helped to lower the concentration of each compound. On the other hand, some compounds may show an antagonistic effect when combined with other compounds (Dhifi et al., 2016).

Combined use of carvacrol and thymol reduced their Fractional inhibitory concentrations against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*, while menthol and geraniol exhibited synergistic effects towards the same food-borne pathogens (Gallucci et al., 2009).

Nowadays, it is a widespread practice to identify the potential of natural compounds as antioxidants as "compounds or molecules able to react with radicals" or "have reducing power potential to overcome the oxidative stress caused by radicals". By definition, "antioxidants are compounds able to slow down or retard the oxidation of an oxidizable material, even at the lowest concentration (less than 1 %, commonly 1-1000 mg/mL) than the amount of material/constituent they have to protect. Focusing on the relevance of antioxidants in food science or biological cells, these compounds can protect food constituents such as fat, protein, sugars (carbohydrates), and other organic molecules that comprise animal or vegetal tissues (Valgimigli & Pratt, 2012; Riccardo et al., 2013). The oxidation takes place through a free radical chain reaction (Figure 2.3), which is initialized by some radicals that are capable of reacting with lipid substrate (RH) to yield an alky radical (R•) (reaction termed as "initiation"). As represented in Figure 2.3, the produced R• radical will react with oxygen at a diffusion-controlled rate to produce peroxy radical (ROO•) (propagation). In a cyclic manner, peroxy radical attacks another molecule of the substrate to create hydroperoxide radical (ROOH) and further another radical. This reaction proceeds for several cycles before any two radicals accidentally scavenge or quench each other in a known termination step of the reaction. In general, initiation occurs through a Fenton-like redox reaction, in which transition metals, including Fe^{2+}, Cu^{+} will catalytically breakdown by electron-transfer peroxides or hydroperoxides such as ROOR, ROOH or HOOH into alkoxy (RO) and hydroxyl (HO) radical.

Under preventive antioxidants, metal chelating agents such as curcumin and phytate and compounds capable of reducing peroxides either catalytically like glutathione peroxidase (GPx) or stoichiometrically, like erucin can prevent the initiation and block the catalytic cycle (Perron & Brumaghim, 2009; Amorati et al., 2013; Riccardo et al., 2013; Amorati et al., 2015). Further, chain-breaking antioxidants, also known as radical-trapping antioxidants, can stop the reaction by reacting with peroxy or hydroperoxyl radicals. The typical phenolic and polyphenolic compounds come under this category, while some non-phenolic compounds like urate and ascorbic acid are also known to stop the propagation reaction (Amorati et al., 2011; Amorati et al., 2015; Amorati et al., 2016). Lastly, a third class of antioxidants was introduced by Riccardo et al. (2013) and Baschieri et al. (2017) group, which can act as termination-enhancing antioxidants but co-oxidizing with the substrate and yield peroxy radicals, which do not propagate the chain while a higher rate of chain termination. Under this category of antioxidants, various non-phenolic terpenoids, such as citral and γ-terpinene, can potentially stop this reaction from proceeding further (Riccardo et al., 2013; Baschieri et al., 2017).

Essential oils can impair this chain reaction through two mechanisms: a) interfering with the initiation process through retardation of the initial formation of radical species (known as preventive antioxidants), and b) slowing down or blocking the autoxidation reaction through the completion of the propagation step (called chain-breaking antioxidants). In order to complete the propagation step, antioxidants react with ROO• faster than the oxidizable substrate to create species that do not proceed the chain. Essential oils as antioxidant agents can reduce oxidative reactions. EOs have different direct or indirect modes of action, including prevention of chain initiation and free-radical scavenging activity (Rodriguez-Garcia et al., 2016). The strong antioxidant activity of essential oils has been linked with the presence of phenolic compounds and some other secondary metabolites structurally similar to phenols (Ribeiro-Santos et al., 2017). Chiorcea-Paquim et al. (2020) reported that a “phenolic

compound could be defined as an antioxidant which can be present in low concentration than the substrate and undergoes oxidation reaction to delay, inhibit and prevent autoxidation or free radical-induced oxidation, and the radical produced after scavenging being stable (Halliwell & Gutteridge, 2015; Chiorcea-Paquim et al., 2020). The antioxidant mechanism of phenolic compounds has been extensively reported through several analytical assays (Robbins, 2003; Chiorcea-Paquim et al., 2020). Different mechanisms can describe the antioxidant activity of polyphenols: free-radical scavenging ability through proton-coupled electron or hydrogen-atom transfer, b) sequential electron and proton transfer, c) sequential proton-loss and electron loss transfer and adduct formation (Dangles et al., 2008; Leopoldini et al., 2011). All the above-discussed mechanisms result in phenoxy radical formation. In addition, metal-chelating characteristics of bidentate phenolic groups, electron or proton donating characteristics of electroactive phenol moieties and binding interaction with biologically active proteins, i.e., oxidoreductases, could be responsible for their antioxidant activity (Rice-Evans et al., 1996; Chiorcea-Paquim et al., 2020).

2.2.3.5. Safety and legal aspects of the use of essential oils in foods

The European Union (EU) is responsible for producing the recommendations, regulations and laws to use plant volatiles like essential oils in food systems to maintain the quality of food products, which are easily prone to harmful and spoilage microbes (Bajpai et al., 2012). By the EU, various essential oil compounds have been registered as flavouring agents to use in food commodities (Burt, 2004). A special E number is designated to all food additives, either approved by the EU or not and labelled with E and a specific number. From 2011, a single database containing all information regarding all approved additive and their acceptable daily intake was listed to be used with the European Union. In the United States, the Food and Drug Administration (FDA) determined all food additives to be labelled as “Generally Recognized

as Safe" (GRAS). This term is still used, and several toxicological assays must be performed for any additive to be listed. Therefore, food additives in this list have changed throughout the years.

In addition, the acceptable daily intake, which measures the amount of a food additive to be ingested orally daily over a lifetime without imposing any appreciable health risk, was introduced in 1961 by the Joint Committee of FAO (Food and Agricultural Organization) and WHO (World Health Organization) on food additives.

The FDA creates a list of natural extracts, oleoresins, and essential oils such as thyme, clove, basil, oregano, and cinnamon for usage in food products (FDA, 2020). Diversification of the chemical composition is one of the main limitations of essential oils that makes it difficult to regulate and standardize ADI (acceptable daily intake) and NOAEL (No Observed Adverse Effect Level) levels. Consumption of essential oil at elevated concentrations and amounts can be toxic if consumed orally (Bhardwaj et al., 2020). Therefore, several studies on the safety and toxicity of essential oils or their compounds have been determined through *in vitro* and *in vivo* methods (Maisanaba et al., 2017). Chen et al. (2016) reported that mānuka and kānuka oils did not have significant toxic effects on THP-1 cells. In the 48-hour exposure treatment test of essential oils at a concentration from 0.1 to 10 %, cell viability (THP-1 cells) exceeded 100 %, and no significant change in the release of TNF-α, cytokine or IL-4 from THP-1 cells was observed (in the absence of lipopolysaccharide stimulation) (Chen et al., 2016). The TNF-α and IL-6 are released from macrophages/monocytes, then activate and encounter antigen-specific natural killer T cells (the process is called Th1), while to respond to allergen exposure, macrophages secrete Th2 cytokines including IL-4 (Mantovani et al., 2004; Liao et al., 2012; Yu et al., 2012). In the past few decades, several research studies have been performed in mice and rats to check the toxicity of rosemary oil and extracts (extracted in different solvents)

(Huang et al., 2020). Independent of the extraction method, a reversible increase in both absolute and relative liver-to-body weights was noticed in many research studies. No other indications of toxicity have been documented (Fiume et al., 2018). In these studies, the doses of tested extracts were as high as 14.1 g/kg body weight and were examined for up to 5 days. Some studies tested doses up to 400 mg/kg body weight for up to 3 months (dietary) (EFSA, 2008a, 2008b). Regarding genotoxicity, which is determined through different assays to measure the induced damage to the DNA by chemical constituents (Pellevoisin et al., 2018), RE has not been identified as genotoxic in the Ames test, gene locus mutation assay or a chromosomal assay in human lymphocytes. Rosemary oil was also found to be non-mutagenic in the Ames test (*Salmonella* test), which is a short-term bacterial reverse mutation test used to detect a wide range of chemical compounds that can result in genetic damage and lead to a mutation in genes (EFSA, 2008a; Žegura et al., 2011; Jonatas et al., 2019; Mortelmans, 2019). The ingestion of larger amounts of rosemary oil or extracts might cause gastrointestinal irritation, renal injury, and neurological effects, as reported by Gwaltney-Brant (2006). The toxicity mechanism is unclear, but gastrointestinal irritation could be the direct irritant effect of excessive quantities of essential oil on the mucosa.

Various clinical assessments have been performed to investigate the health effects of rosemary oil/extracts (Bloomer et al., 2016; Nematolahi et al., 2018). Samman et al. (2001) investigated the effect of rosemary or green tea extracts against the absorption of prooxidant metals like non-heme-iron in young women. The results reported that meals containing rosemary and green tea extract declined the iron absorption in subjects from 7.5 ± 4.0 % to 6.4 ± 4.7 % and 12.1 ± 4.5 % to 8.9 ± 5.2 %, respectively (Samman et al., 2001). In another study, the effect of oral rosemary (dried aerial part in the capsule form, 500 mg) on anxiety, depression, memory performance, and sleeping quality was tested on university students. The results documented that rosemary had a significant effect on reducing anxiety and depression, increasing memory

performance, and improving the sleeping quality of university students (Nematolahi et al., 2018). This study recommended the potential of rosemary as a substitute for abusing stimulant drugs (Nematolahi et al., 2018).

2.3. Overview of meat and ground meat products

Meat and meat products occupy a reserved place in the human diet owing to their energy-dense and high-value protein nature. The demand for this high-value-based protein source is growing continuously (Pellissery et al., 2020). Worldwide meat consumption has doubled over the past two decades to achieve 360 billion tonnes in 2018 and is anticipated to increase over the upcoming years, owing to the increasing population and household income. In 2021-2022, New Zealand exported worth 11 NZD billions of beef, sheep meat and their co-products, achieving a 20 % increase compared to 2020-2021 (Meat Industry Association, 2022). This year, New Zealand exported beef to 71 different countries of about 484769 tonnes of valuing about $ 4.6 billion, which was 28 % higher than the last year, contributing remarkably to the nation's economy (Meat Industry Association, 2022). Globally, poultry meat is the highest (14.7 kg/capita/year) consumed meat, followed by pork (11.1 kg/capita/year) and beef and veal (9.6 kg/capita/year). Sheep meat has only 1.8 kg/per capita/ year consumption (OECD/FAO, 2020). Meat has a reserved place in the human diet because of its energy-dense and protein-affluent nature. Meat is an extremely valuable source of minerals (such as iron, zinc and, phosphorus, selenium), vitamins (like vitamin B_{12}, niacin, vitamin B_6, riboflavin, pantothenic acid and potentially vitamin D), and a multitude of endogenous antioxidants and bioactive substances (like taurine, carnitine, carnosine, ubiquinone, glutathione and creatine) (Jiménez-Colmenero et al., 2001; Williams, 2007; McAfee et al., 2010; Aarti et al., 2020). Several meat and meat products are grouped into two broad groups: white meat and red meat. In red meat, pork and beef are included, while in white meat, chicken, fish, duck and others are included. The raw

meat is further processed using different technologies to produce cooked, semi-cooked and processed meat and meat products like ham, bacon, sausages, meat patties, and others, which are well renowned for their organoleptic characteristics (Lu et al., 2022; Wang et al., 2022). As meat is a rich source of essential nutrients and is highly perishable, it is more prone to microbial degradation, autolytic, enzymatic breakdown, and oxidative reactions (Cassens, 2004; Dave & Ghaly, 2011). In order to enhance the flavour and colour characteristics of meat products and improve their shelf life, meat products are transformed by using the process of smoking, curing, and fermentation, known as "processed meats". These meat products are usually made up of pork or beef meat; however, meat by-products like blood or poultry can also be utilised (IARC, 2018).

Ground meat products, especially ground or minced beef, are one of the most widely purchased forms of meat (Brewer, 2012). Ground meat products are produced using mechanical processes such as high-speed chopping/grinding of boneless beef and trimmings. The commonly available ground meat products are hamburgers and meat patties (Lonergan et al., 2019). Meat paste is also a ground meat product having a homogenous texture but is understudied than other ground meat products.

2.3.1. Meat paste

Meat pastes are an important material used in the preparation of meat products like such as sausages, hamburgers, meat pies, meatballs and dried meat slices, owing to their high nutritional value and convenient use (Amiri et al., 2019). Meat pastes are ground meat products (paste) prepared with one or more kinds of meat, farinaceous material, flavourings, and other wholesome food (Parliament, 1913). Among different meat products, meat paste is especially renowned for its protein content, affability and characteristics. Meat pastes have a spreading consistency and can be packaged in containers and casings. As per the standards, meat pastes

are of two types, i.e., a) meat pastes made up of at least 20 % of muscle tissue, and b) meat-containing pastes made up of 0 and 20 % of muscle tissue.

These kinds of products originated in French cuisine, and a few examples are (cooked in casings), *rillette* (prepared with meat and liver), *mousse* (including eggs to produce a foamy texture), and *terrine* (hot-moulded in recipients) (Guerrero-Legarreta et al., 2010).

Due to the overexposure of meat surfaces to oxygen and catalysts (metals) and the distribution of microbes during the grinding process, ground meat products like meat pastes are more prone to spoilage oxidative reactions and microbial growth (Amiri et al., 2019). Other than the loss of colour, flavour, texture and nutritional values, these deteriorating reactions can produce free radicals and pathogenic microbes, which may be harmful and pathogenic to human health (Devatkal et al., 2010). Antioxidant agents' usage is one of the most promising and practical approaches to reduce oxidative reactions and prolong the shelf of meat products. According to the available literature, researchers are interested in adding plant ingredients (extracts or essential oils), antioxidants, vitamins, and amino acids to meat products to improve their functional, technological, and nutritional characteristics (Bazhenova et al., 2020). The antioxidant characteristics of lychee seed water extracts in raw meat paste were studied during 15 days of refrigerated storage, and further sensory characteristics of cooked meat paste added with different amounts of extracts were examined (Qi et al., 2015). The supplementation of antioxidants reduced the lipid oxidation in meat pastes without adversely influencing the organoleptic properties of cooked meat products (Qi et al., 2015).

2.3.2. Spoilage reactions in meat products and their impact on meat quality

Meat spoilage is defined as a dynamic change in meat quality that renders it unsuitable and undesirable for consumption, usually judged by losses of physical structure, texture, appearance and chemical deterioration (Odeyemi et al., 2020; Shao et al., 2021). Food

spoilages incur substantial economic losses for farmers, producers, retailers, and consumers (Mageswari et al., 2015; Bradford et al., 2018; Ioannidis et al., 2018; Ndraha et al., 2018; Wang et al., 2018; Odeyemi et al., 2020). As per the Food and Agriculture Organization report, approximately 21 % of the food losses are attributed to meat and meat products (FAO, 2011; Höll et al., 2016). Various factors such as pH, water activity, storage temperature, indigenous microflora, improper storage, processing, handling, and transportation influence the food spoilage rate. Soilage is a complex process, broadly categorised as chemical, microbial, and physical (Petruzzi et al., 2017). Despite extensive efforts, meat spoilage due to microorganisms' cross-contamination is unavoidable (Shao et al., 2021).

Microorganisms are too small to be observed with the naked eye and are one of the most ubiquitous reasons for meat spoilage. Due to the invisibility of microbes with the naked eye, excluding moulds, colonization of foods exposed to bacteria and yeast may remain undetected (Hammond et al., 2015). The microbial quality of meat is strongly affected by the hygienic conditions during production and handling (Osama & Kassem, 2011). The exterior surface of healthy living cattle is generally contaminated, whereas muscles are naturally sterile. As per Featherstone (2003), a one-centimetre area of live animals' hide contains 10^7 microbes. In addition, inner organs like the digestive tract, respiratory tract, and faecal are held by various microflora, and among these, the faecal could be a major cause of spoilage of carcass through direct and indirect contact (Unc & Goss, 2004; Ukut et al., 2010). Indirect contact with faecal to transfer microbes to carcasses includes uncleaned and contaminated slaughtering equipment, surface, installations, air, liquids and slaughterhouse personnel (Omoruyi et al., 2011). Contamination may occur during the slaughtering, cutting, processing, storage and distribution of meat (Clarence et al., 2009), which is why cold chain and hygiene maintenance during meat shipping is extremely important (Adzitey, 2011). In general, microflora is in the range of around 2-4 log colony-forming units (cfu) in the initial chilled meat, while it can rapidly

increase to 6.5-7 log cfu/g (spoilage threshold). However, owing to the heat and sterilization processing, this initial microbial load is low for processed meat products, excluding fermented meat products, and spoilage usually takes place at 3.5 -5 log cfu/g (Doulgeraki et al., 2012; Chen et al., 2020). Lactic acid bacteria, also known as LAB, a group of microaerophilic microbes, is predominant in vacuum-packed meat (Jones, 2004). Pathogens microbes such as *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus* may be found in different meat products, including beef, pork, poultry, lamb, and mutton (Li et al., 2020). As per the European food safety report, Shiga-toxin-producing *Escherichia coli* (also known as STEC) commonly occur on beef and is associated with 30 % of illness in Europe and cause severe diseases including blooding diarrhoea and haemolytic-uremic syndrome (Tilden et al., 1996; Paton et al., 1996; Frenzen et al., 2005; Karch et al., 2005; Panel et al., 2020). Similarly, *Listeria monocytogenes*, linked with human listeriosis, commonly occur in fresh meat and meat products with a long shelf life (Frenzen et al., 2005; Sofos, 2008).

Recently, the presence of this microbe in food processing environment has been noted explicitly in deli meats (especially sliced and packed) and is responsible for about 83 % of all listeriosis incidents in the United States (Buchanan et al., 2017; Li et al., 2020). However, in China, *Staphylococcus aureus* was detected in almost 35 % of the retail meat, including raw fresh, quick frozen and ready-to-eat meat, collected from 39 different cities in this country during 2011-2016. However, these meat products do not remarkably contribute to foodborne intoxication with staphylococcal enterotoxins (Wu et al., 2018; Li et al., 2020). It has been reported that *Salmonella Typhimurium* (DT104) incidences have been associated with meat paste, chicken, pork sausages, and several other food products like unpasteurized milk products and fresh apple cider. These products represented around 38 % of human salmonellosis in 2000 in Canada (Dore et al., 2004; Anany et al., 2015).

The predominant meat spoilage microbes are *Actinobacteria*, *Firmicutes* and *Proteobacteria*, in which *Carnobacterium* spp., *Brochothrix thermosphacta* and lactic acid bacteria are the most ubiquitous on raw and packaged meat products and form a large part of the core meat community on meats (Ercolini et al., 2006; Chaillou et al., 2015; Rouger et al., 2018).

Under chemical spoilage, oxidation of meat products is one of the most important reasons for food quality spoilage and degradation, leading to the loss of colour and nutritional value of meat products (Estévez & Cava, 2006; Fasseas et al., 2008). Due to high concentrations of unsaturated fatty acids (lipids), metal catalysts, heme pigments, and oxidizing agents in the muscle tissue, the meat becomes prone to oxidation (Falowo et al., 2014). Various studies have reported that amounts of metal ions present in enzymes, metalloproteins, or those migrating from the processing equipment (either by abrasion or acid dissolving of meat from the surface) could stimulate the rate of oxidation. In addition, the diet of animals during the production stage also influences the susceptibility of meat to post-mortem oxidation (Rulíšek & Vondrášek, 1998; Jacobsen et al., 2008). Generally, the oxidation of meat/meat products is determined by measuring the concentrations of thiobarbituric acid-reactive substances (TBARS), peroxide value, carbonyl and sulfhydryl groups produced during the process (Falowo et al., 2014). This analysis is performed using spectrophotometric, chromatographic (head-space gas chromatography (GC), high-performance liquid chromatography (HPLC) and liquid chromatographic-mass spectrophotometric (LC-MS)), and 2,4 dinitrophenylhydrazine (DNPH) methods.

Various preservation methods have been employed to prevent these spoilage reactions, and chemical additives are one of the most frequently used methods in the meat industry.

2.3.3. Usage of chemical preservatives in meat products and their association with adverse health implications

The most widely used antimicrobial compounds in meat products are sodium chloride, nitrites, organic acids and sulfides. Sodium chloride kills the microbes by reducing water activity and causing plasmolysis through water withdrawal from the cells (Marshall et al., 2016). However, nitrites prevent microbial growth through different mechanisms, including a) blockage of sulfhydryl groups (interference with sulphur-nutrition), b) reaction with α-amino groups of the amino acids, c) reaction with iron compounds (interference with iron nutrition), d) interference with membrane permeability (limited transport across the cells) (Ray & Bhunia, 2004). These are used to prevent the growth of toxin-producing *Clostridium botulinum*, *Yersinia enterocolitica,* and *Staphylococcus aureus*, which can grow in vacuum-packaged meats under anaerobic conditions. In addition, these agents effectively control lipid oxidation and the colour of meat products. Due to these purposes, sodium and potassium salt of nitrates and nitrites have been utilized in processed meat products (Sindelar & Houser, 2009; Dave & Ghaly, 2011). However, sulphites are an antimicrobial agent in ground meat products like sausages to control *Enterobacteriaceae* and pathogenic *Salmonella* (D and Dave & Abdel E Ghaly, 2011). In addition, organic acids such as lactic, sorbic, benzoic and ascorbic are also employed in meat industries (Theron & Lues, 2007; Mani-López et al., 2012).

Recent epidemiological studies linked meat intake, particularly processed meat intake, with the prevalence of various emerging diseases such as various tumours, diabetes, heart diseases, and obesity (Micha et al., 2011; Micha et al., 2012; Rouhani et al., 2014; IARC, 2018; Alshahrani et al., 2019; Virtanen et al., 2019). World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) provided an updated meta-analysis of prospective cohort studies and documented convincing evidence on high consumption of processed and red meat with increased possibilities of colorectal cancer. In 2015, the International Agency for Research on Cancer categorised processed meats as “carcinogenic to humans” (group 1 carcinogen) and red meat as “probably carcinogenic to humans” (group 2 A). As per the report, every increase

of 50 g of processed meat intake per day elevates the chances of colorectal cancer by 18 %, whereas every increment of 100 g of red meat intake level, rises the risk of colorectal cancer by 17 % (Bouvard et al., 2015; Zeng et al., 2019). The cancer precursors may be the curing ingredients (salt, nitrates, nitrites etc.), compounds produced on smoking (heterocyclic amines, polyaromatic hydrocarbons) and excess fat, protein and iron (Botez et al., 2017; Kaur & Sharma, 2019). Several components, including nitrates, haem iron, heterocyclic amines, polycyclic hydrocarbons (PAH) and high fat, have been implicated as potential causes of colorectal cancer; however, nitrites have emerged as the leading candidate accountable for the association of processed meats with cancer (Crowe et al., 2019) (Figure 2.2).

Nitrites are added in meats, such as ham, bacon, and sausages, during the curing process, and these are effective antimicrobial agents to retard the unique growth of C*lostridium botulinum* and to improve the colour and flavour of processed meats. However, these agents can form endogenous N-nitroso compounds (NOCs), a few of which are carcinogenic (Cantwell & Elliott, 2017; De Mey et al., 2017). When nitrites are taken into the body, NOCs are formed by the reaction of dietary nitrates or nitrites with amines and amides, which are identified to cause cancer in animals (Figure 2.2) (Veena & Rashmi, 2014; Xie et al., 2016; Crowe et al., 2019). Moreover, the consumption of nitrites has been associated with methemoglobinemia, especially in infants, because it reacts with haemoglobin and interferes with its ability to carry oxygen (Bedale et al., 2016). Furthermore, nitrates also affect the environment hazardously, mainly aquatic life. Nitrite consumption at high concentrations can lead to functional impairment of red blood cells, while these are safe on approved levels (Hotchkiss & Cassens, 1987).

2.3.4. Consumer perceptions about meat products (Need for an alternative)

Recently, there has been enormous interest towards developing natural alternatives and other preservation technologies that are relatively safer than synthetic preservatives. This interest is due to the pressure generated from the consumer demand for reduced nitrite or salt levels in meat products. Owing to the controversy over the association of nitrates with various diseases, people are commencing to pay more attention towards their diet and seeking health-related aspects through the diet (Angulo & Gil, 2007; Fonseca & Elisabete, 2008; Tobin, 2013). They are more attracted towards meat products with high nutritional value, which are free of chemical preservatives but safe to consume (Kaur & Sharma, 2019). In parallel, consumer demands are gearing towards high-quality meat products, which are prepared with the novel concept of "all-natural" and "clean label" (Jayasena & Jo, 2013; Alahakoon et al., 2015)

2.3.5. Usage of essential oils in meat products

The utilization of essential oils as natural preservatives has been reported to reduce the rate of oxidative reactions and microbial growth, thereby enhancing the shelf life of meat and meat products (Thales et al., 2011; Šojić et al., 2019; Tomović et al., 2020). So, essential oils are the suitable candidates that can exert a synergistic effect with reduced amounts of chemical preservatives (like nitrite) to produce chemicals-free or reduced-chemicals meat products.

Several research studies discussed the plant oils' potential to replace nitrite in meat products, as presented in Table 2.5. A recent study by Tomović et al. (2020) reported that the addition of *Juniperus communis* essential oil (0.01 and 0.05 µL/g) with a reduced concentration of sodium nitrite (75 mg/kg) prevented lipid oxidation of high-fat (25 %) fermented sausages. Moreover, no growth of foodborne microbes like *Escherichia coli, Listeria monocytogenes, Salmonella* spp. and sulfite-reducing *Clostridia* was observed during 225 days storage period at 15±1 °C (Tomović et al., 2020).

Bakhtiary et al. (2018) documented the antimicrobial potential of five types of essential oils, either used alone or in combination with sodium nitrite (0, 100, and 200 mg/kg), in beef fillets against the *Clostridium perfringens* and *Clostridium sporogenes*. The *Satureja bachtiarica* Bunge oil had the highest inhibition effect, followed by the *Zataria multiflora Boiss,* oregano, mint (*Mentha pulegium*) and rosemary oil. The antimicrobial effect of these essential oils was increased when utilized in combination with sodium nitrite in beef fillets during 30-day storage period at room temperature (Bakhtiary et al., 2018). As per the research study of Moarefian et al. (2012), all sausages prepared with varying quantities of the *Mentha piperita* essential oil and sodium nitrite provided desirable characteristics. The best results, including the strongest lipid oxidation activity, were demonstrated by 20 ppm essential oil and 100 ppm nitrite-added sausages than the 120 ppm of sodium nitrite alone added samples. The study also reported that partial (50 %) replacement of nitrite with this oil successfully reduced the growth of *Clostridium perfringens* during the storage of cooked sausages (Moarefian et al., 2012). In their following study, the partial replacement of nitrites with cinnamon essential oil led to no growth of *Escherichia coli* and *Clostridium perfringens* in cooked sausages during one-month refrigerated storage. In terms of oxidative stability, samples were acceptable after 30 days, and the colour was better than control sausages prepared without essential oil addition (with 120 ppm nitrite) (Moarefian et al., 2013).

Thales et al. (2011) reported the antimicrobial effect of winter savoury (*Satureja montana* L.) essential oil (0, 0.78, 1.56, and 3.125 % concentrations) and sodium nitrite (0, 100, and 200 ppm) against *Clostridium perfringens* in mortadella type-sausage during (30 days of storage at 25 °C). The mortadella, with 100 ppm of sodium nitrite and all three concentrations of essential oil, reduced the targeted microbes throughout the storage period than the control mortadella prepared without the incorporation of essential oil and nitrite (Thales et al., 2011). Their following study reported that combined treatment of 15.60 µL essential oil and 100 ppm nitrite

exerted a synergistic effect against lipid oxidation than other treatments. However, an antagonistic effect was noticed in samples prepared with 15.60 and 3.125 µL/g essential oil and 200 mg/kg nitrite. The possible reason could be the interaction between the essential oil's phenolic compounds and nitrites by linking a portion of the aromatic ring, and thus antagonism may impair the antioxidant efficacy of nitrite and essential oil (Thales et al., 2012). These studies reported the potential of combined use of savoury essential oil and reduced quantities of sodium nitrite to control the *Clostridium perfringens* in meat products, as per the current market trends, where customers are looking for natural products (Thales et al., 2011; Thales et al., 2012; Jonatas et al., 2017; Jonatas et al., 2019).

The abovementioned studies show that essential oils can partially replace the nitrates/nitrites in processed meat products. Nevertheless, some studies have reported the potential of plant oils/extracts for the complete replacement of nitrites. For instance, Salvaneschi et al. (2020) showed that thyme essential oil exerted antibacterial activity similar to nitrites in salami against *Listeria innocua*. *Listeria innocua* is a non-pathogenic microbe with similar morpho-cultural traits to *Listeria monocytogenes* (Salvaneschi et al., 2020). In another study, the effects of nitrate (200 ppm), sodium lactate (SL) (1.5 %) and thyme essential oil (TEO) (100 ppm) against *Listeria monocytogenes* on sausages stored at 8 °C (for 41 days) and 30 °C (for 14 days) was reported. At low temperatures, the SL and nitrate treatment exerted the highest antibacterial effect, but at high temperatures, *Listeria* was most inhibited by SL, followed by TEO and nitrate treatments (Blanco-Lizarazo et al., 2017). This difference in the antimicrobial effect of essential oil at high temperatures could be related to the generation of low pH at elevated temperatures and the increase in solubility and stability of essential oil that enhanced their antimicrobial effect (Burt, 2004).

Al-Hijazeen and Al-Rawashdeh (2019) compared the effects of various treatments (rosemary extract (300 ppm and 350 ppm), L-ascorbic acid (300 ppm), sodium nitrite (200 ppm) and BHA (14 ppm)) on stability and quality of cooked chicken meat. It was reported that throughout the storage period (4 °C for 7 days), both RE (350 ppm) and sodium nitrite (200 ppm) exhibited the highest effect on maintaining the low carbonyl values. However, no significant difference was reported between all other treatments and control samples with no added preservatives (Al-Hijazeen & Al-Rawashdeh, 2019). Similarly, the combined usage of rosemary and citrus extracts in cured meat products like Spanish chorizo as an alternative to produce clean, natural-label meat products free of artificial additives has been reported by Martínez et al. (2019). Therefore, these studies concluded that rosemary extract could be a natural antioxidant for partial replacement or substitution for synthetic antioxidants in meat products. The oregano essential oil (2.5 %) presented the highest anticlostridial activity against *Clostridium perfringens* in mortadella during 20 days of storage at 25 °C, where sodium nitrite (control treatments of 75 and 150 ppm) remained ineffective against this microbial strain (Dias et al., 2015).

Table 2.5. Application of essential oils as natural preservatives against different microbes in meat and meat-based products.

Meats	Microbe Tested	Essential oil	Observations	References
Mortadella	*Clostridium perfringens*	Oregano essential oil	✓ Essential oil (2.5 %) presented the highest anticlostridial activity against the tested microbe, while sodium nitrite remained ineffective against this microbial strain	Dias et al. (2015)
Beef Fillets	*Clostridium perfringens, Clostridium sporogenes*	*Satureja bachtiarica Bunge, Zataria multiflora Boiss,* Oregano, *Mentha pulegium,* Rosemary	✓ *Satureja bachtiarica Bunge* oil had the highest inhibition effect, followed by the *Zataria multiflora Boiss,* Oregano, *Mentha pulegium* and rosemary oil ✓ Antimicrobial effect of these essential oils was increased when utilized in combination with sodium nitrite in beef fillets during the storage period	Bakhtiary et al. (2018)
Cooked Sausages	*Clostridium perfringens*	Peppermint essential oil	✓ Reduction in the growth of *Clostridium perfringens* ✓ Strongest lipid oxidation stability	Moarefian et al. (2012)
Cooked Sausages	*Clostridium perfringens, Escherichia coli*	Cinnamon essential oil	✓ No growth of *Escherichia coli* and *Clostridium perfringens* was observed ✓ Improved colour and oxidative stability	Moarefian et al. (2013)
Salami	*Listeria innocua*	Thyme essential oil	✓ Essential oil exerted antilisterial activity similar to that of the nitrites	Salvaneschi et al. (2020)
Mortadella	C*lostridium perfringens*	*Satureja montana* L. essential oil	✓ Essential oil, at all three concentrations, reduced the targeted microbes throughout the storage period	Thales et al. (2012)
Fermented Sausages	*Escherichia coli, Listeria monocytogenes, Salmonella spp.* and sulphite-reducing *Clostridia*	*Juniperus communis* essential oil	✓ No foodborne microbes were observed in any of these samples during 225 days of storage at 15±1 °C ✓ Lipid oxidation of high-fat (25 %) fermented sausages was prevented	Tomović et al. (2020)

2.3.6. Challenges associated with the use of essential oils in food products

There is no question that essential oils under controlled conditions (*in vitro* studies) show a broad range of bioactivity; however, this efficacy is reduced when used in food formulations, posing a challenge for their use for food preservation (Silva et al., 2022). In addition, the extrinsic factors (microflora type, microbial concentration and environment), and several intrinsic factors of the food matrix, including fat, water activity, pH, protein, and salt concentration, can influence the bioactivity of essential oil or their compounds (Hyldgaard et al., 2012; Prakash et al., 2018). The heterogeneous meat structure can give rise to regions with varied physiochemical characteristics, which can influence the interaction of bioactive compounds with specific targets (Gutierrez et al., 2009). One of the significant challenges of using pure essential oils in raw and processed meats is the diluting effect of essential oil compounds in meat fat (Hyldgaard et al., 2012). Raw meat contains 2-5 % fat, which varies depending on the animal species, age, gender, genetics, muscle location and animal management (López-Bote, 2017). The fat content of meat products may be higher for some formulations, depending on their sensory and technological characteristics (Silva et al., 2022).

Due to the lipophilic nature of essential oils or their bioactive compounds, they show an affinity towards meat fat. Wang et al. (2020) studied the partitioning of antimicrobial compounds, i.e., carvacrol, in food packaging systems between food products (beef with 5 and 12 % fat contents, headspace and polylactic acid films. Their results exhibited that carvacrol absorption was 1.3-fold higher in high-fat beef (12 %) than the carvacrol concentration in low-fat beef (3 %), indicating that active compounds show high affinity towards fat and are more easily absorbed in high-fat meats (Wang et al., 2020). In addition, food formulation with lower pH values (≤ 5) may increase the hydrophobicity/lipophilicity and decrease the solubility of essential oils in the water phase of meat products (Juven et al., 1994; Gutierrez et al., 2009). Meat proteins can

reduce the bioactivity of essential oils through electrostatic or hydrophobic interactions (van der Waals Forces) with essential oil constituents (Weiss et al., 2010). Other meat nutrients can facilitate the microorganisms' recovery, which has gone through some types of stress due to the antimicrobial action of essential oils. It is clear that meat matrix constituents and several other factors can hinder the efficacy of added essential oils.

The low water-solubility, strong odour, and instability under extreme conditions, especially volatility and easily degradable nature, restrict essential oil's direct application in the meat industry (Chouhan et al., 2017). Other than this, heat liable or volatile bioactive compound content of essential oils may be affected by the processing method (e.g., cooking) and storage period (Kaur & Kaur, 2020).

The abovementioned factors profoundly influence the efficacies of directly added essential oils in meat matrices. Therefore, it is imperative to employ other approaches or technologies involving essential oils to overcome the limitations of using them in food formations as a natural preservative (Silva et al., 2022). This problem could be overcome through encapsulation, an emerging solution to the demerits of essential oils used in foods (Chouhan et al., 2017; Molina et al., 2019). Encapsulation is one of the pioneering and efficient methods to preserve the beneficial properties of natural preservatives like rosemary oil by isolating them from external factors (Hussein et al., 2017; Abandansarie et al., 2019).

There is no doubt that food preservation practices are of considerable importance when considering both food safety and security through the shelf-life extension, quality, and safety improvement of food commodities. Food researchers and processers constantly strive to improve food formulations' shelf life, safety, and quality through a wide range of conventional and modern techniques.

2.4. Overview of encapsulation

Encapsulation is a process that involves the covering of an active compound (core material) with a covering material (wall material) (Assadpour & Jafari, 2019; Amaral et al., 2019). The ingredient/drug/bioactive compounds inside a capsule are known as an encapsulant, payload phase, core material, fill, and internal phase, whereas the outer covering is ascribed to wall material, carrier material, shell, membrane shell, external phase, matrix, or coating material (Madhavi & Usha, 2014; Jafari, 2017). The commonly utilized wall materials for the encapsulation process of food products are gum arabic, gelatin, maltodextrin, whey protein, sodium caseinate, chitosan, and modified starches (Gómez et al., 2018). The selection of suitable carrier material is an enormously imperative factor that protects the bioactive ingredient(s) from deterioration and should also be compatible with the food formulation, especially during processing, along with having desired mechanical strength and controlled release properties (Gharsallaoui et al., 2007). This interdisciplinary approach requires in-depth knowledge of colloid and interface chemistry and material science and an understanding of active agents' stability (Vinceković et al., 2017). In this process, carrier material protects the active material from unfavourable processing or storage conditions, such as high temperature, moisture and oxygen, certain pH levels and light, thereby prolonging their stability and shelf life, as well as helping to hide sensory characteristics that are not preferred by the food consumers (Castro-Rosas et al., 2017)

To encapsulate the bioactive ingredients, numerous techniques like freeze-drying, spray-drying, liposomes, molecular inclusion, coacervation, extrusion, supercritical fluids, polymeric micelles, nanostructured lipid matrices, and solvent evaporation are currently employed (Aguiar et al., 2016; Franco et al., 2017; Gómez et al., 2018). The selection of an appropriate encapsulation method depends upon several factors, like the physicochemical characteristics of the core and shell material and the required application in the product (Ubbink & Krüger, 2006). It should achieve high encapsulation efficiency, homogeneous distribution in the

spherical capsules/ spheres, high loading capacity, and the required controlled release characteristics. It should also be a low-cost process and must be operative under mild and simple processing conditions (Kailasapathy, 2009; Vinceković et al., 2017).

Encapsulation technology holds the creditably to enhance the stability (physical, chemical and thermal), bioactive activity, and controlled release and targeted delivery of bioactive compounds (Chandrakasan et al., 2019; Martínez et al., 2019; Kaur & Kaur, 2020). Owing to these advantages, encapsulated compounds perform better when incorporated into food products. Generally, the encapsulated particles can be classified as nanocapsules (particle size smaller than 0.2 µm), microcapsules (0.2 µm-5000 µm) and macrocapsules (particle size bigger than 5000 µm) (Jafari, 2017). Nanoencapsulation, an innovative and practical branch of nanotechnology in the food industry, deals with the encapsulation of particles on a nanometer scale (Jafari, 2017).

2.4.1. Nanoencapsulation

In 1959, famous physicist Richard Feynman introduced the idea of nanotechnology and later, in 1974, "nanotechnology" term was first used by a scientist Norio Taniguchi. Nanotechnology deals with particle size between 1-100 nm and is applied in various fields, including physics, chemistry, biology and materials engineering (Rashidi & Khosravi-Darani, 2011). However, the definition of engineered nanomaterials also incorporates structured nanoentities with a size of more than 100 nm, which possess properties that are the characteristics of nanoscale materials (EFSA, 2021). As per the Novel Food Regulation (EU no 2015/22837) and Provision of Food Information to Consumers (EU No 1169/20118), engineered nanomaterials may be defined as intentionally fabricated materials, which has one or more dimensions of the order of 100 nm or maybe less and made up of discrete functional parts, either inside or at the outer surface, many o which have size in the order of 100 nm or less including structures

agglomerates and aggregates, which have size more than the 100 nm but possess the characteristics that are properties of nanoscale materials (European Union, 2011a, 2011b, 2015).

The use of nanotechnology has made it possible to ameliorate the macroscale food characteristics, i.e., texture, taste, appearance, and durability (McClements et al., 2007; Huang et al., 2010). The increased surface area of nano-size particles enhances the practical application of bioactive compounds (Rashidi & Khosravi-Darani, 2011). The undesired flavour and taste of the compound could be masked by encapsulation, and the compound remains stable during harsh physical, chemical, and thermal food processing conditions (Lee et al., 2019). Several studies addressed the application of nanoencapsulation of bioactive compounds such as essential oils, antimicrobial drugs, and other active compounds, including nutrients (minerals, vitamins), flavouring, colouring, antioxidant agents, and probiotics (Shahidi, 2006; Hsieh & Ofori, 2007).

2.4.2. Nanocapsules

In recent years, nanocapsules have acquired a widespread interest in different areas, including physics, chemistry, and biology, due to their applications in food technology, pharmaceuticals, and materials engineering (Liu et al., 2017; Kong et al., 2019; Wu et al., 2020). Nanocapsules are vesicular-type systems that consist of active molecules in an inner core, which is encapsulated or surrounded by a polymeric wall/membrane or shell (Xu et al., 2016; González-Reza et al., 2021). Capsules can be categorized into two basic categories., single core (single wall and multi wall) and multicore (single wall and multi wall), as presented in Figure 2.4. The nanocapsules of core-shell material are promising carriers to encapsulate bioactive compounds like essential oils in a polymer shell, protect them from harsh external conditions, and facilitate their release when required (Liu et al., 2017; Yang et al., 2019). In addition, the encapsulation

of bioactive compounds in nanocapsules can overcome their limitation of poor dispersibility and water-solubility in an aqueous environment, thereby ameliorating their bioavailability and bioactivity (Zhang et al., 2018; Wu et al., 2020). Various methods, such as chemical (complex coacervation, polymerization-induced phase separation, and interfacial polymerization) and physical (fluid bed coating, one and two-step emulsification), are employed to prepare nanocapsules (Hede et al., 2008; Nori et al., 2011; Kim et al., 2015; Lee et al., 2016; Sang et al., 2018; González-Reza et al., 2021). Keeping in view the potential offered by polymer chemistry today, only a limited number of polymers can be used for designing nanoentities like nanocapsules and nanoparticles to deliver bioactives efficiently and adequately (Vauthier et al., 2004; Nair & Laurencin, 2007; Vauthier & Bouchemal, 2009). Under the natural polymer category, alginate, chitosan, gelatin and albumin, while in colloid stabilizers, Tween® 20 or Tween ®80 dextran, poly (vinyl alcohol), and copolymers are the most widely used for nanoencapsulation and nanoemulsification (Vauthier & Bouchemal, 2009). Alginate nanocapsules can be prepared by complexation reaction using cross-linked like calcium chloride or by mixing with oppositely charged polyelectrolyte solutions such as chitosan. Several studies have developed alginate nanoparticles or nanocapsules to encapsulate essential oil or their bioactive compounds. For instance, Li et al. (2022) produced the alginate/chitosan nanoparticles (200 nm) containing ε-poly-lysine-epigallocatechin gallate with ionotropic pre-gelation and polyelectrolyte complexation method, which can be used to inhibit *Escherichia coli, Staphylococcus aureus* and the aquatic products dominant spoilage bacterium *E3* strains (Li et al., 2022).

Hussein et al. (2017) confirmed that nano-encapsulation improved the thermal stability of the rosemary oil and minimized aroma degradation and loss throughout the food processing and storage conditions, thereby making it ideal for use in food products. A cinnamon oil containing nanoparticles was prepared with a hot homogenization method and further microencapsulated

in sodium alginate-calcium chloride (Yostawonkul et al., 2021). The nano/micro composite particles showed antibacterial activities against foodborne pathogens (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Salmonella enterica* and *Vibrio cholerae*, and resistant to gastric fluids and sustained release in the intestinal system (Yostawonkul et al., 2021). Similarly, Natrajan et al. (2015) optimized the influence of heat, alginate and chitosan concentration on the encapsulation of turmeric and lemongrass essential oil in nanocapsules. Their results exhibited that 0.3 mg/mL concentration of sodium alginate and 0.6 mg/mL of chitosan resulted in minimum-sized nanocapsules of particle size less than 300 nm, with good stability (Natrajan et al., 2015). However, citronella essential oil was first loaded in copolymer Pluronic® F127 nanoparticles and then covered with a chitosan-alginate complex. The encapsulation efficiency of 80 % for essential oil was achieved by this method, and particles were also in the nano-range (Talebian et al., 2022).

A recent study by Rahnemoon et al. (2021) encapsulated pomegranate (*Punica granatum* L.) peel extract with water in oil emulsification and external gelation (calcium chloride). The optimized particles had a particle size of 205.1 ± 0.1 nm and an encapsulation efficiency of 83.90 %. Nanospheres in innovative coating preserved the fresh chicken and showed inhibitory effects against microbial growth than free extract and alginate-containing extract-treated chicken samples throughout two weeks of storage (4 °C). *Ziziphora clinopodioides–Rosmarinus officinalis* essential oil containing-alginate nanoparticles of size varying from 159.14 to 256.14 nm have been used for the preservation of lamb burger patties. Nanoparticles inhibited the growth of *Escherichia coli and Staphylococcus aureus,* delayed lipid oxidation than control and free oil-containing patties. In addition, nanoparticles reduced discolouration and the development of off-odours in patties (Karimifar et al., 2022).

Similar to alginate, whey proteins have been reported as excellent and effective emulsifiers to stabilise the essential oils droplets in food emulsions, owing to their amphiphilic characteristics (Hebishy et al., 2022). The amphiphilic nature of these proteins allows their absorption on to oil droplets surface and thus stabilises the prepared food emulsions (Tirok et al., 2001; Ven et al., 2001*)*. Ribes et al. (2017) obtained an essential oil (lemon, cinnamon and bergamot) nanoemulsion using 1 % anionic whey protein isolate (WPI) or tween 80, 3 % weight essential oil and 1 % sunflower oil (ripening inhibitor), which was stable over 7 days during accelerated ageing at 35 °C (Ribes et al., 2017).

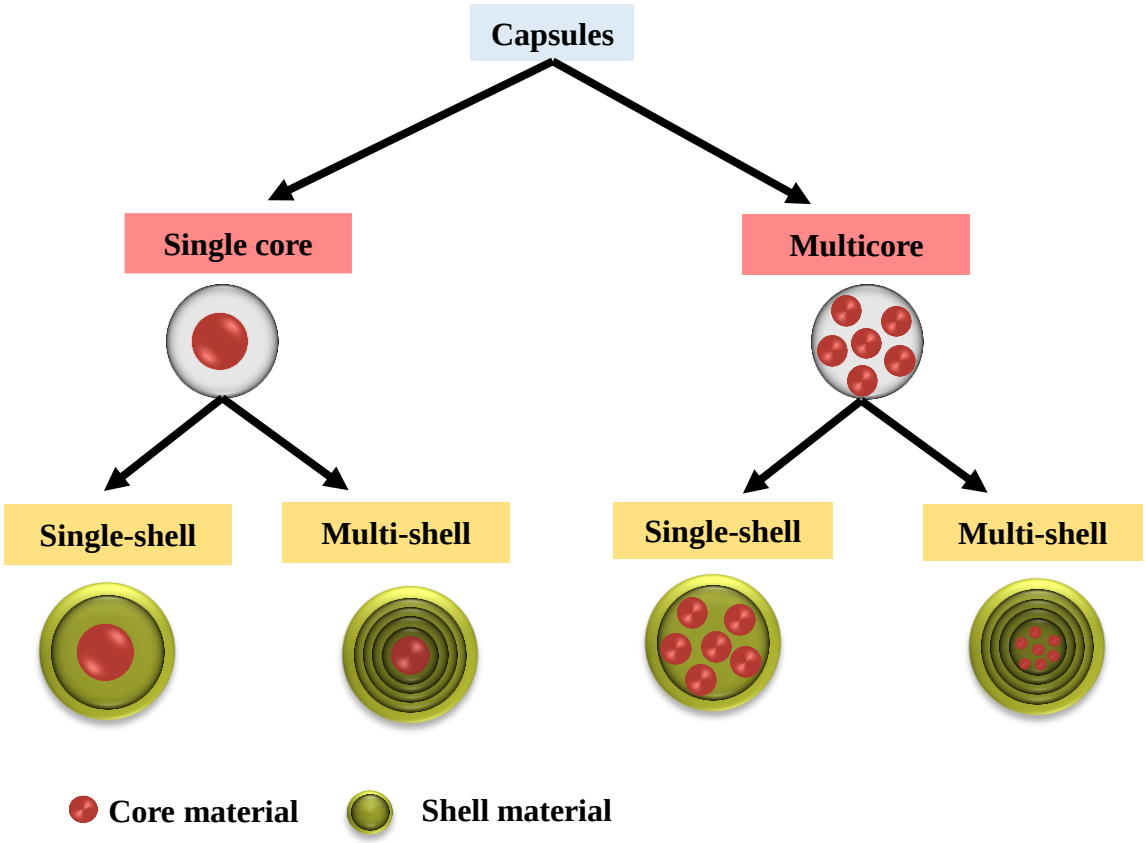


Figure 2.4. A schematic representation of single and multicore capsules.

This figure was adapted from Katouzian and Jafari (2016) with copyright permissions.

2.4.3. Nanoemulsions

Nanoemulsions are defined as metastable colloidal dispersions of one fluid in the form of droplets, size varying between 10 and 100 nm (more than 100 nm in some cases), in another immiscible fluid (Pathak, 2017). Emulsions-based systems are produced using emulsifier-covered oil droplets dispersed in an aqueous phase (Nile et al., 2020). Nanoemulsions are a nonequilibrium mechanism produced with the help of an internal or external energy source. Nanoemulsions contain three phases, i.e., continuous phase, dispersed phase, and emulsions stabilizer, also known as emulsifier or surfactant (Solans et al., 2005). The reduced size of nanoemulsions helps to produce a large surface area, which may be imperative for strong interaction with several bioactive compounds transported in the gastrointestinal (GI) tract (Salvia-Trujillo et al., 2013). Nanoemulsions possess the considerable potential to encapsulate, deliver and protect hydrophobic and hydrophilic bioactive compounds. The potential advantages of nanoemulsions include stability (especially physical), optical clarity, small particle size, and sustained release activities with prolonged duration (Mostafa et al., 2017). In addition, nanoemulsions exhibit a higher digestion rate than conventional emulsions because of the availability of more binding sites for digestive enzymes, i.e., lipase and amylase, in the GI tract (Salvia-Trujillo et al., 2013).

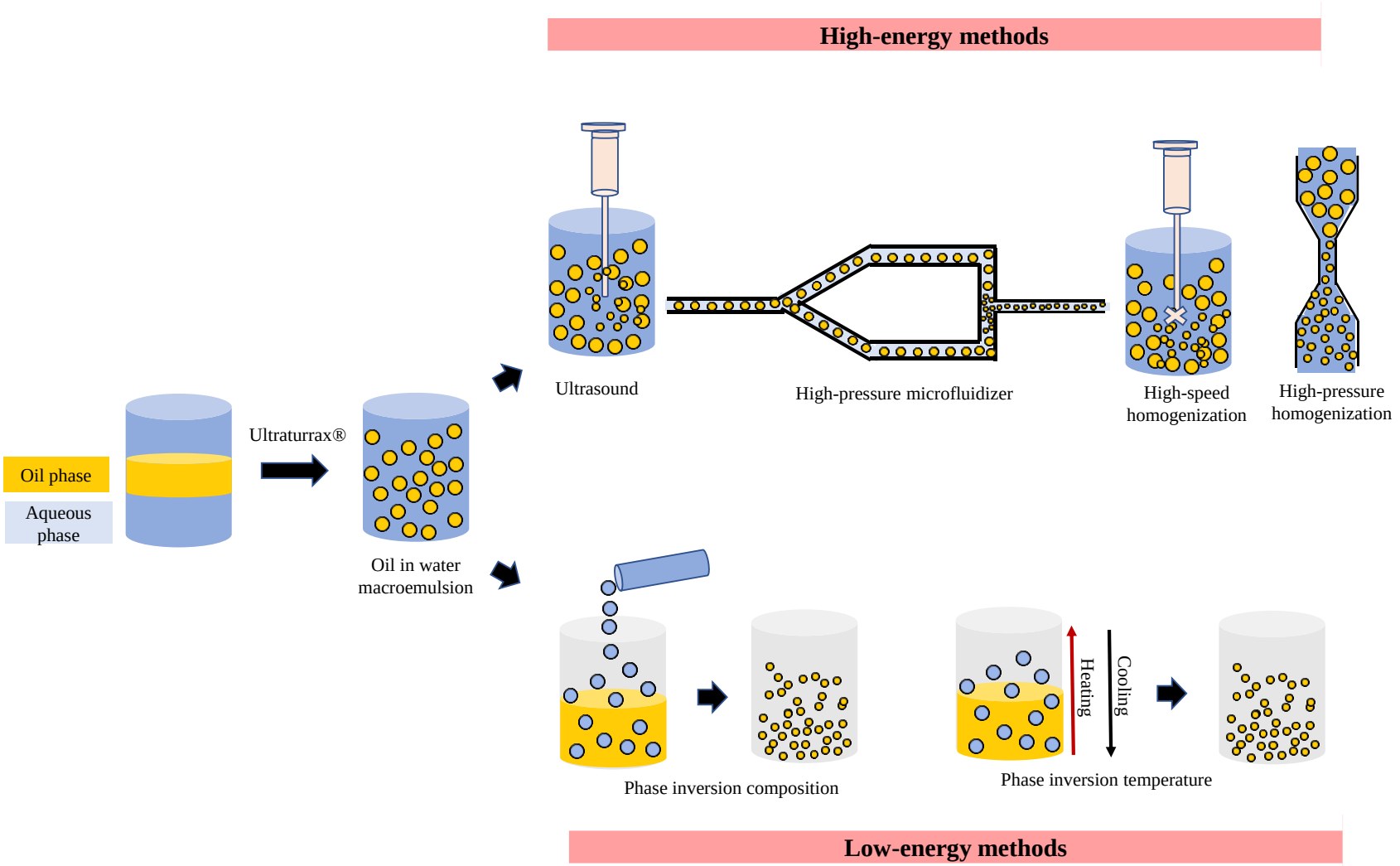


Figure 2.5. A schematic representation of the methods used to fabricate nanoemulsions, correlating their mechanisms. This figure was adapted from Silva et al. (2022) with copyright permissions.

2.4.3.1. Nanoemulsification methods

In general, two different methods: low- and high-energy, are employed to prepare emulsions (Mohamed & Shahira, 2018). In high-energy methods, mechanical devices create powerful disrupting forces and break down the oil and water droplets, producing tiny oil droplets. Various mechanical devices that produce nanoemulsions include microfluidizers, ultrasound homogenizers, high-pressure valve homogenizers, and other high-speed devices (Swathy et al., 2018). Nanoemulsion formation using involves two steps: a) conventional emulsion (droplet size between 500-1000 nm) formation by mixing oil, water and emulsifier using Ultraturrax® homogenizer, and b) droplet diameter reduction with the application of a microfluidizer, ultrasound, and high-speed homogenizer (Barradas & Silva, 2021). These mechanical devices have different mechanisms of action, as shown in Figure 2.5. In high-pressure homogenization, fluid is continuously compressed and passed through a micrometric channel at elevated pressures (between 50 and 4000 MPa). The fluid passage generates intense mechanical stresses such as shear, turbulence, and cavitation in the channel, which reduces the droplet size of dispersed phase particles in the continuous phase (Bai & McClements, 2016).

In a microfluidizer, coarse emulsion passes through a narrow band within the chambers, with homogenization pressures varying between 3.5 and 150 MPa (Dammak et al., 2020). In this device, the inlet splits into small branches to form a 'Y' or 'T' type junction between 50 and 300 µm, as shown in Figure 2.5. The interaction channels are designed in such a way to produce emulsion currents that collide with each other, creating turbulence, cavitation, and shear disruptive forces, which reduce the droplet size by rupturing it (Bai & McClements, 2016). However, in low-energy methods, tiny droplets are produced spontaneously in the presence of oil in a water mixture emulsifier when there is a change in solution or environmental conditions like temperature and composition change of the solution and need little external energy for

droplet formation (Sneha & Kumar, 2022). These methods are based on the release of internal energy due to the phase transition of surfactant/emulsifier throughout the emulsion process, which can be induced by composition (phase inversion composition (PIC)) or temperature change of the emulsion system (phase inversion temperature (PIT)) (Solans & Solé, 2012). Hien and Dao (2021) formulated the black pepper essential oil nanoemulsions using the PIT and emulsion phase inversion method. The emulsion produced using the PIT method was more stable throughout the heating-cooling cycles, while during storage of 2 weeks and 4 weeks, it separated and produced creamy layers, respectively. However, the phase inversion nanoemulsions were stable for up to 4 weeks. In addition, the high loading efficiency of nanoemulsion phase inversion nanoemulsions (92.13 %) was observed more than the PIT nanoemulsions (81.52 %) (Hien & Dao, 2021).

For instance, a recent study reported that the particle size of cinnamon oil nanoemulsion was 102 nm at 0.8×10^8 Pa homogenization pressure, and the formed emulsion showed no slick and sedimentation after 25 days of storage under refrigerated conditions (Liu et al., 2021). Similarly, Sichuan pepper essential oil nanoemulsion prepared and optimized using high-pressure homogenization and Tween-80 (T-80) and capric/caprylic triglyceride as emulsifiers exhibited long-term stability. After 31 days of storage (at 4 and 25 °C), the particle size of the nanoemulsion changed from 125.07 nm to 134.53 nm, and nanoemulsification showed no weakening of antioxidant activity and improvement in inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* (Shi et al., 2022). In ultrasonic homogenization, the cavitation phenomenon induced by pressure flocculations of the sound waves, turbulence, and physical shear of the ultrasonicator causes disturbances at the oil/water interface by rapid creation and collapse of vapour bubbles (Kumar et al., 2019; Sneha & Kumar, 2022). Due to the collapsing of bubbles, intense sound waves with pressures of up to 100 MPa are generated, which can travel throughout the emulsion and induce rupture of the oil/water interface, thereby

reducing droplets' size to the nanometric range (Donsì & Ferrari, 2016; Barradas & Silva, 2021). An optimal clove oil nanoemulsion produced using a microfluidizer at 10,000 psi, 1 % clove oil, and 3 % (v/v) surfactant (Tween® 80) had a droplet size of 30.76 nm, as reported by a recent study of (Pilong et al., 2022). The droplet size of nanoemulsions can be reduced by increasing the pressure and number of cycles. Fennel oil nanoemulsions prepared using xanthan gum and 12 or higher cycles of microfluidizer at the highest homogenization pressure (140 MPa) had an average droplet size below 10 nm (Llinares et al., 2021).

In addition, nanoemulsions containing essential oils and polysaccharides (like alginate) prepared with microfluidizer have great potential to be used to produce an edible film with functional characteristics (Acevedo-Fani et al., 2015). Hasheminya and Dehghannya (2022) produced *Froriepia subpinnata* (Ledeb.) Baill essential oil nanoemulsion with a particle size below 100 nm using surfactants (Tween 80 and Span 80) and high-intensity ultrasound and was stable up to 45 days. Not only the antioxidant and antimicrobial (against *Escherichia coli* and *Staphylococcus aureus*) activities of this nanoemulsion were higher than free oil, but these characteristics were maintained for 45 and 60 days, respectively (Hasheminya & Dehghannya, 2022). The antimicrobial activity of nanoemulsions is increased by a reduction in particle size, which facilitates the transport of essential oils compounds across the cell membranes of microbes and increases the surface area of contact with multiple targets in the microbial cells (Moraes-Lovison et al., 2017) (Table 2.6). Moraes-Lovison et al. (2017) reported that oregano (*Origanum vulgare*) essential oil nanoemulsion prepared using the phase inversion temperature method showed no loss of its antimicrobial efficacy against *Escherichia coli* and *Staphylococcus aureus* even after 90 days of storage. In chicken pâté, after 8 days of storage, the antibacterial activity of oregano essential oil nanoemulsion prepared using phase inversion temperature method was higher than the free oil and synthetic preservatives (sodium nitrite and BHT)-added chicken pâté against *Escherichia coli* (Moraes-Lovison et al., 2017).

Although new and exciting encapsulation/emulsification technologies have been developed over time, the potential of some understudied areas must be discovered for sustainable production. For instance, the particle size distribution of the bioactive components in the core material and the application of encapsulated antimicrobials in the food formulation also need to be explored. More research studies emphasizing the interaction of food components and ingredients with the encapsulated antimicrobial need further investigation. Modelling the controlled or sustained release of the antimicrobials encapsulated in the carrier material also establishes greater accuracy for further research. It is also worth looking at the numerous unclear and uncomprehensive areas where encapsulation technology could provide needed innovations.

Table 2.6. Studies on the antimicrobial effectiveness of encapsulated essential oils and their application in meat and meat-based products.

Meat Product	Microbe tested	Bioactive/Essential oil	Encapsulation method	Encapsulating material	Findings	References
Chicken meat	Total microbial count, yeast, mould, and psychrophilic species	Pomegranate (*Punica granatum* L.) peel extract	Water in oil emulsification and external gelation (calcium chloride)	Alginate	• Particle size in a nano range (205.1 ± 0.1 nm) • Encapsulation efficiency 83.90 %. • Nanospheres in the form of innovative coating showed inhibitory effects against microbial growth than other treatments throughout two weeks of storage (4 °C)	Rahnemoon et al. (2021)
-	*Escherichia coli*, *Staphylococcus aureus* and aquatic products' dominant spoilage bacterium *E3* strain	ε-poly-lysine-epigallocatechin gallate	Ionotropic pre-gelation and polyelectrolyte complexation (chitosan)	Alginate/Chitosan	• Particle size in a nano range (200 nm) • Encapsulation efficiency 78.2 %. • Nanoparticles inhibited the tested microbes in zone inhibition and growth curve assay	Li et al. (2022)
Lamb burger patties	*Escherichia coli* and *Staphylococcus aureus*	*Ziziphora clinopodioides–Rosmarinus officinalis* essential oil	Spray drying	Alginate	• Mean particle size varies from 159.14 to 256.14 nm • Nanoencapsulated oil decreased the lipid oxidation and growth of *Escherichia coli* and *Staphylococcus aureus* more than control and free oil-containing patties. • Nanoparticles reduced discolouration and development of off-odours in patties	Karimifar et al. (2022)

Chicken Meat	*Salmonella* Enteritidis	Thyme oil	Thin film dispersion method	Soy lecithin and cholesterol liposomes	• Encapsulated oil had a more bacteriostatic effect against the inhibition of tested microbe than free thyme oil.	Cui et al. (2017)
Beef	*Escherichia coli* O157:H7	*Zataria multiflora* Boiss essential oil	Modified Mozafari method (heating method)	Liposome	• Low minimum inhibitory concentration in the case of encapsulated oil	Khosravi-Darani et al. (2016)

2.5. Gaps in the literature

The literature survey has presented valuable insights into the use of essential oils in food preservation. The present review has identified numerous research gaps that open opportunities for future research. In order to explore the potential of natural preservatives in meat products, studies documenting the application of essential oils are abundant in the literature. Among the essential oils, rosemary, thyme oil, oregano, tea tree, lemon, lavender, eucalyptus, and peppermint oil has been widely studied, but no study has explored the use of mānuka oil in food products. As per the available literature, mānuka oil possesses antimicrobial and antioxidant characteristics, indicating that this oil has the potential to be used in food products. Likewise, limited research is available on the characterization of their antimicrobial activity against food pathogenic and spoilage microflora. For instance, no research has been performed on mānuka oil-added food products. Limited studies on the effect of food composition on the activity of essential oils have been documented, most of which are not deeply investigated. The dilution or absorption of essential oils in food fats is poorly understood.

A few studies have evidenced that essential oils can be added into nanoemulsions or nanocapsules without influencing the characteristics of essential oils and carrier materials. More research studies in this arena are needed to incorporate essential oils into nanoemulsions or nanocapsules made of food-grade wall materials. Consequently, future research investigations are needed to fill these gaps in the literature.

Chapter 3 Characterisation of the antioxidant and antimicrobial potential of the mānuka, kānuka and rosemary oils

Abstract

Essential oils possessing antioxidant and antimicrobial characteristics have acquired broad interest as an alternative to synthetic food preservatives. This research hypothesizes that mānuka and kānuka oils may possess antimicrobial and antioxidant characteristics and have the potential to be used as natural preservatives for food applications. In this study, mānuka (with 5, 25 and 40 % triketone content), rosemary and kānuka oils were characterised and screened through DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric radical absorbing power) assays for their antioxidant efficacies. Initial experimentation was conducted to characterize mānuka oils (with 5, 25, and 40 % triketone contents), rosemary oil along with kanuka oil for their antibacterial efficacy against selected Gram-negative (*Salmonella* spp. and *Escherichia coli*), and Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) bacteria through disc diffusion and broth dilution assays. All mānuka oils showed higher phenolic content and antioxidant activities than kānuka and rosemary oils. All mānuka oils showed a higher antimicrobial effect against *Listeria monocytogenes* and *Staphylococcus aureus* with a minimum inhibitory concentration below 0.04 %, compared with kānuka oil (0.63 %) and rosemary oil (2.5 %). In chemical composition, α-pinene in kānuka oil, 1, 8 cineole in rosemary oil, calamenene, and leptospermone in mānuka oil were the major compounds, confirmed through Gas-chromatography-mass spectrometry analysis. The result of this study shows the potential of mānuka oil as a natural antioxidant agent and antimicrobial agent, specifically against *Listeria monocytogenes* and *Staphylococcus aureus* and antioxidant agents.

3.1. Introduction

The utilisation of medicinal and aromatic plants in foods and traditional medicines around the globe continues to play an imperative role in food preservation, providing nutritive support and treating diseases (Xu et al., 2020; Ćavar et al., 2008). These plants can be applied in fresh, dry, essential oil, extract, herbal tea, or pharmaceutical forms (like capsules and tablets) (Napoli & Vito, 2021; Xu et al., 2020). Essential oils are volatile nature mixtures obtained from different parts of plants like leaves, seeds, buds, flowers, roots, fruits, bark, twigs, herbs and wood. These oils are a complex mixture of various bioactive compounds, including monoterpenes, sesquiterpenes, oxygenated terpenes phenolic, and phenylpropanoid compounds (Basak & Guha, 2018; Napoli & Vito, 2021). These plant materials are primarily used due to their promising antimicrobial and antioxidant efficacies and other biological characteristics. These oils have been reported to possess antimicrobial activities against food spoilage and foodborne pathogenic microbes (Ramli et al., 2021).

Pathogenic bacteria, especially *Salmonella*, *Escherichia coli* and *Campylobacter,* are of major concern and significantly affect the safety of raw meat and poultry (Doyle & Erickson, 2006). It has been reported that *Salmonella* and *Escherichia coli* are among the most predominant microbes found in human and animal intestines and can be found in wastewater bodies due to faecal contamination (Mpundu et al., 2019). However, *Listeria monocytogenes* are of most concern in ready-to-eat processed meats (Sofos, 2008). Chemical preservatives such as sodium nitrates/nitrites have been utilized to produce safe, stable and longer shelf-life meat products (Kaur et al., 2021). On the other hand, recent research controversies on the use of chemical preservatives with chronic diseases like colorectal cancer moved the consumers and meat industry's interest towards the usage of natural or plant-based preservatives (Dellavalle et al., 2014; Cantwell & Elliott, 2017). Oliveira et al. (2012) found that alleviated levels of

thiobarbituric acid values were noticed in mortadella samples prepared with mg/mL of winter savoury (*Satureja montana* L) essential oils and without added nitrites. Among the different tested sodium nitrite levels, 1000 mg/kg was enough to provide characteristics red to the mortadella-type sausages (Oliveira et al., 2012). Several research studies have reported the antioxidant and antimicrobial effect of essential oils as a complete or partial replacer to sodium nitrate/nitrite or other synthetic preservatives in meat products (Oliveira et al., 2012; Tomović et al., 2020; Lages et al., 2021; Pinelli et al., 2021).

Leptospermum scoparium (mānuka) and *Kunzea ericoides* (kānuka) are New Zealand's indigenous plants belonging to the *Myrtaceae* family. Like the Australian tea tree oil, essential oils obtained from these plants are known as New Zealand tea tree oils. Owing to higher UV exposures and geographical isolation, these native plant species of New Zealand contain various health-promoting constituents (Zhang & Björn, 2009; Alsaud et al., 2021). In the past, early settlers used mānuka leaves and foliage to make tea and infusions (Porter & Wilkins, 1999; Klink et al., 2005). The *in vitro* antimicrobial potential of mānuka oil against the Gram-positive microbe has also been reported in the literature due to the presence of β-triketones (Harkenthal et al., 1999; Jeong et al., 2009).

Various *in vitro* studies have shown that mānuka and kānuka oils possess antimicrobial characteristics. In light of this, this research hypothesises that mānuka and kānuka oils may be used as natural alternatives to synthetic antimicrobial agents. This study aimed to characterise the chemical compositions and antioxidant and antimicrobial characteristics of mānuka and kānuka oil and compare them with rosemary oil. The antimicrobial effect of these oils against selected pathogenic Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative (*Salmonella* spp. and *Escherichia coli*) bacteria of pathogenic concern was evaluated.

3.2. Materials and Methods

3.2.1. Materials

The mānuka oils with different triketone contents, i.e., 5, 25 and 40 %, and kānuka oil, were purchased from Tairawhiti Pharmaceuticals Ltd. (Te Araroa, New Zealand), while rosemary oil was brought from "Now Foods" (Auckland, New Zealand). All oil samples were stored at 4 °C in amber-coloured glass bottles. The bottles were packed in black plastic bags to prevent the effect of temperature and light on the volatile profile of essential oils. The samples were kept at room temperature for 30-45 minutes before the analysis. Mānuka oil with triketone content of 5, 25 and 40 % have been referred to as mānuka oil 1, 2, and 3, respectively. Each oil sample was dissolved in dimethyl sulfoxide (DMSO) to test *in vitro* antimicrobial effect of oils against microbial growth.

3.2.2. Characterisation of mānuka (with different triketone contents), kānuka and rosemary oils

3.2.2.1. Chemical constituents analysis of essential oils

The gas chromatography-mass spectrometry (GC-MS) analysis of all oil samples was performed using the method of Van Vuuren et al. (2014). The TG-5MS (Thermo Fisher) GC column (30m × 0.25 mm ×0.25 µm) was used in this analysis. The essential oil samples were first diluted in hexane and then injected using a split ratio of 100:1 and oven temperature of 220 °C. The initial temperature used was 60 °C (for 10 min), then rising to 220 °C (at a rate of 4 °C/min), held for 10 min and again increasing to 240 °C (at a rate of 1 °C/min). The detector conditions, such as temperature and ionisation mode, were set at 250 °C and electron impact, respectively. Pure triketones were used as an internal standards and mass spectra library (NIST 05) was used to compare the obtained peaks.

3.2.2.2. Fourier transform infrared spectrometer (FTIR) analysis

The infrared spectroscopy analysis of mānuka, kānuka and rosemary oils was performed with an iDr 7 ATR-FTIR spectrophotometer (Thermo Fisher Scientific, USA). The oil samples were put directly on the surface of platinum-diamond crystal using glass transfer pipettes, and spectra were obtained from 400 to 4000 cm^{-1}. The background correction was performed to avoid the background effect. The obtained data were analysed using OMNIC™ software (Thermo Scientific, Auckland, New Zealand).

3.2.2.3. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was conducted with a thermal analyser (TGA, model STA 449 F5 Jupiter) to check the thermal stability of mānuka, kānuka and rosemary oils. Each oil sample of 10 mg was heated from 30 °C to 300 °C with a heating rate of 10 °C/min. The heating curve data were analysed using the NETZSCH ASC software (NETZSCH, Selb, Germany).

3.2.2.4. Total phenolic content (TPC) determination

The total phenolic content of all the essential oils (mānuka, kānuka and rosemary) was determined by following the method of Viuda-Martos et al. (2010) with slight modifications. Firstly, the essential oil mixture was prepared by dissolving the 100 µL of essential oil in methanol. This mixture (0.1 mL) was diluted with 0.4 mL of rosemary oil water. Then, 1 mL of Folin-Ciocalteu reagent solution (1:10 in RO water) was mixed with the sample mixture (0.5 mL). It was mixed thoroughly and left at room temperature for 10 min. After this, 2 mL of 15 % sodium bicarbonate solution was added, and the mixture was incubated at room temperature for 1 hour in the dark. UV-Vis spectrophotometer (Evolution 201) equipped with INSIGHT™ software (Thermo Scientific™, United States) was used to observe a decrease in absorbance at 740 nm in test samples against a blank prepared without any added oil. Gallic acid was used as a standard, and the calibration curve was plotted using different concentrations of gallic acid

(0.01-1 mg/mL) in methanol. After observing the absorbance at 740 nm, the concentration of phenolics (expressed as µg GAE/ 100 µL) was determined from the calibration curve.

3.2.3. Determination of antioxidant potential of oils

3.2.3.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of oils

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of all the oils (mānuka, kānuka and rosemary) was determined by using the method of Torres-Martínez et al. (2018) with some modifications. Firstly, 100 µL of the essential oil (at different concentrations of 0.1, 1 and 100 % (pure oil) in methanol was mixed with 2 mL of DPPH solution (0.5 mmol/L). The prepared mixture was shaken vigorously and left in the dark for 20 min for incubation at room temperature. The absorbance was measured against blank at 515 nm using a UV-Vis spectrophotometer (Evolution 201) equipped with INSIGHT™ software (Thermo Scientific™, United States).

3.2.3.2. FRAP radical scavenging activity of oils

The FRAP assay was also performed using the method of Torres-Martínez et al. (2018) with modifications. The oil samples (mānuka, kānuka and rosemary) were dissolved in methanol (at different concentrations of 0.1, 1 and 100 % (pure oil)), and 250 µL of this mixture was mixed with 1.25 mL of each 50 mM sodium phosphate buffer (pH 7) and 1 % potassium ferricyanide solution. After incubating for 20 min at 50 °C in the dark, 1.25 mL of 10 % trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm (1107 × g) for 10 min. The supernatant obtained after centrifugation was mixed with 2.5 mL of 0.1 % ferric chloride. Then, absorbance was read at 700 nm with a UV-Vis spectrophotometer (Evolution 201) equipped with INSIGHT™ software (Thermo Scientific™, United States). Gallic acid was used as standard control at a concentration ranging from 0-300 mg/L. The results were expressed in terms of µg gallic acid equivalents per mL of oil.

3.2.4. Determination of antimicrobial potential of oils

3.2.4.1. Bacterial strains

Four bacteria, *Listeria monocytogenes* (NZRM 4230), *Staphylococcus aureus* (ATCC 25923, NZRM 917), *Escherichia coli* (ATCC 25922, NZRM 916) and *Salmonella* spp. (NZRM 4030) were tested in this study. The powdered Nutrient, Tryptic-Soy and Mueller Hinton broths were obtained from Sigma Aldrich (Saint Louis, MO, USA). All broths were prepared and sterilised before use. The selective agar plates, i.e., brilliant green modified agar for *Salmonella* spp., eosin methylene blue (EMB) for *Escherichia coli*, oxford agar for *Listeria monocytogenes* and Baird Parker agar plates for *Staphylococcus aureus*, and Mueller Hinton agar plates were purchased from Fort Richard (New Zealand). *Escherichia coli* and *Salmonella* were grown and maintained in nutrient broth, and *Listeria monocytogenes* and *Staphylococcus aureus* were grown in tryptic soy broth (TSB) for 24 hours at 37 °C.

3.2.4.2. Disc diffusion assay

The disc diffusion assay of essential oils diluted in 0.01 % dimethyl sulfoxide (DMSO) (essential oil at 5 % concentration) was performed according to the method described by Jeong et al. (2018) with some modifications. We checked that DMSO at this concentration had no antibacterial effect against all tested microbes (data not shown). Firstly, the overnight grown bacterial cultures were adjusted to 0.5 McFarland turbidity standard (around 10^8 cfu/mL), and the bacterial suspension was uniformly swabbed on Mueller Hinton agar plates using sterile cotton swabs. The sterilised paper discs (about 6 mm diameter) were placed in the centre of inoculated Mueller Hinton agar plates, and 40 µL of the prepared oil samples were added to the discs. Negative controls (with DMSO added onto the discs) for each tested microbe were also used. The prepared agar plates were incubated at 37 °C for 24 hours, and antimicrobial efficacy was determined by gauging the diameter of the zone of inhibition (in millimetres)

around the discs. Inhibition zones were measured in horizontal and vertical directions at four different places, and then the average diameter was noted. Each oil was tested in three different replicates.

3.2.4.3. Minimum inhibitory concentration (MIC) determination

Microplate turbidimetric growth inhibition assay was performed according to the method described by Pahalagedara et al. (2020) to determine the minimum concentration of oils required to inhibit bacterial growth. In brief, the oil sample (50 µL) diluted in DMSO (0.01 %) was added to the 96-well microlitre plates (Thermo Fischer Scientific, Denmark) containing 50 µL of Mueller-Hinton broth (MHB). The overnight grown bacterial culture was diluted, and 100 µL of this suspension was added to each well containing approximately 10^5 cfu/mL cells. As shown in supplementary Figure 3.1, 96-well microlitre plates containing the final concentration of essential oils in each well 5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.08, and 0.04 % were prepared. The plates were incubated in a microplate spectrophotometer (multiskan GO, Thermo Fischer Scientific, United States) at 37 °C, and optical density was measured at 595 nm wavelength for 24 hours. An increase in optical density (turbidity) indicates bacterial growth in that well. The background correction was performed using the appropriate blanks (growth medium and oil).

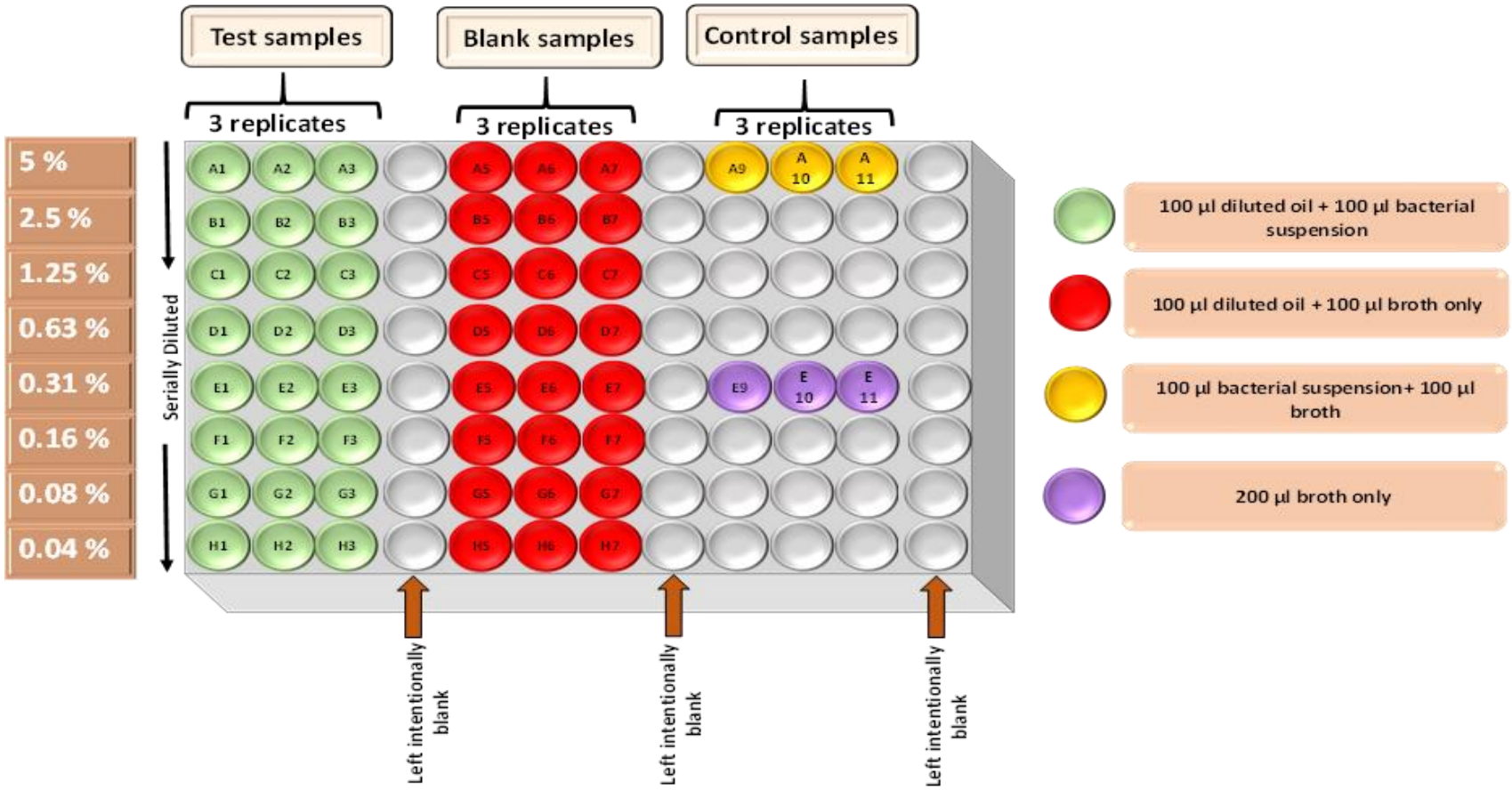


Figure 3.1. The layout of the 96-well plate experiment was used for the broth dilution method.

The plates were covered with a Breathe-Easy® sealing membrane (Diversified Biotech, United States) to prevent loss of the volatiles and provide aerobic conditions to the bacteria. A control (untreated) containing only microbial suspension in the MHB was also prepared.

3.2.5. Statistical analysis

Statistical evaluation was performed using a general linear model in Minitab Version 19.2020.2.0 (Minitab Inc., State College, PA, USA) to compare the values among different essential oils. One-way analysis of variance (ANOVA), followed by the *Tukey* method analysis at a 95 % confidence interval, was done to determine the significant difference ($p \leq 0.05$) between treatments. Each experiment was carried out on three different replicates.

3.3. Results and Discussion

3.3.1. Chemical composition of essential oils

The GCMS analysis results displayed that α-pinene, β-pinene, calamene, α -terpinene and α-terpineol are the common chemical constituents present in all mānuka, kānuka and rosemary oils. However, in mānuka oils, the concentration of other compounds decreased as the concentration of triketones increased. The highest concentration of triketones, i.e., flavesone, leptospermone, and isoleptospermone, were found in mānuka oil 3. The GCMS results showed that the primary compound in rosemary oil was 1,8 cineole and α-pinene. Similarly, in kānuka oil, α-pinene was the predominant monoterpene in the highest concentrations, comprising about 60 % of the composition (Table 3.1). The significant difference in mānuka and kānuka oil composition was the highest level of alpha-pinene and absence of the triketones in the latter oil than the former oil, as represented in Figure 3.2 (a and b). The moderate presence of sesquiterpenes such as calamenene, viridiflorene, ledol and viridiflorol was also found.

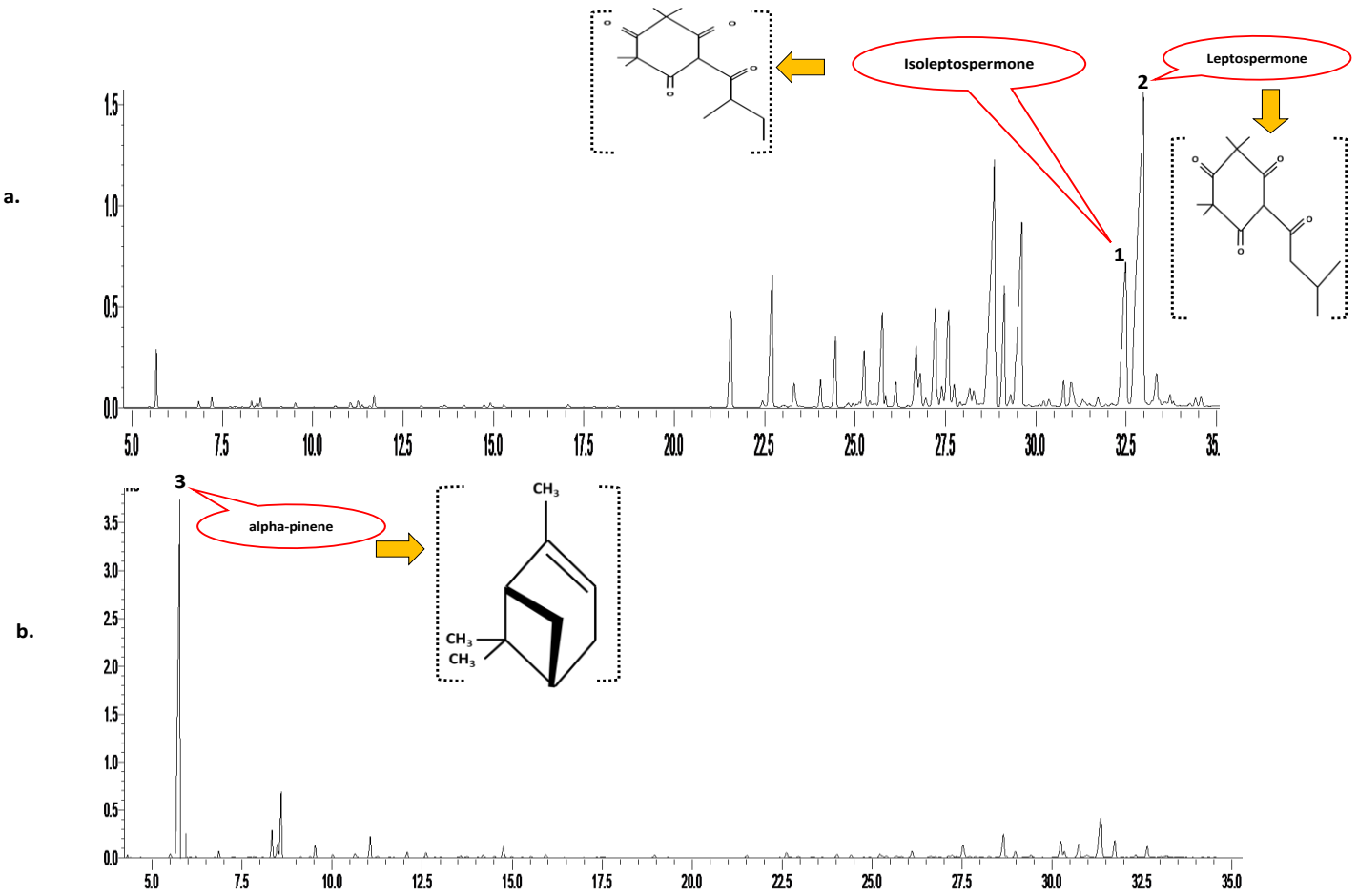


Figure 3.2. Gas chromatography-mass spectrometric (GC-MS) analysis of the (a) mānuka and (b) kānuka oils.

Peaks 1 and 2 in Figure 2 a. represent compounds isoleptospermone and leptospermone in mānuka oil 3, respectively. Peak 3 in Figure 2 b. represents the presence of compound alpha-pinene as a major compound in kānuka oil.

Table 3.1. The main constituents in mānuka, kānuka and rosemary oil were identified through gas chromatography and mass spectrometry analysis.

Chemical compound name	Mānuka oil 1 (Area %)	Mānuka oil 2 (Area %)	Mānuka oil 3 (Area %)	Rosemary oil (Area %)	Kānuka oil (Area %)
α-Pinene	1.32	1.19	0.92	19.09	64.25
β-Pinene	0.37	0.17	0.15	5.31	0.71
β -Myrcene	0.35	0.3	0.27	3.14	
Γ-Terpinene	0.44	0.15	0.14	2.34	1.52
Limonene	0.7	0.12	0.1	0.2	1.79
Linalool				2.91	2.76
α-Terpinol	0.21	0.2	0.22		
p- Cymene	0.44	0.08	0.15		3.28
1,8 Cineole	0.35	0.23	0.22	50.75	6.6
β -Cryophyllene	3.7	2.56	2.4	6.46	
Aromadendrene	2.51	2.24	1.85		
α-Gurjuene	0.32	0.26	0.24		
δ-Cardinene	8.26	5.49	6.1		
Alloaromadendrene	1.16	0.96	0.81		

Cardia- 3,9 diene	4.15	4.97	4.67	
B-Elemene	0.4	0.39	0.38	
α-Farnesene	1.75	2.2	2.04	
α-Cedrene	6.05	4.39	4.22	
Calamene or Cardia 1,4 dinene	16.42	13.23	11.01	3.78
Flavesone	1.89	6.26	8.57	
γ-Elemene	1.73	1.33	1.28	
β-Selinene	5.26	0.08	0.92	
α-Cubebene	4.88	3.59	3.41	
Ylangene	0.36	0.3	0.26	
Copaene	6.01	4.88	4.19	
Cubenol	0.65	0.17	0.13	
Globulul	0.49	0.28	0.22	
Leptospermone	1.09	5.19	4.89	
Isoleptospermone	3.89	12.37	18.39	
Verdiflorol	2.61	2.41	1.94	8
Spanthul	1.23	1.24	1.11	2.53

Grandiflorone	0.23	0.56	
Aliphatic ester	0.23	0.18	
n-Amyl isovalerate	0.1	0.3	
α-Thujene			0.68
Nerolidol			2.53

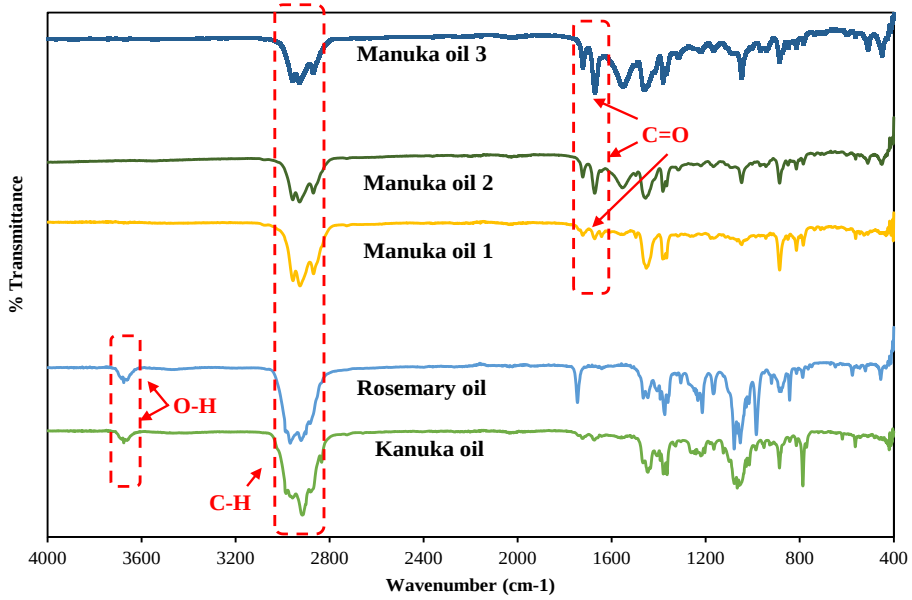
The results on the mānuka oil composition of this study agree with the study of Perry et al. (1997), who examined the effect of different geographical variations on the mānuka oil composition and reported that the highest triketones are found in the East cape and Marlborough Sounds region of New Zealand. Maddocks (2021) also reported that all samples of kānuka oil from different locations in New Zealand contained alpha-pinene at a minimum of 60 % of their volume. The chemical composition of rosemary oil has already been reported by the study of Jiang et al. (2011), in which 1,8 cineole was the primary compound, followed by the alpha-pinene, camphor and camphene.

3.3.2. FTIR analysis of essential oils

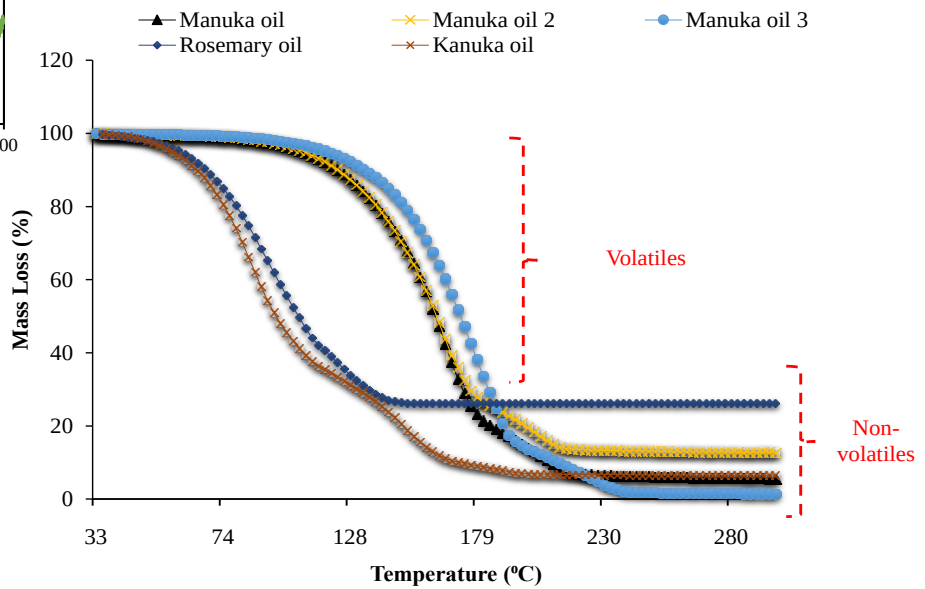
The FTIR spectra of mānuka (1, 2 and 3), kānuka and rosemary oils obtained in the IR region of 400 to 4000 cm^{-1} are shown in Figure 3.3. Each essential oil showed three intense peaks between 2800-3000, possibly due to the C-H stretching vibration of aliphatic CH_2 bonds in essential oil molecules (Kim et al., 2022). However, due to H-bonded O-H group stretching, an intense peak between 3640 and 3720 cm^{-1} was observed in the spectra of kānuka and rosemary oil. It shows the presence of alcoholic compounds, like 1,8 cineole, linalool and terpinol, which are higher in kānuka and rosemary than all mānuka oils (Kinninmonth et al., 2013). In kānuka oil spectra, an intense peak was observed at 885 cm due to the carbonyl (CH and CH_2) groups bending, which may be due to the presence of α-pinene, the major compound present in the oil. Mānuka and rosemary oils also exhibited this peak but were sustainably lower than the kānuka oil because alpha-pinene is present in lower quantities in the former oils (Kinninmonth et al., 2013). The presence of these chemical compounds was also confirmed by gas chromatography-mass spectrometry (GCMS).

In the present study, due to the solid stretching vibration of the carbonyl group, a characteristic absorption band between 1690-1720 was noticed in the spectrum of all mānuka oils. This peak

could be identified with C=O stretching and attributed to the presence of β-triketones, including leptospermone, isoleptospermone, flavesone and grandiflorone (Liu et al., 2021). Liu et al. (2021) and Kim et al. (2022) also showed the presence of aliphatic and conjugated triketones bands between 1724 and 1674 cm^{-1} in mānuka oil.



1 (a)



1 (b)

Figure 3.3. Fourier-transform infrared (FTIR) spectrum (a) and thermogravimetric analysis (b) of mānuka 1 (5 % triketones), 2 (25 % triketones), 3 (40 % triketones), kānuka and rosemary oils.

3.3.3. Thermogravimetric analysis of essential oils

The thermogravimetric weight loss of essential oil samples with an increase in temperature is shown in Figure 3.3. In the case of kānuka and rosemary oils, major weight loss was observed between 50 and 150 °C. However, the mānuka oils were more thermostable and started losing weight after 100 °C. In the present study, the ash content of essential oils follows the order of rosemary oil > mānuka oil 2 > kānuka oil > mānuka oil 1 > mānuka oil 3. Interestingly, only mānuka oil 3 achieved the baseline (decreased to zero), while other oils showed constant weight. Herculano et al. (2015) reported that ash content could be related to the formation of heat-labile complexes between different bioactive compounds of the essential oils, which would not decompose below 300 °C. The results on the thermostability of essential oils agree with the study of Riabov et al. (2020).

The higher weight loss of rosemary and kānuka oils could be attributed to the volatilisation/decomposition of the bioactive compounds in essential oils, such as phenolic diterpenes (Riabov et al., 2020). Chambre et al. (2020) reported that the thermostability of essential oils could be related to their chemical composition. From the GCMS results, it can be noticed that kānuka oil contains around 50-60 % of the monoterpenes, i.e., α-pinene and beta-pinene. The boiling point of alpha-pinene and beta-pinene under normal pressure is 155 °C and 165 °C, respectively. Thereby, kānuka oil lost a major part of its weight between 100-150 °C. The TGA curves of mānuka oil showed that it might be a thermostable antimicrobial agent and can have the potential to be used in cooked meat products.

3.3.4. Total phenolic content of the essential oils

The total phenolic contents (TPC) of mānuka (with different triketone contents- 5, 25 and 40 %), kānuka and rosemary oils are illustrated in Figure 3.4 (a). Amongst all the tested oils, mānuka oil containing the lowest triketone content (5 %) showed the highest TPC. The

phenolic content decreased as the triketone content of the mānuka oils increased. The mānuka oil having the highest triketone content (25 %), exhibited the lowest total phenolic content but was not lower than the rosemary oil. The phenolic content and composition of rosemary oil have been widely studied (Viuda-Martos et al., 2010; Kaur et al., 2021). The values for total phenolic content for rosemary oil reported in the current study are similar to those reported previously (Viuda-Martos et al., 2010).

3.3.5. Antioxidant potential of the essential oils

The antioxidant potential of the oil samples was evaluated by determining their DPPH and FRAP radical scavenging activities, as shown in Figures 3.4 (b and c). The antioxidant assays measure the ability of antioxidants to quench free radicals by single electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms. The DPPH method uses both mechanisms as radicals are scavenged by either SET or HAT, while FRAP is SET-based (Liang & Kitts, 2014).

The results of the DPPH test exhibited that essential oils had a potent concentration-dependent DPPH radical scavenging activity (Figure 3.4 (b)). The higher the test compound concentration higher the activity. Rosemary oil had lower DPPH radical scavenging activity than the other tested samples at all tested concentrations, and kānuka oil possessed lower DPPH activity than the BHT and mānuka oils at all tested concentrations. However, BHT showed significantly higher ($p \leq 0.05$) DPPH radical scavenging activity than the oils at its highest concentration. At all the other tested concentrations, mānuka oils, particularly mānuka oil containing 40 % triketone content, showed better DPPH radical scavenging activity than all other tested samples. No significant effect ($p \leq 0.05$) of the triketone content was observed on the DPPH radical scavenging activity at the lowest tested concentration (0.1 %) of mānuka oils. Various types of research in the literature have reported a positive linear correlation between the total phenolic content and antioxidant activity of plant essential oils (Sethi et al., 2020). It has also

been reported that antioxidant activity is due to the synergistic effect of major and minor components, including phenolic and non-phenolic compounds present in essential oils (Bassolé & Juliani, 2012). The essential oils may exhibit stronger antioxidant activity than their individual isolated compounds (Bassolé & Juliani, 2012). Moreover, essential oils may contain phenolic and conjugated double groups associated with antioxidant potential (Bassolé & Juliani, 2012). Kwon et al. (2013) reported that the antioxidant activity of mānuka oil is due to sesquiterpene compounds. However, when individual components in the mānuka oil were tested for their antioxidant potential, only γ-terpinene and terpinen-4-ol showed antioxidant activity (Lis-Balchin, 2006). No literature evidence has been found on the antioxidant potential of triketones such as leptospermone.

The FRAP assay measures the antioxidant potential by measuring the degree of residual Fe^{2+} produced after reducing Fe^{3+} (Liang & Kitts, 2014). Among all tested oils, mānuka oil with the lowest triketone content had the highest FRAP values across all concentrations (Figure 3.4 (c)). The possible reason could be the presence of higher amounts of phenols and flavonoids in oils with lower triketone contents, which could have contributed to an enhanced FRAP activity. In our results, this observation is contrary to the observation made for the DPPH assay, especially at the lowest (0.1 %) and highest concentrations (100 %, pure oils). The possible explanation could be that some phenolic compounds may act as prooxidants at some concentrations; for instance, eugenol may act as an antioxidant at low concentrations; however, it may act as a prooxidant at high concentrations (Bezerra et al., 2017). Another reason could be that DPPH radicals can react with other radicals, and consequently, the time to reach the stable state is not linear to the concentration ratio of the antioxidant, which is one of the limitations of the DPPH assay mentioned by Santos-Sánchez et al. (2019).

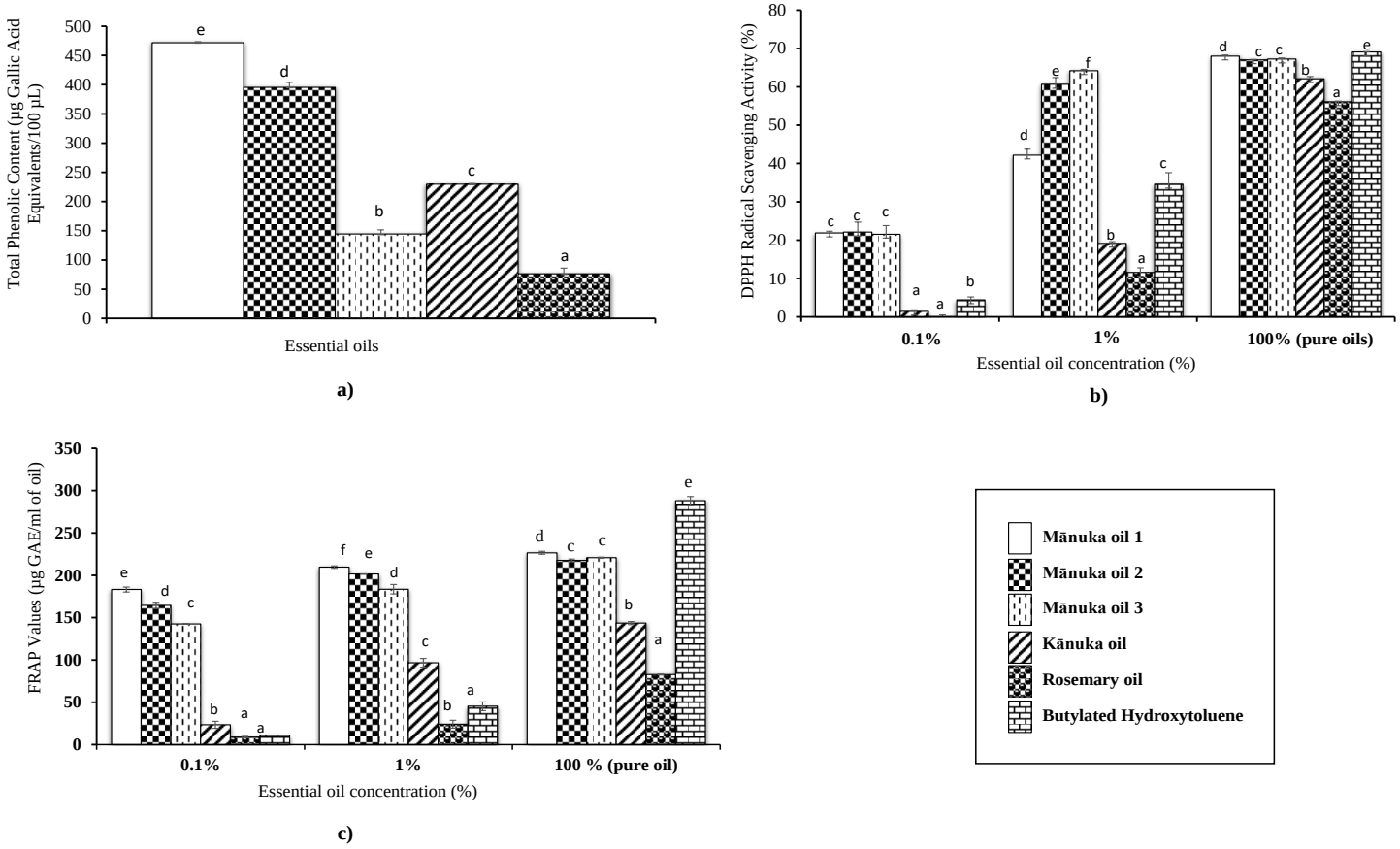


Figure 3.4. Total phenolic contents and antioxidant effects of mānuka (1 (5 % triketones), 2 (25 % triketones), 3 (40 % triketones)), kānuka and rosemary oils a) total phenolic content, b) DPPH radical scavenging activity, and c) FRAP radical scavenging activity.

Lowercase letters represent the statistically significant difference between the samples at the same concentration ($p \leq 0.05$).

Similar to the observation for the DPPH assay, at the highest concentration, BHT exhibited the highest FRAP values than any of the other samples. However, the values dropped drastically with a decrease in its concentration (Figure 3.4 (c)). The decrease in FRAP values with a decrease in concentration was observed to be lowest for mānuka oils, suggesting their suitability as an antioxidant agent at even low concentrations. The kānuka oil exerted lower antioxidant activity than mānuka oils in both assays at all tested concentrations. The higher antioxidant potential of mānuka oil than that of kānuka oil has been reported previously (Lis-Balchin & Hart, 1998; Lis-Balchin, 2006). In these studies, the mānuka oil exerted a more consistent antioxidant effect on mice's skin than tea tree (*Melaleuca alternifolia*) or kānuka oils. These studies tested the individual components in mānuka oil for their antioxidant potential on mice's skin against photoaging. The results showed that γ-terpinene and terpinen-4-ol exhibited antioxidant activities, and the antioxidant potential of mānuka oil was greater than that of the kānuka and Australian tea tree oil (*Melaleuca alternifolia*) (Lis-Balchin, 2006).

3.3.6. In vitro antimicrobial potential of the essential oils

It can be observed from the disc diffusion assay results that the antimicrobial effect of mānuka oils was significantly higher ($p \leq 0.05$) than the kānuka oil but lower than the rosemary oil against selected Gram-negative bacteria (*Salmonella* and *Escherichia coli*) (Table 3.2). Rosemary oil was more effective against *Salmonella* and *Escherichia coli* and had the highest value of inhibition zone diameter than other oils. Table 3.2 shows no statistically significant effect of the triketone increase against chosen Gram-negative bacteria was observed. On the other hand, Gram-positive microbes (*Listeria monocytogenes* and *Staphylococcus aureus*) were more sensitive to all mānuka oils and thereby had a higher value of inhibition zone than other oils treatments (Table 3.2).

Interestingly, a significant increase ($p \leq 0.05$) in the inhibition zone was observed with increased triketone content of mānuka oils. The mānuka oil 3 (containing the highest triketone content (40 %), produced the largest inhibition zone diameter against both tested Gram-positive bacteria.

Table 3.2. The inhibition zone values observed for mānuka, kānuka and rosemary oils against different microorganisms.

Oil type	**Zone of Inhibition (mm)**			
	Listeria monocytogenes	***Staphylococcus aureus***	***Escherichia coli***	***Salmonella* spp.**
Rosemary oil	6.9 ± 0.5^{e}	6.2 ± 0.3^{e}	17.5 ± 0.5^{a}	18.27 ± 1.6^{a}
Kānuka oil	9.83 ± 0.2^{d}	9.7 ± 0.5^{d}	11.43 ± 0.5^{c}	14.17 ± 0.7^{b}
Mānuka oil 1	17.53 ± 0.5^{c}	11.38 ± 0.3^{c}	11.27 ± 1.1^{c}	14.2 ± 0.7^{b}
Mānuka oil 2	23.5 ± 0.5^{b}	16.01 ± 0.1^{b}	14.06 ± 1.0^{b}	15.1 ± 0.8^{b}
Mānuka oil 3	26 ± 0.2^{a}	17.69 ± 0.4^{a}	14.27 ± 1.1^{b}	15.3 ± 0.5^{b}

Different superscripts within a column represent a statistically significant difference ($p \leq 0.05$).

Figures 3.5, 3.6, 3.7 and 3.8 show the antimicrobial effect of mānuka oil, kānuka and rosemary oils (concentration from 0.04 to 5 % and time from 0 to 24 hours) against *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* and *Escherichia coli* in broths. Consistent with the disc diffusion assay results, the broth dilution assay showed that all types of MOs showed a strong antimicrobial effect against tested Gram-positive bacteria than the rosemary and kānuka oils (Figures 3.5 and 3.6).

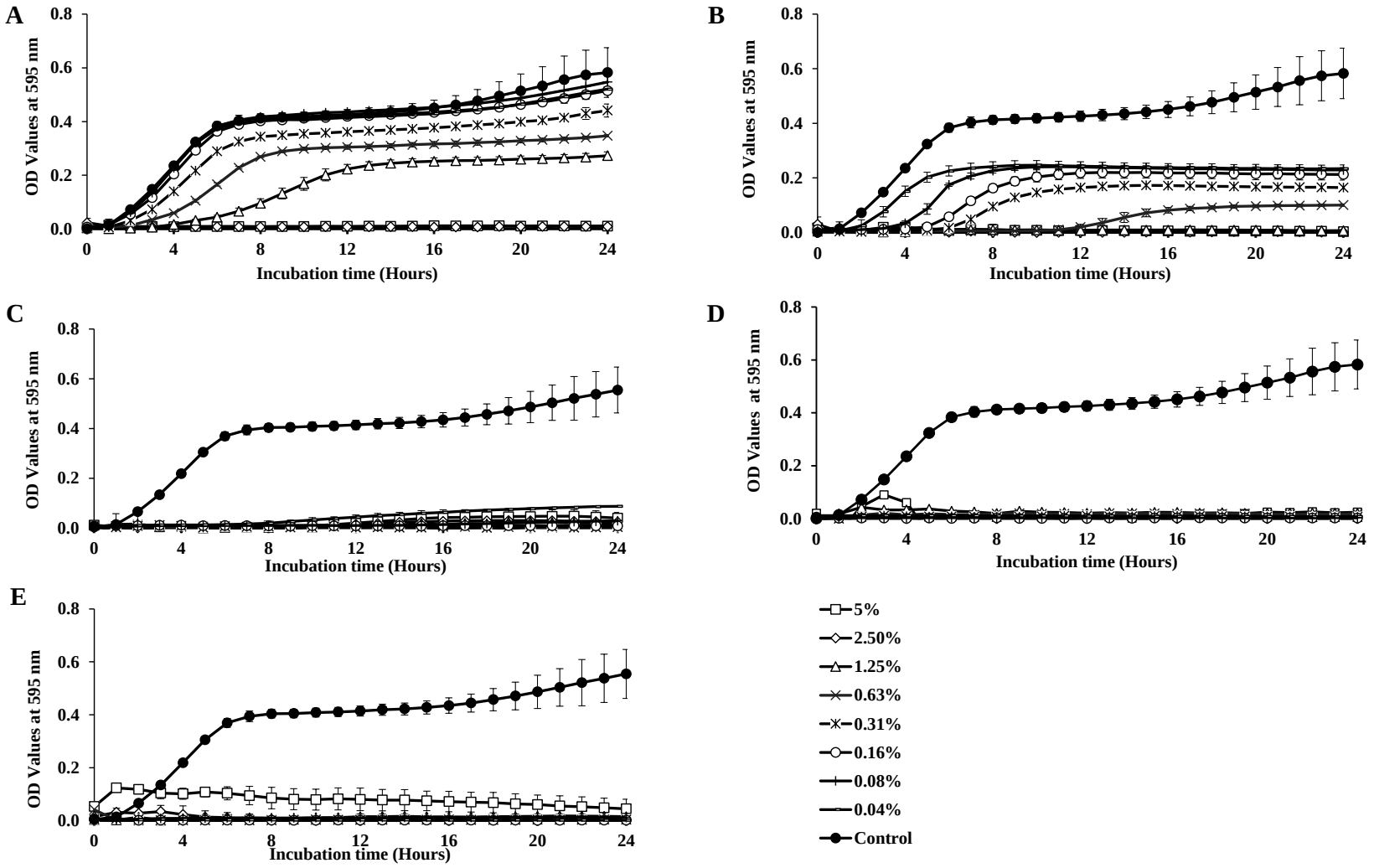


Figure 3.5. *Staphylococcus aureus* growth curves for A) rosemary oil; B) kānuka oil; C) mānuka oil 1 (5 % triketone content); D) mānuka oil 2 (25 % triketone content); and E) mānuka oil 3 (40 % triketone content) at different concentrations.
The control sample contained no oil but the test organism, *Staphylococcus aureus.*

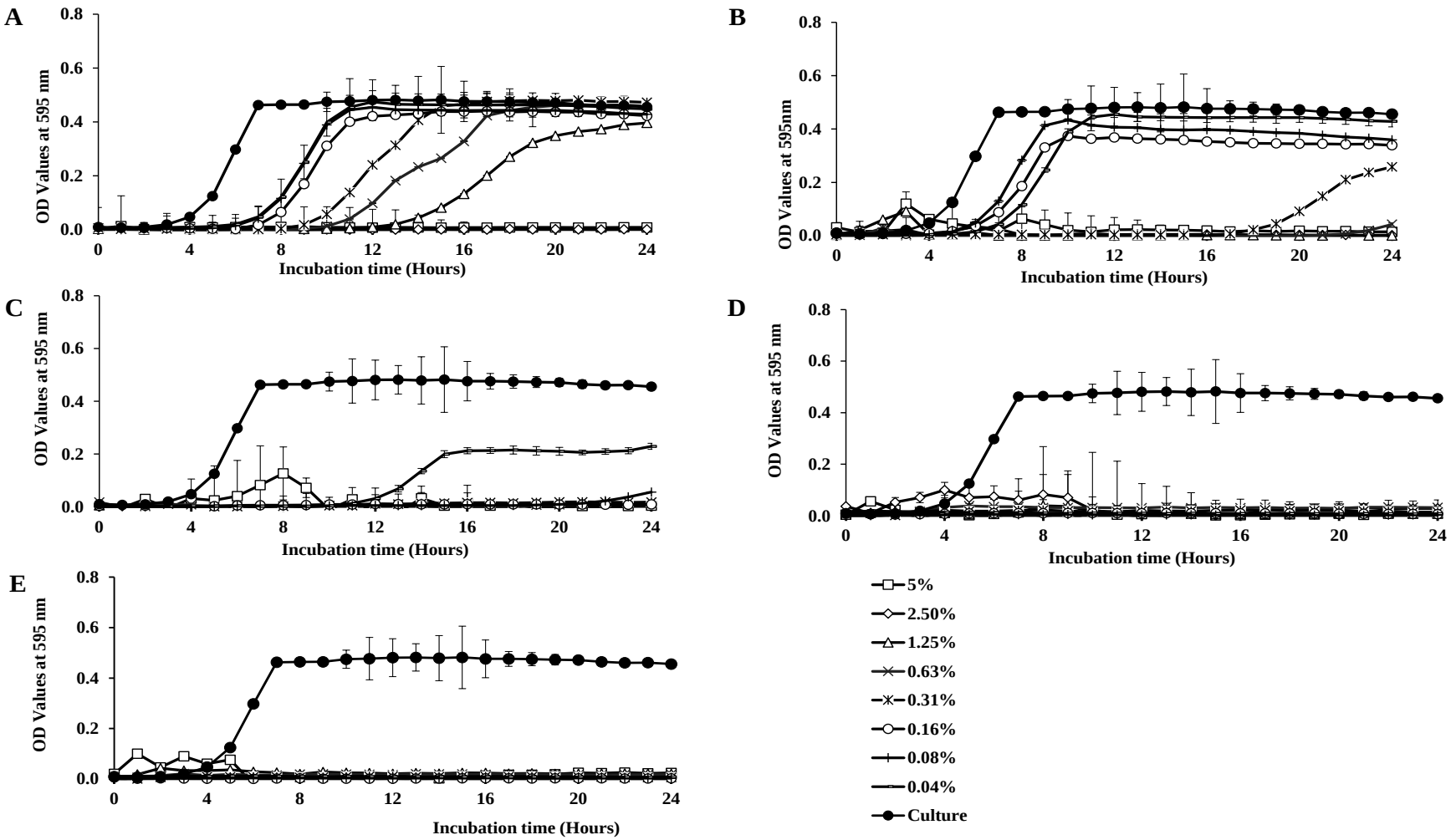


Figure 3.6. *Listeria monocytogenes* growth curves for a) rosemary oil; b) kānuka oil; c) mānuka oil 1 (5 % triketone content); d) mānuka oil 2 (25 % triketone content); and e) mānuka oil 3 (40 % triketone content) at different concentrations.
The control sample contained no oil but the test organism *Listeria monocytogenes.*

For *Listeria monocytogenes* and *Staphylococcus aureus*, at least 0.16 % concentration of mānuka oil 1 was reported as MIC value. In mānuka oils 2 and 3 (having 25 and 40 % triketone contents, respectively), no microbial growth of both microbes was observed even at the lowest tested concentration (0.04 %) (plate counting was done for this analysis, but data is not shown). This indicates that concentrations lower than 0.04 % of mānuka oil 2 and 3 can inhibit selected Gram-positive microbes' growth. In contrast, a higher concentration (around 2.5 %) of mānuka oils was required to inhibit Gram-negative microbes, *Escherichia coli* and *Salmonella,* than the rosemary oil. The MIC value obtained for the rosemary oil was 2.5 % (v/v) for all tested bacteria (Figures 3.5, 3.6, 3.7 and 3.8). The antimicrobial effect of kānuka oil was less than mānuka oil and rosemary oil. For kānuka oil, 0.63 and 2.5 % were the recorded MIC values against tested Gram-positive and Gram-negative microbes, respectively. As per the available literature, monoterpene, especially alpha-pinene, can be responsible for the antimicrobial effect of kānuka oil (Porter & Wilkins, 1999). However, the antimicrobial potential of mānuka oil could be attributed to the presence of β-triketones, including leptospermone, flavesone, and iso-leptospermone (Chen et al., 2016).

Similar to our results, Klink et al. (2005) documented that different triketones isolated from mānuka oil were ineffective in inhibiting Gram-negative *Pseudomonas aeruginosa*. The possible explanation could be that the outer membrane of Gram-negative bacteria, made up of phospholipids, lipoproteins, lipopolysaccharides etc., may serve as a barrier, so triketones may not penetrate the cell membrane and pose any antimicrobial effect (Klink et al., 2005). Harkenthal et al. (1999) have reported that the antimicrobial effect of mānuka oil was higher than that of the Australian tea tree oil, with MIC values of 0.12 % against *Staphylococcus aureus*.

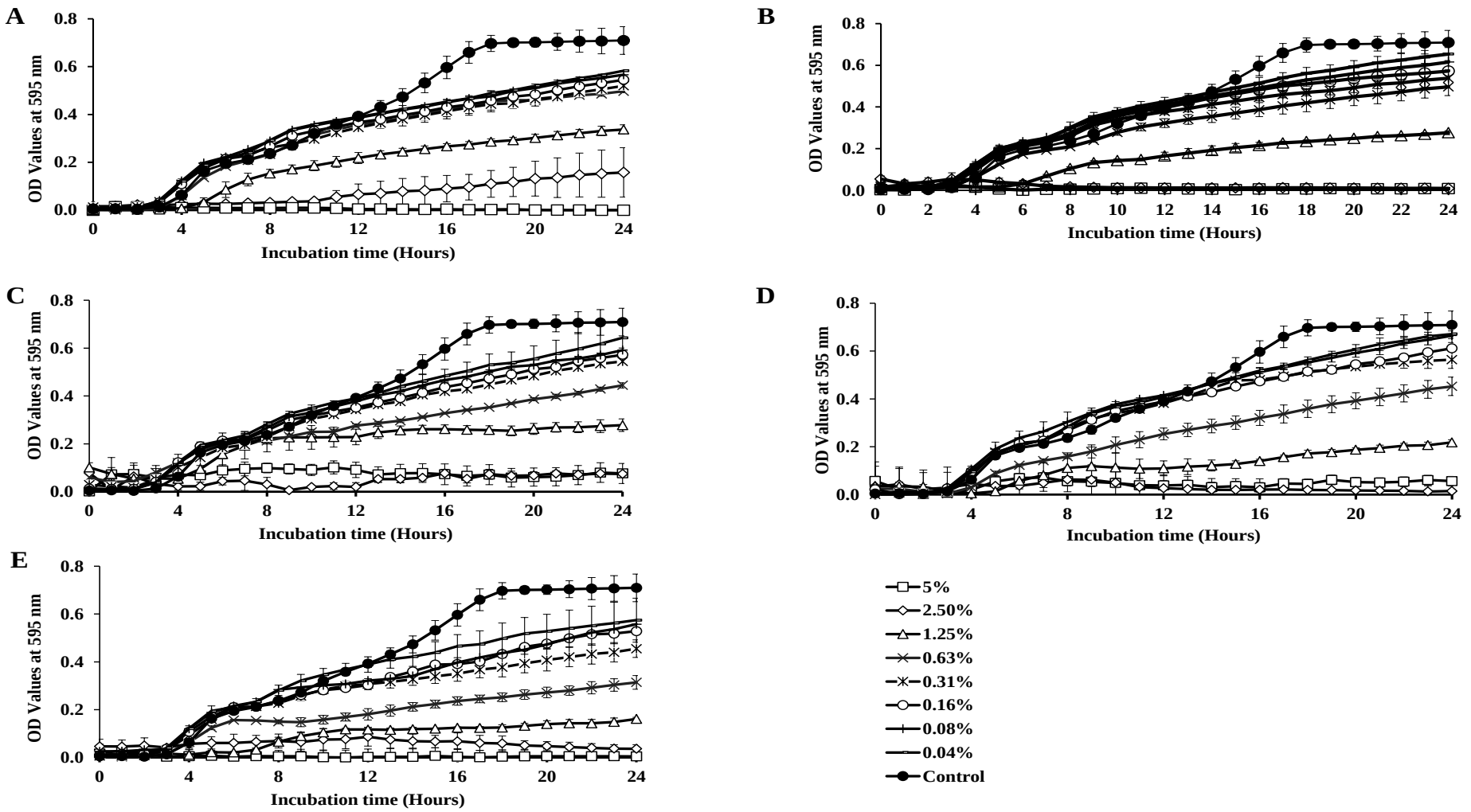


Figure 3.7. *Salmonella* spp. growth curves for A) rosemary oil; B) kānuka oil; C) mānuka oil 1 (5 % triketone content); D) mānuka oil 2 (25 % triketone content); and E) mānuka oil 3 (40 % triketone content) at different concentrations.
The control sample contained no oil but the test organism *Salmonella* spp.

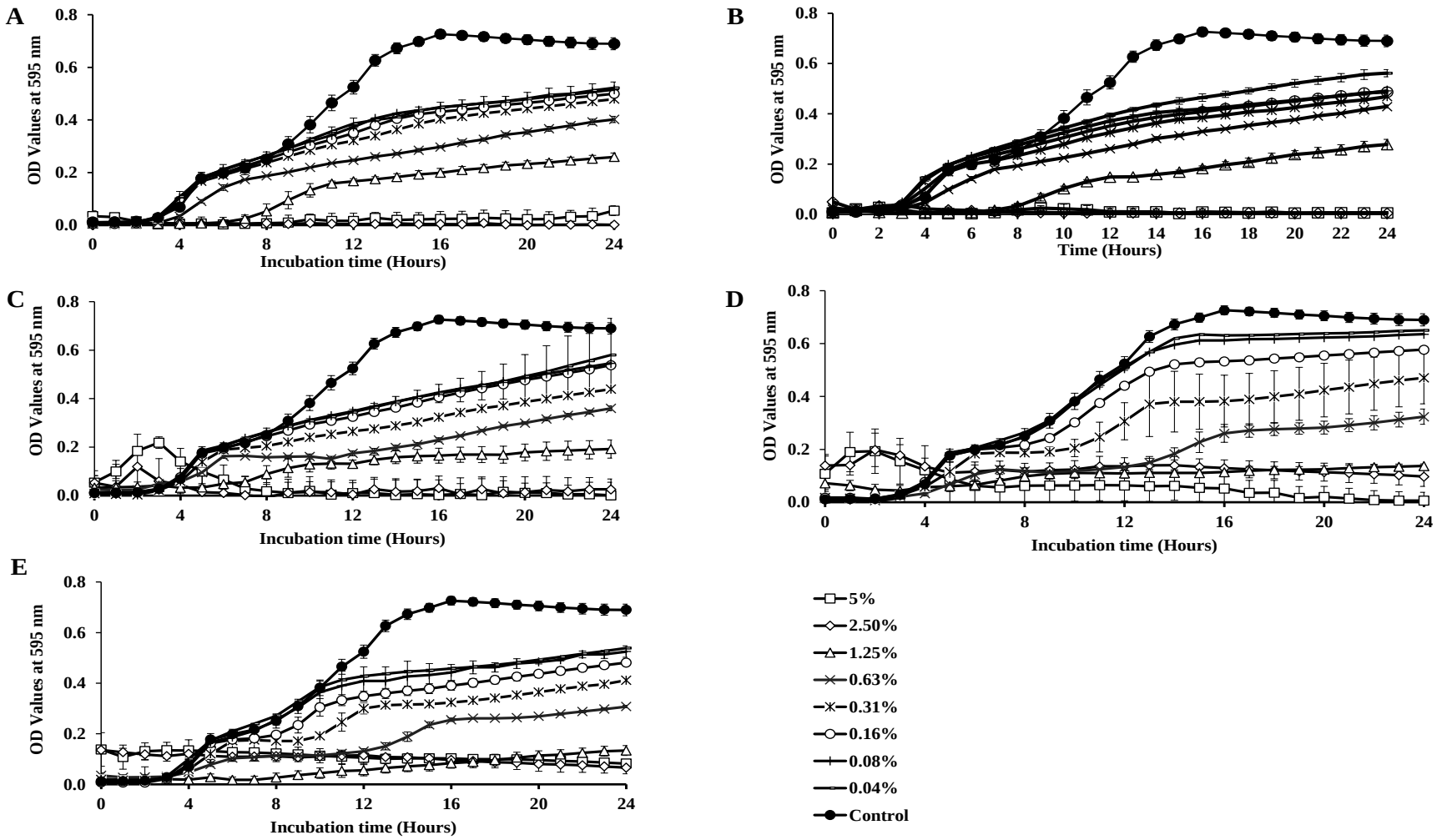


Figure 3.8. *Escherichia coli* growth curves for a) rosemary oil; b) kānuka oil; c) mānuka oil 1 (5 % triketone content); d) mānuka oil 2 (25 % triketone content); and e) mānuka oil 3 (40 % triketone content) at different concentrations.
The control sample contained no oil but the test organism *Escherichia coli.*

Several studies have reported a varying MIC of rosemary oil against Gram-negative bacteria. Barbosa et al. (2016) reported MIC values of 0.005 % (5 µL/mL) for rosemary oil against *Escherichia coli* and 0.010 (10 µL/mL) against *Salmonella* Enteritidis. It is essential to mention that the above-mentioned studies have used different methodologies and microbial strains, which could explain the variations in the reported MIC values for the rosemary oil.

3.4. Conclusions

In conclusion, the results of the present study exhibit that mānuka oils possessed higher antioxidant activity than rosemary or kānuka oils in all the antioxidant assays performed. A concentration-dependent increase in the antioxidant efficacy of essential oils and BHT was observed in these free radical scavenging assays.

The antimicrobial results of the study revealed that all mānuka oils (triketone contents 5, 25, and 40%) exerted better antimicrobial activities than kānuka oil against Gram-negative and Gram-positive microbes, as exhibited by the *in vitro* results. As per the disc diffusion and broth dilution assay results, *Listeria monocytogenes* and *Staphylococcus aureus* exhibited more sensitivity to mānuka oils than tested Gram-negative microbes. Increased antimicrobial efficacies were observed with increased triketone content of mānuka oils against selected Gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*). However, rosemary oil inhibited *Salmonella* and *Escherichia coli* more effectively than the kānuka and mānuka oils.

The results point toward the potential of mānuka oil as a green and natural preservative for completely replacing synthetic antioxidant agents, such as BHT, in food systems. In addition, mānuka oil can be preferred for use in food products as a natural antimicrobial agent against *Listeria monocytogenes* and *Staphylococcus aureus* at low concentrations when compared to rosemary oil, while rosemary oil can be used against *Salmonella* and *Escherichia*

coli (at high concentrations). However, the effect of essential oils can be influenced by food constituents like fat and water, which can affect the partitioning of their bioactive compounds into food systems. The partitioning of these oils and their bioactive compounds can be studied by determining their octanol-water partition coefficient, which is commonly used to measure the lipophilicity of the chemical constituents and explain their partition between cell membranes and water. This has been discussed in Chapter 4.

Chapter 4 Partitioning of essential oil compounds in beef fat and water: using an octanol-water partition coefficient approach

Abstract

Plant essential oils have gained widespread interest as natural preservatives for food applications, while the fat content of the food matrix can significantly reduce their effect. Essential oil compounds added to foods will partition between the different phases, so lipophilic compounds may be absorbed into the fat components. The octanol-water partition coefficient is commonly used to characterise the lipophilicity of volatiles. Octanol is used for approximate membranes, and the partition coefficient is used to evaluate toxic effects on cellular systems. The portioning between water, octanol and air can be potentially used to explain the effectiveness of essential oils as antimicrobial agents in foods of different compositions. There is a large amount of research on octanol-water partition coefficients, but the concept is rarely applied to antimicrobial systems applied to foods. This study determined the octanol-water partition coefficient (log *P*) of mānuka oil beta-triketones (leptospermone, isoleptospermone, and flavesone), α-pinene and γ-terpinene experimentally using the shake flask method and through prediction using the EPI software. Further, the concentration of these compounds partitioned in water from beef fat (3 and 12 %) was evaluated. All compounds showed a lipophilic nature and log *P* values ≥2.5. Higher concentrations of these compounds were found in water from the low-fat than in the high-fat systems. The results suggest that the tested compounds are inclined to accumulate in the fatty phase of food than the water phase, limiting their effectiveness in high-fat foods.

4.1. Introduction

Plant essential oils have acquired significant scientific interest as a natural preservative for food applications. The essential oils are a complex mixture of various bioactive compounds, including phenolic, monoterpenes, sesquiterpenes, oxygenated sesquiterpenes and monoterpenes (Tongnuanchan & Benjakul, 2014; Hanif et al., 2019). These bioactive compounds have been reported for their wide range of antimicrobial and antioxidant properties in the literature (Christaki et al., 2021; Kaur et al., 2021). However, the chemical nature (hydrophobicity and volatility) of bioactive compounds, environmental conditions (relative humidity and temperature), and food composition and structure are the essential factors which can influence the activity of these bioactive compounds (Wang et al., 2020).

The release and retention of these compounds in foods under equilibrium conditions depend on their partitioning between the different phases of the food. This partitioning characterises the lipophilicity/hydrophilicity, i.e., solubility in fats/oils and the aqueous phase (Landy et al., 1996; Malone et al., 2000; Shojaei et al., 2007). An octanol-water partition coefficient (K_{ow}) measures the hydrophilicity or hydrophobicity of the chemical compound and is extensively utilised to explain how chemical compounds partition between cell membranes and water. Because the magnitude of K_{ow} can vary over many orders of magnitude, it is usually reported as log (K_{ow}) or log P. Bioactive compounds with log P (K_{ow}) higher than 1 are hydrophobic and thus have more absorption in the cell membrane than in water (Cumming & Rücker, 2017)

The octanol-water partition coefficient is commonly used to approximate absorption into cellular systems or tissue to evaluate likely drug delivery or ecotoxicity effects (Hermens et al., 2013; Harris & Logan, 2014). By approximating the properties of the cell wall, absorption into octanol simulates uptake by cell membranes that might facilitate transport into the body or accumulation in cell membranes which may impair cell viability. Compounds with high log P

are likely to be good antimicrobial agents. Because absorption into lipid membranes is similar to absorption into bulk lipids or fats, it follows that essential oil volatiles with high log *P* may also partition into fats and lipids, reducing their availability as antimicrobial agents or requiring much higher concentrations in order to be effective.

Some recent studies have shown that higher concentrations of compounds such as carvacrol, thyme oil, and limonene are required to inhibit microbial growth in high-fat foods. For instance, Singh et al. (2003) showed a higher bacterial reduction by thyme oil (1 mL/L) in zero and low-fat hotdogs than in full-fat hotdogs. At 10 mL/L, thyme oil reduced *Listeria* populations in zero-fat samples while being less effective in samples containing full-fat and low-fat. This suggests that fat composition may affect the retention of essential oils in the different phases. Wang et al. (2020) reported that the antimicrobial activity of carvacrol against *Listeria monocytogenes* was lower in regular beef than in lean beef. They suggested that it might be related to the influence of fat on bioactive migration in the food matrix and the high log *P* (3.52) value of carvacrol. Due to the retention of carvacrol in the fat, there would be less interaction with microbes present in the water phases of the food (Wang et al., 2020).

Leptospermum scoparium, well known as mānuka, is the most widely distributed and environmentally tolerant flowering shrub or small tree indigenous to New Zealand. Historically, mānuka leaves were utilised to make an infusion, which acted as an effective tea supplement to cure numerous internal complaints such as back stiffness, breast inflammation, and eye-related problems (Chen et al., 2016). An essential oil obtained from a specific mānuka plant line is being developed as an antimicrobial product (Brooker et al., 1987; Riley, 1994; Perry et al., 1997), particularly against antibiotic-resistant strains (Douglas et al., 2004). The existing research has reported that the antimicrobial characteristics of mānuka oil are attributed to triketones (Killeen et al., 2016; Zhang et al., 2017).

Various researchers have reported the antimicrobial characteristics of different essential oils and their bioactive compounds, while there is a literature gap in their partitioning behaviour between different phases. In addition, the influence of varying food constituents' concentration (especially fat) on the retention and partitioning of bioactive compounds within foods is anticipated to be an essential factor that has rarely been studied. This study hypothesises that bioactive compounds with different log *P* values may exhibit different aqueous phase concentrations in low and high-fat foods. Thus, this study aimed to determine the log *P* of the bioactive compounds of mānuka oil, i.e., leptospermone, isoleptospermone, flavesone, α-pinene and γ-terpinene, experimentally using the shake-flask method and through prediction with the EPI suite software. In addition, the solubility, air-water partition coefficient, and chemical structure characteristics of all compounds present in mānuka oil were determined using the EPI suite. Further, the concentration of these compounds in the aqueous phase in equilibrium with low (3 %) and high fats (12 %) was determined.

4.2. Materials and Methods

4.2.1. Materials

The mānuka, pure triketones (mixture of leptospermone, isoleptospermone and flavesone) and kānuka oil samples were kindly provided by Tairawhiti Pharmaceuticals Ltd. (Te Araroa, New Zealand). Oil samples were stored in a black box under 4 °C to avoid the effect of temperature and light on the volatiles. Oils were normalised at room temperature for 45-60 minutes before use. All additional chemicals, such as α-pinene, γ-terpinene, octanol, hexane, dichloromethane, and sodium sulfate, were of analytical grade (Sigma Aldrich).

4.2.2. Octanol-water partition coefficient determination using the shake flask method

The octanol-water partition coefficient of bioactives was determined using the shake flask method, as described by Zhu et al. (2022). Before the beginning of this equilibrium experiment,

both phases (octanol and water) were presaturated. Briefly, 50 mL of the water was filled in a volumetric flask, and then 100 µL of n-octanol containing the test compounds was mixed. After 2 days of shaking and 2 days of resting (in a shaking incubator) at 25 °C, both phases were separated for analysis. A syringe was used to collect the octanol phase, and approximately 30 mL of water was withdrawn from the bottom separately. The octanol phase was diluted in hexane, spiked with internal standards and analysed using gas chromatography-mass spectrometry (GCMS). The 30 mL water samples (with internal standards) were extracted with solvents (hexane: dichloromethane (1:1)) three times and then concentrated under nitrogen up to 1 mL. Extracted and concentrated samples were diluted in hexane and analysed by GCMS. All hexane-diluted samples (octanol and water) were passed over anhydrous sodium sulfate to remove any traces of free water.

4.2.3. Quantification using Gas chromatography-mass spectrometry (GCMS)

Gas-chromatography-mass spectrometry (7890A, Agilent Technologies, USA) equipped with a VL MSD triple-axis detector was used to quantify the concentration of bioactives in both phases. The column and split ratio used were HP 5 MS (30 m long, 0.25 mm diameter and 0.1 um film thickness) and 200:1, respectively. The inlet temperature was set at 250 °C, and the pressure was 24.79 psi. The initial temperature was adjusted to 60 °C (for 10 min) and then rose to 150 °C at a rate of 4 °C/min and again increased to 240 °C at a rate of 1 °C min. The detector conditions, such as temperature at 250 °C and ionisation mode at electron impact, were used. Calibration curves of leptospermone, isoleptospermone, flavesone, α-pinene and γ-terpinene were used to quantify the concentration of compounds partitioned in the octanol and water phase.

4.2.4. Octanol-water partition coefficient prediction using the EPI suite

Different parameters, including water solubility, air-water partition (log K_{aw}), octanol-air partition (log K_{oa}), and octanol-water partition coefficient (log *P*) values, were determined using the EPI suite (Tamaru et al., 2018). Various physiochemical characteristics of the chemical compounds were calculated after entering the chemical compound's name in the EPI software. The preciseness of this software in calculating partition coefficient in comparison to other programs (CAChe, MOE and Hyperchem) has been reported by Shojaei et al. (2007) and Tamaru et al. (2018).

4.2.5. Determination of partition in beef fat and water

The whole experiment was performed at 55 °C to maintain the liquid state of beef fat. Similar to the octanol-water coefficient protocol, the water was transferred to a flask, followed by the addition of beef fat (3 and 12 % fat)-containing test compounds. After 2 days of shaking and 2 days of resting at 55 °C, water phases were separated for analysis. The bottom part of the water was collected using a syringe (30 mL) and extracted with solvents (hexane: dichloromethane (1:1)) three times and then concentrated under nitrogen. Extracted and concentrated samples were diluted in hexane, and analysis was carried out by GCMS, as explained above.

4.2.6. Estimation of the fat-water partitioning coefficient and concentration of the compounds present in fats using mass balance equation

In this approach, the mass balance equation was applied to estimate the concentration of compounds dissolved in fats. Then, the concentration of compounds present in fat and water was used to determine the fat-water coefficient. Here C_f =concentration of compounds in the fat, C_w =concentration of compounds in the water, C_T = total concentration of compounds (1 %), K_{fw} is a fat-water partitioning coefficient X_w = fraction of the water, X_w = fraction of the fat

$$X_f + X_w = 1$$

$$C_wX_w + C_f(1 - X_w) = 1 \times C_T$$

$$C_f = \frac{1 \times C_T - C_wX_w}{(1 - X_w)} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Eq\ 4.1$$

$$K_{fw} = \frac{C_f}{C_w} = \frac{1 \times C_T - C_wX_w}{(1 - X_w)\ C_w}\ _w$$

Estimation of fat-water partitioning coefficient by rearranging the equations

$$Log\ P = Log_{10}(K_{fw}) = Log_{10}\{\frac{C_f}{C_w}\} = Log_{10}\{\frac{1 \times C_T - C_wX_w}{(1 - X_w)\ C_w}\} \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots..Eq\ 4.2$$

After applying the mass balance equation, the concentration of the compounds separated in water was estimated using log *P* (method I) and log K_{fw} (method II), and values were compared with the experimental values:

The use of log *P* data using the same mass balance,

$$Log\ P = Log_{10}(K_{fw}) = Log_{10}\{\frac{C_f}{C_w}\}$$

$$C_f = C_W 10^{LogP}$$

$$C_wX_w + C_f(1 - X_w) = 1 \times C_T$$

$$C_w = \frac{1 \times C_T}{\{X_w + (1 - X_w) \times 10^{LogP}\}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Eq\ 4.3$$

4.2.7. Statistical analysis

All experiments were carried out in triplicate. Statistical evaluation was done using IBM SPSS (Armonk, NY: IBM Corp) to determine significant differences between the samples ($p \leq 0.05$).

4.3. Results and Discussions

4.3.1. Experimental octanol-water partition coefficient values of leptospermone, isoleptospermone, flavesone, α-pinene and γ-terpinene

To address the first objective of the study, the log *P* values of leptospermone, isoleptospermone, flavesone, α-pinene and γ-terpinene were determined. As shown in Table 4.1, the average log *P* values of tested compounds were greater than 3.5, indicating that the transfer and solubility of these compounds from the octanol phase to the aqueous phase were weak. Among all tested compounds, the leptospermone showed the highest log *P* values (5.02), followed by its isomer (Isoleptospermone), while α-pinene showed the lowest coefficient values. It can be observed that both terpenes had lower octanol-partition values than the triketones.

The octanol-water partition coefficient of chemical compounds can be related to their chemical structure (Vilas-Boas et al., 2022). The octanol-water partition coefficient of α-pinene and γ-terpinene have been reported by various studies using different methods (Griffin et al., 1999), whereas research studies on leptospermone, isoleptospermone, and flavesone are non-existent in the literature. A study by Griffin et al. (1999) reported the use of the shake flask method and reversed phases of high-performance liquid chromatography (HPLC) to determine the octanol-water partition coefficient of various terpenoids. The observed log *P* values for α-pinene and γ-terpinene were 4.44 and 4.36, respectively.

Table 4.1. Predicted and experimental values of octanol-water partition coefficient of tested compounds.

Compound name	Experimental log *P*	Estimated log *P* (using EPI software)
α-pinene	3.99 ± 0.13	4.44
γ-terpinene	4.06 ± 0.11	4.50
Flavesone	4.43 ± 0.10	4.70
Isoleptospermone	4.93 ± 0.24	5.19*
Leptospermone	5.02 ± 0.04	5.19

4.3.2. Comparison between estimated (using EPI suite) and experimental log P values

Due to the limitations associated with the experimental methods, such as massive expenditure, time and equipment requirement, developing in silico models has become an imperative method to predict log *P* values of chemical compounds. The EPI suite and ACD/ACD Absolv are two widely utilised computational software to predict log *P*, physiochemical, toxicity and many other properties of chemical compounds.

In this study, the log *P* values of the tested compounds were predicted using the EPI suite software. As shown in Table 4.1, the experimental values of all compounds were lower than the predicted values. Similar to these results, Zhu et al. (2022) determined the octanol-water partition coefficient of liquid crystal monomers using the shake-flask method and reported that experimental values were lower than predicted values using the EPI software. It could be attributed to the limitation of the shake-flask method that the partition coefficient of compounds containing more than two rings in their structures is usually underestimated.

4.3.3. Correlation of the concentrations of the compounds in fat systems to the octanol-water partition coefficient

The concentration of the key mānuka essential oil compounds in the water phase in equilibrium with 3 and 12 % beef-fat systems was measured as shown in Table 4.2. The concentration of compounds in water decreased with an increase in fat content from 3 to 12 %. The concentration of leptospermone in the water fraction of the 3 % fat system was 0.14 %, while it was not present in water from the 12 % fat system. Similarly, two other ketonic compounds (isoleptospermone and flavesone) were absent in the water phases from 12 % fat systems. However, α-pinene and γ-terpinene showed 0.05 and 0.39 % release in water from a low-fat system than compared to 0.02 and 0.04 %, respectively, in the high-fat. This behaviour is consistent with these compounds' high log *P* values and low water solubilities. Indeed it has

been documented that lipophilic compounds with lower water solubilities are released in lower quantities under such conditions than hydrophilic compounds (Sadovoy et al., 2013). A study by Tamaru et al. (2019) reported that the release rates of different compounds such as α-terpineol, nonanal, limonene, benzaldehyde, ethyl hexanoate, geraniol, and ethyl benzoate from emulsions into the headspace reduced with increasing the oil content in the emulsions. Consistent with our results, Wang et al. (2020) reported that the carvacrol absorption in fatty beef from polylactic acid films was significantly higher than the low-fat (lean) beef.

Table 4.2. The tested compounds' concentration (%) in the water of fat systems containing 3 % and 12 % beef fat.

Compound name	3 % fat	12 % fat
α-pinene	0.05 ± 0.01	0.02 ± 0.01
γ-terpinene	0.39 ± 0.49	0.04 ± 0.01
Flavesone	0.07 ± 0.01	nd
Isoleptospermone	0.02 ± 0.01	nd
Leptospermone	0.14 ± 0.10	nd

Nd= 0 means no quantities were found.

4.3.4. Estimation of the fat-water partitioning coefficient and concentration of the compounds present in fats using the mass balance equation

The mass balance was used to determine the concentration of compounds absorbed in fats and then the fat-water coefficient. It has been observed from the results that log K_{fw} obtained from the mass balance equation were significantly lower ($p \leq 0.05$) than the experimental log P (Table 4.3).

Table 4.3. Estimated values of the fat-water partitioning coefficient and concentration of the compounds present in fats using a mass balance (*Eq.* 4.1).

Compounds	Concentration in water (%)	Fat fraction (%)	Concentration of compounds in fat calculated using the mass balance equation *Eq* 4.1	log (K_{fw})
α-pinene	0.05	3	31.7	2.80
α-pinene	0.02	12	8.2	2.61
γ-terpinene	0.39	3	20.7	1.72
γ-terpinene	0.04	12	8.0	2.30
Flavesone	0.07	3	31.1	2.64
Isoleptospermone	0.02	3	32.7	3.21
Leptospermone	0.14	3	28.8	2.31

Table 4.4. Estimation of the compounds present in water phases using log *P* (octanol-water partition coefficient) using *Eq*. 4.3 and comparison with experimental values.

Compounds	Experimental log *P*	Fat (%)	Estimated C_w (%)	Experimental C_w (%)
α -pinene	3.99	3	0.003	0.05
α -pinene	3.99	12	0.000	0.02
γ -terpinene	4.062	3	0.002	0.39
γ -terpinene	4.062	12	0.000	0.04
Flavesone	4.428	3	0.001	0.07
Flavesone	4.428	12	0.000	0.00
Isoleptospermone	4.933	3	0.000	0.02
Isoleptospermone	4.933	12	0.000	0.00
Leptospermone	5.018	3	0.000	0.14
Leptospermone	5.018	12	0.000	0.00

It has been observed from the results that the estimated concentration of compounds in water using log *P* values was significantly lower than the concentrations calculated using log K_{fw} and approximately closer to the experimental C_w values (Table 4.4 and 4.5). The possible reason for this could be the complex structure and composition of the beef fat, which might interact with the compound; thus, a lesser concentration of these compounds was found in the water. It has been reported by Waring (2010) that chemical compounds with higher values of log *P* often show off-target lipophilic interactions and hydrophobic collapsing. Thus, it could be related to the complex fats and lipids in beef fats and the high log *P* values of these compounds. In addition, octanol partitioning has been studied as a crude surrogate to mimic the separation of compounds into cell membranes and aqueous environments (Summerfield et al., 2007; Gleeson, 2008; Lindsley, 2010). Studies emphasizing the partitioning of compounds in fats and its relation to log *P* are not available in the literature. Riéra et al. (2006) showed that among the several non-volatile food constituents, fats were most efficient in absorbing the volatiles molecules. When the volatile retaining effect of individual fat constituents was examined, a minor amount of phospholipid could effectively absorb the volatiles (Riéra et al., 2006). Likewise, Wang et al. (2020) observed that the carvacrol absorption in fatty beef from polylactic acid films was significantly higher than the low-fat (lean) beef, thus the higher antimicrobial activity of carvacrol in lean-beef than the fatty liver was observed.

Table 4.5. Estimation of the compounds present in water phases using log (K_{fw}) (fat-water partition coefficient) using *Eq*. 4.3 and comparison with experimental values.

Compounds	**log (K_{fw})**	**Fat (%)**	**Estimated C_w (%)**	**Experimental C_w (%)**
α-pinene	2.61	3	0.07	0.05
α-pinene	2.80	12	0.01	0.02
γ-terpinene	2.30	3	0.14	0.39

γ-terpinene	1.72	12	0.13	0.04
Flavesone	2.64	3	0.07	0.07
Flavesone	2.64	12	0.01	0.00
Isoleptospermone	3.21	3	0.02	0.02
Isoleptospermone	3.21	12	0.00	0.00
Leptospermone	2.31	3	0.14	0.14
Leptospermone	2.31	12	0.03	0.00

4.3.5. Estimations of air-water partition (log K_{aw}), octanol-air partition (log K_{oa}), and octanol-water partition coefficient (log P) values of major compounds present in mānuka oil

The partition coefficients are important physiochemical parameters to provide insights into the retention of volatile compounds like essential oil bioactives in different food phases. In addition to experimental log *P* values, the EPI suite was used to determine the log *P* values and air-water partition coefficient values of all chemical compounds in mānuka oil.

Water solubilities

In the present study, the water solubility of alcoholic compounds such as α-terpineol, borneol, eucalyptol, and ketones (camphor) was significantly higher than (≥ 100 mg/ L at 25 °C) the other compounds (Table 4.6). However, the mānuka oil triketones (leptospermone, isoleptospermone, flavesone) showed lower water solubility, possibly due to their higher log *P* values. Terpenes like α-pinene, β-pinene, myrcene, terpinolene, limonene, thujene and terpinene exhibited water solubility values between 2 and 10 mg/L, as shown in Table 4.3. In contrast, other terpenes such as cubenene, selinene, ylangene, copanene, aromadendrene, humulene, cadinene, and gurjunene had very low solubilities, even less than 1 mg/L. Dai et al.

(1998) documented that the increase in polarity and water solubility of compounds are related to the decrease in log *P* values.

*Air-water partition coefficient (*log K_{aw}*) values*

All alcoholic and ketonic compounds showed negative values of air-partition coefficients, indicating an affinity of these compounds to water as opposed to the air phase. Similarly, all terpenes had low log $K_{aw} \leq 2$. The partitioning of compounds in the atmosphere is controlled by food and the air above the food. As most foods contain 50-90 % of water, it could be related to the air-water partition (Buttery et al., 1965). However, other solutes, such as proteins and fat, also affect the partitioning of compounds between air and water (van Ruth & Villeneuve, 2002).

Octanol-air partition coefficient (log Koa) values

In addition, all compounds exhibited octanol-air partition coefficient (log Koa) values of more than 1, showing that these compounds prefer the octanol/fat phases over the air phase. In the presence of fat, their ability to go into the headspace will be less. Mānuka oil triketones had the maximum log K_{oa} values of ≥ 16. Generally, for hydrophobic/lipophilic compounds, increasing fat would make a higher coefficient value- thereby making compounds less likely to be in the air. Several studies have reported that lipophilic compounds with low water solubilities show lower release rates from lipid matrices than from aqueous or carbohydrate solutions (Seuvre et al., 2006; Perreault et al., 2010). In addition, amounts of hydrophobic compounds retained in emulsions increased with an increase in the oil content of the emulsions (Bayarri et al., 2006).

Table 4.6. Chemical structure, octanol-water partition coefficient (log *P*), air-water partition coefficient (log K_{aw}), octanol-air partition coefficient (log K_{oa}) and water-solubilities of the major terpenes present in mānuka oil (estimated using EPI suite).

Compound name	**Log *P***	**Log Kaw**	**Log Koa**	**Water Solubility**
α-Pinene	4.44	1.080	3.360	4.071
β-Pinene	4.16	0.818	3.342	7.061
Myrcene	4.17	0.420	3.750	6.92
Limonene	4.38	0.115	4.265	4.581
Γ-Terpinene	4.50	-0.036	4.536	3.618
Terpinolene	4.47	-0.242	4.712	3.838
α-Cubenene	6.73	0.902	5.828	0.0214
Gurjunene	6.18	0.973	5.207	0.06382
Caryophyllene	6.30	1.450	4.85	0.05011
Γ-Cadinene	6.27	1.450	4.820	0.05378
α-Humulene	6.95	1.824	5.126	0.01396
Selinene	6.30	1.450	4.850	0.05011
B-Cubebene	6.13	1.078	5.052	0.07057
Ylangene	5.71	0.902	4.808	0.1614

Thujene	4.61	0.641	3.969	2.91
Copanene	5.71	0.902	4.808	0.1614
Aromadendrene	6.13	1.078	5.052	0.07057
Δ-Cadinene	6.32	1.595	4.725	0.04863
p-Cymene	4.10	-0.347	4.447	27.88
Isoamyl isovalerate	3.66	-1.285	4.945	44.59
Spanthulenol	4.63	-3.359	7.989	12.44
Viridiflorol	4.63	-3.302	7.932	11.98
Globulol	4.63	-3.302	7.932	11.98
A-Terpineol	3.30	-3.190	6.490	360.2
Camphor	2.74	-2.480	5.220	339.1
Isoleptospermone	5.19	-11.827	17.017	0.7124
Flavesone	4.70	-11.951	16.651	2.242
Borneol	2.69	-3.249	5.939	1186
Eucalyptol	2.74	-2.347	5.087	332.1
Leptospermone	5.19	-11.827	17.017	0.7124

4.4. Conclusions

In this work, the octanol-water coefficient of mānuka oil beta-triketones (leptospermone, isoleptospermone, and flavesone), α-pinene and γ-terpinene were determined using a shake flask (GCMS) and EPI suite. All tested compounds demonstrated a strong lipophilic nature, and the octanol-water coefficients were greater than 3.5 value. However, the obtained experimental partition coefficient values were lower than the estimated partition values (using the EPI suite). In addition, water solubility, air-water partition (log K_{aw}), and octanol-air partition (log K_{oa}) coefficient values were determined using the EPI suite. The low water solubility and high octanol-air partition coefficient values of all tested compounds confirmed the lipophilic nature and their retention in the fat phase than the aqueous and air phases, respectively. The concentration of compounds in water decreased with increased beef-fat content from 3 to 12 %. Mānuka oil beta-triketones showed very low amounts in the water of the low-fat system, while they were absent in the water of the high-fat system. However, both terpenes were released in higher concentrations in water from 3 % beef fat than 12 % beef fat.

In the future, the experimental determination of partition coefficients of other chemical compounds in mānuka oil using novel instrumental (chromatographic spectroscopic) and software approaches will provide concrete proof of their liposoluble nature. The partitioning coefficients can be used to accurately predict the release/retention of bioactives compounds in different phases of foods under equilibrium conditions. The unavailability or lower availability of the essential oil compounds in an aqueous environment where microbes usually grow indicates that oils may not exhibit antimicrobial effects in high-fat meat systems. On the other, due to their interactions with fats, oils can effectively reduce lipid oxidation in high-fat meat systems. This has been discussed in the next chapter.

Chapter 5 Influence of different meat matrices on the antioxidant and antimicrobial potential of essential oils

Abstract

Essential oils possessing antimicrobial characteristics have acquired considerable interest as an alternative to chemical preservatives like sodium nitrate in food products. Based on the obtained results (from chapter 3), the mānuka oil with 25% triketone content and rosemary oil was chosen to study their antioxidant and antimicrobial effects in pastes prepared from commercial breed (3 % fat) and wagyu (12 % fat) beef during refrigerated storage (7 days). These effects were compared with sodium nitrate and control (without added oil)-pastes during refrigerated shelf life. In both meat types, compared with the sodium nitrate -treated and control samples, lower growth of *Listeria monocytogenes* and *Staphylococcus aureus* in mānuka oil - and rosemary oil - treated samples was observed. However, for *Salmonella* and *Escherichia coli*, rosemary oil treatment inhibited microbial growth most effectively. In antioxidant results, no significant effect of the oils was observed on lipid oxidation in commercial breed pastes during storage. However, mānuka oil significantly reduced ($p \leq 0.05$) lipid oxidation in wagyu pastes, showing the potential of mānuka oil as a natural antioxidant in high-fat meat products. The results suggest the potential use of mānuka oil as a partial replacement for synthetic preservatives like sodium nitrate in meats, especially against *Listeria monocytogenes* and *Staphylococcus aureus*.

5.1. Introduction

Beef paste is an important material used in the preparation of meat products such as sausages, hamburgers, meat pies, meatballs, and dried meat slices, owing to its high nutritional value and convenient use. It is more susceptible to microbial growth and oxidative reactions, as it promotes the distribution of microbes and oxygen by disrupting muscle membrane integrity and increasing surface area (Qing et al., 2021). It has been reported that *Salmonella Typhimurium* (DT104) incidences have been associated with meat paste, chicken, pork sausages, and other food products like unpasteurised milk products and fresh apple cider. These products represented around 38 % of human salmonellosis in 2000 in Canada (Dore et al., 2004; Anany et al., 2015). Pathogenic microbes such as *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus* may be found in meat products, including beef, pork, poultry, lamb, and mutton (Li et al., 2020). The predominant meat spoilage microbes are Actinobacteria, Firmicutes and Proteobacteria, in which *Carnobacterium* spp., *Brochothrix thermosphacta* and lactic acid bacteria are the most ubiquitous on raw and packaged meat products and form a large part of the core meat community on meats (Ercolini et al., 2006; Chaillou et al., 2015; Rouger et al., 2018; Li et al., 2020). These products require the addition of preservatives to prevent the growth of food pathogenic and spoilage bacteria. In addition, the grinding and comminution of meat products also expose meat tissues to iron (which behaves as a potent oxidation catalyst), enzymes and water, making them more susceptible to deteriorative oxidative changes (Lee et al., 2005; McBride et al., 2007).

The meat industry has long used the addition of chemical antioxidants, such as butylated hydroxytoluene (BHT), propyl gallate, *tert*-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA), to alleviate these quality spoilage reactions (Lee et al., 2005). Chemical preservatives like sodium/potassium nitrate and nitrites have been used in raw and processed

meats to alleviate lipid oxidation and pathogenic and spoilage microbes' growth (Elias et al., 2020; Nunzio et al., 2022; Kaur et al., 2022; Zhang et al., 2023). These are added to processed meats such as ham, bacon, raw meats, and sausages (uncooked raw, semi-smoked, frankfurter, boiled-type, liver pâtě mortadella) to prevent the growth of *Clostridium botulinum* and other pathogenic microbes (Zhang et al., 2023). In addition, nitrates/nitrites retard the spoilage oxidative reactions and ameliorate the colour and flavour of meat products. However, besides desirable effects, the consumption of nitrates/nitrites can form endogenous N-nitrosamine, a few of which have been reported as carcinogenic (Pegg & Shahidi, 2008; Kalaycıoğlu & Erim, 2019; Balaraman et al., 2021; Nunzio et al., 2022; Zhao et al., 2022). Hence, driving the need to use preservatives of natural or plant-based origin in meat products.

In this context, plant essential oils, which have been reported to possess antimicrobial activities against microorganisms associated with food spoilage, could be a promising alternative to chemical preservatives (Badia et al., 2020; Pateiro et al., 2021). Various plant essential oils such as thyme, clove, oregano, ginger, rosemary, and basil have been reported for their use in fresh, processed, minced, and cooked meat products (Alfonzo et al., 2017; Rodrigues et al., 2017; El-Sayed et al., 2017; Sharma et al., 2017; Zahid et al., 2018; Pateiro et al., 2021; Das et al., 2023). However, no research report is available on the usage of mānuka oil (MO) as a preservative in meat and meat products, even though some studies on this oil's *in vitro* antimicrobial potential have been reported (Porter & Wilkins, 1999; Douglas et al., 2004; Chen et al., 2016; Fratini et al., 2019).

Leptospermum scoparium (mānuka) and *Kunzea ericoides* (kānuka) are New Zealand's indigenous plants belonging to the *Myrtaceae* family. Like the Australian tea tree oil, essential oils obtained from these plants (mānuka and kānuka oils) are known as New Zealand tea tree oils. The antimicrobial potential of mānuka oil against a wide range of microbes has been

reported in the literature (Harkenthal et al., 1999; Chen et al., 2016). The existing research documented that the powerful antimicrobial characteristics of the mānuka oil are due to the presence of triketones (Harkenthal et al., 1999). As per the available literature, the antimicrobial effect of kānuka oil could be attributed to the monoterpenes, especially alpha-pinene (Porter & Wilkins, 1999).

It has been reported that antimicrobial efficacies of essential oils are lowered in food matrices than *in vitro* systems (in broth media), possibly due to the interactions of the oil with food constituents (Silva et al., 2022). The probable reasons could be the lower water content of foods than the laboratory media, which may hamper the progress of antimicrobial agents like essential oils to the target site in the microbial cell. Additionally, levels of fat present in food may dissolve the essential oils; thereby, they may be relatively less available to make contact with microbe existing in the aqueous phase of food. The solubilising effect of lipids in foods may influence the bioactive and antimicrobial activity of essential oils (Burt, 2004). For instance, the study of Singh et al. (2003) reported that the incorporation of thyme oil could not show an appropriate effect in full-fat hotdogs, whereas, in low and zero-fat hotdogs, it reduced the *Listeria monocytogenes* numbers. Similarly, Wang et al. (2020) reported a decreased antimicrobial activity of carvacrol in regular beef (12 % fat) than in lean beef (5 % fat) due to the absorption of carvacrol in the fat of regular ground beef.

Black Japanese cattle, also known as wagyu, is an extensively marbled (intramuscular fat) beef product renowned for its unique flavour and tenderness (Bermingham et al., 2018). The higher intramuscular fat content and unsaturated fatty acid composition of wagyu beef than Angus and other beef breeds have been reported in various studies (Smith et al., 2006; Bermingham et al., 2018; Bermingham et al., 2021).

Due to increased consumer consciousness, there is a growing demand for chemical additive-free meat products. This study examined the antimicrobial potential of mānuka and rosemary oils as an alternative to chemical preservatives like sodium nitrate (SN) in low (3 %)- and high (12 %)-fat meat products. Kānuka oil was not selected to test in meat pastes due to its lower antimicrobial activity than mānuka and rosemary oils.

5.2. Materials and methods

5.2.1. Materials

The vacuum-packed meat samples of wagyu beef and commercial breed beef tenderloins were purchased from Gourmet Butchery, Napier (New Zealand). The meat samples were stored at -20 °C in a freezer and thawed overnight before the analysis. Mānuka oil with triketone content of 5, 25 and 40 % have been referred to as mānuka oil 1, 2, and 3, respectively.

5.2.2. Meat pastes preparation and storage conditions

'Meat paste' was chosen as a meat matrix to maintain a uniform fat distribution throughout the meat systems, a. fresh meat pastes using commercial breed and wagyu beef were prepared in the FoodPilot plant, School of Food and Advanced Technology Massey, University (NZ). Meat samples (wagyu and commercial breed beef) were cut into small cubes using a sharp knife and then passed through a mincer (Mainca, PM-98, Spain) with a plate of 8 mm diameter holes. The minced samples were transferred into Hobart meat bowl Chopper (Ohio, USA), attached with a knife and ground for about 15 min to obtain a uniform paste. The prepared paste was further used for the different treatments.

5.2.2.1. Proximate composition analysis

The moisture content of the meat paste samples was checked according to the method of (AOAC, 1995). The Dumas method (AOAC, 1995) was used to determine the protein content.

The obtained values were multiplied with the factor of 6.25 to calculate protein content. For the estimation of fat content, the Soxhlet method was used (AOAC, 2000, 2006).

5.2.3. Preparation of essential oils-added meat systems

Preliminary experimentation was performed to select the concentration of essential oils to exert an antioxidant and antimicrobial effect in meat pastes. The paste was divided into six different lots for treatments with 2.5 % mānuka oil, 2.5 % rosemary oil, 300 mg/kg sodium nitrate, and 0.02 % BHT, followed by mixing in a mixer (Kogan, 1600 W, New Zealand) for about 15 min at room temperature. A control sample without any preservative/treatment was also prepared from each meat system. All prepared samples were packed in zip-lock bags (100 mm × 155 mm) and stored at 4 °C in a dark room. Samples were removed at different time intervals of 0, 1, 4, and 7 days and analysed for colour, pH and lipid oxidation.

5.2.3.1. Lipid oxidation analysis

The 2-Thiobarbituric acid (TBA) values were determined per Botsoglou et al. (1994) method with some modifications. Briefly, 2 grams of the meat sample were homogenised in 5 mL of BHT (0.8 % in hexane) and 8 mL of trichloroacetic acid (5 %). The upper hexane layer was removed, and the remaining liquid was filtered through filter paper. The supernatant was adjusted to a volume of 10 mL using trichloroacetic acid (5 %). Lastly, 2.5 mL of the liquid was mixed with 1.5 mL of TBA solution and incubated at 70 °C for 30 min. The absorbance reading was noted against a blank at 521.5 nm. To plot the calibration curve, 1, 1, 3, 3-tetraethoxypropane (TEP) was used as a standard. The results were expressed as mg of malonaldehyde per kg meat sample.

5.2.3.2. pH

The changes in the pH of meat paste samples during the 0- and 7-day storage period were estimated by following the AOAC method (AOAC, 1995). One gram of the meat paste samples was homogenised in deionised water (9 mL) using an ultra-turrax homogeniser (T25, Selangor, Malaysia) for 2 min. Then, the pH of the meat samples was checked and recorded at room temperature. All readings were taken in triplicates.

5.2.3.3. Colour

The meat paste samples' colour was checked using the Minolta colourimeter (Chroma meter, CR 400, Hong Kong, China). Briefly, equipment was calibrated first using a white tile, and each meat sample was scanned for colour values (L^*, a^* and b^*). The results were expressed as the average value of the triplicates.

5.2.4. Preparation of essential oil-added and microbial cultures-inoculated meat pastes

Preliminary experimentation was performed to select the concentration of essential oils to exert an antimicrobial and antioxidant effect in meat pastes. The meat paste was divided into four different lots and mixed with the bacterial culture of 10^4-10^5 cfu/g of *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes* or *Staphylococcus aureus*, respectively, using a bench mixer (Kogan, 1600 W, New Zealand). The pastes were left at room temperature for 15 minutes to ensure the microbes' attachment to the meat paste. Concentrations of mānuka and rosemary oils used against Gram-positive microbes in the meat paste were 1.25 % (for each oil), while 300 mg/kg of meat of sodium nitrate was used. To test the efficacy of essential oils against Gram-negative microbes, the pastes were mixed with oils/sodium nitrate at a concentration of 2.5 % (w/v) for mānuka oil, 2.5 % (w/v) for rosemary oil (for Gram-negative microbes), and 300 mg/kg sodium nitrate. A control sample for each bacteria type was also prepared and used for each meat system (commercial breed and wagyu), which contained a bacteria culture but without any preservatives. All prepared samples were vacuum-packed into

bags (90 mm × 120 mm) using a vacuum packer machine and stored at a temperature of 4 °C for further analysis. Samples were removed at different time intervals of 0, 4, 10, and 16 days and analysed for microbial growth.

5.2.4.1. Microbial growth analysis in meat

At selected storage time intervals, meat samples were transferred to a stomacher bag, and 45 mL of peptone water was added. The meat samples were homogenised in a stomacher bag mixer at 200 rpm for 2 min. Serial dilution of each sample was prepared and spread on selective agar plates (brilliant green modified agar for *Salmonella* spp., Eosin Methylene blue (EMB) for *Escherichia coli*, oxford agar for *Listeria monocytogenes* and Baird Parker agar plates for *Staphylococcus aureus*). Inoculated plates were incubated at 37 °C for 48 hours, and colonies were enumerated. The results were expressed as log cfu/g. Each treatment was tested in triplicate meat samples.

5.2.5. Statistical analysis

Statistical evaluation was performed using a general linear model in Minitab Version 19.2020.2.0 (Minitab Inc., State College, PA, USA) to compare the effects of different treatments in commercial breed and wagyu meat. The comparison was made between different meats (between commercial breed and wagyu), storage periods (between the 0^{th} and 7^{th} day of storage), and treatments (between mānuka oil and rosemary oil, mānuka oil and sodium nitrate, mānuka oil and butylated hydroxytoluene, and mānuka oil and control). The analyses were performed for each meat and storage day separately. One-way analysis of variance (ANOVA), followed by the *Tukey* method analysis at a 95 % confidence interval, was done to determine the significant difference between treatments ($p \leq 0.05$). Each experiment was carried out on three different replicates.

5.3. Results and Discussions

5.3.1. Essential oil selection for adding in meat systems

Among the three mānuka oils, only mānuka oil 2 with 25 % triketone content was chosen to examine its antimicrobial effect against microbial growth in the meat systems. This selection was based on the results of preliminary experimentation (Chapter 3) involving the thermostability, antioxidant, and antimicrobial characteristics of these oils. As rosemary oil inhibited Gram-negative (*Escherichia coli* and *Salmonella*) and Gram-positive bacteria (*Staphylococcus aureus* and *Listeria monocytogenes*) at 2.5 % concentration; thus, the same concentration of mānuka oil was selected to compare their antibacterial effect. However, due to the strong antibacterial effect of mānuka oil against *Staphylococcus aureus* and *Listeria monocytogenes* (confirmed through broth dilution assay), lower concentrations of mānuka oil can be employed to prevent these microbes. Kānuka oil had lower antioxidant and antimicrobial properties than mānuka oil, so it was not examined in meat pastes.

In the chemical composition of meat pastes, commercial breed paste's initial fat, protein, and moisture contents were found to be 3.4 %, 20.5 % and 72.2 %. However, the wagyu beef paste had lower moisture content (64.8 %) and higher fat content (12.3 %). The protein content of wagyu beef (19.4%) was almost similar to commercial breed beef. Comparing the fat and moisture content of both beef pastes, it can be noted that fat content increased proportionally with a decrease in moisture content.

5.3.2. Effects of the essential oils against lipid oxidation in meat systems

The changes in TBARS values of meat systems treated with and without essential oils and chemical preservatives (sodium nitrate and BHT) on the 0 and 7^{th} days of the storage period are presented in Table 5.1.

Comparing the TBARS values of the control wagyu and commercial breed beef pastes, the former meat system appeared more vulnerable to lipid oxidation throughout the storage period than the latter due to its higher fat content. The higher unsaturated fatty acid and neutral lipid content in cooked wagyu beef have already been reported by Boylston et al. (1996), which could also be linked to its more lipid oxidation vulnerability.

The addition of essential oils to commercial breed and wagyu meat pastes resulted in lower lipid oxidation values than their respective controls on the 0^{th} day, indicating the antioxidant effects of these preservatives against lipid oxidation. The reason might be that fat is exposed to oxygen during the paste preparation, such as grinding, chopping, and mixing. The added preservatives showed their immediate effect on inhibiting lipid from the initial day of storage and thereby had lower TBARS values than the control samples.

The mānuka oil-treated commercial breed beef pastes exhibited a significant increase ($p \leq 0.05$) in lipid oxidation values during the 7-day storage period, similar to the control, rosemary oil and sodium nitrate-treated meat pastes. For Wagyu beef pastes, mānuka oil and BHT treatments were most effective in controlling lipid oxidation during storage, followed by rosemary oil and sodium nitrate. No treatment, except the mānuka oil treatment, differentiated between commercial breed and wagyu beef pastes, as shown by their similar effects in both meat types. The reason may be the highly lipophilic nature of the bioactive compounds of mānuka oil, which easily get absorbed in the higher-fat meat samples. The lipophilicity can be related to the octanol-water partition coefficients of the main compounds of mānuka oil, which were above 4, as shown in the results of chapter 4. These values indicate the higher affinity of bioactive compounds towards the fat part than the aqueous phase of meat. A recent study by Tomović et al. (2020) reported a higher antioxidant potential of *Juniperus communis* essential oil in 25 %-fat containing dry-fermented sausages than the 15 % fat sausages (Tomović et al.,

2020). Additionally, the lower lipid oxidation values observed in essential oil-added samples than their respective controls could be due to the presence of bioactive compounds like phenolic compounds and sesquiterpenes in rosemary and mānuka oil, which have been documented in the literature for their promising health properties, including antioxidant effects (Porter & Wilkins, 1999; Kaur et al., 2021).

Comparing the antioxidant potential of rosemary and mānuka oil, the former oil inhibited lipid oxidation in both meat pastes, while the latter oil was more effective in wagyu beef paste than the commercial breed beef paste ($p \leq 0.05$). The difference in the chemical composition of essential oils could also be responsible for differences in their antioxidant activities. Oliveira et al. (2015) reported that the presence of phenolic compounds in rosemary oil could be linked to its antioxidant activity. Phenolic compounds can scavenge free radicals and donate atoms with electrons, thus preventing the degradation of active oxidising forms like malonaldehyde (Oliveira et al., 2015). Regarding mānuka oil, triketones are responsible for its antimicrobial properties have been reported, but research emphasising the antioxidant effect of mānuka oil triketones such as leptospermone, isoleptospermone, and flavesone is not available in the literature (Porter & Wilkins, 1999; Chen et al., 2016). However, some studies reported that bioactive compounds, which contained β-β triketones moiety in their structure, possess antioxidant properties.

It is also important to mention that all treatments led to some reduction in lipid oxidation during storage compared to the control, and this effect was more pronounced in the wagyu beef. The possible reason for the observed differences could be the different fat content and fatty acid compositions of the two beef pastes (Oliveira et al., 2013; Oliveira et al., 2015; Barbosa et al., 2016; Badia et al., 2020). The difference in the fatty acid compositions could affect the physical state of lipids and, thereby texture of the prepared product. Thus, there may be changes in the

dispersion of the essential oils in both meat systems, resulting in different antioxidant activities (Estévez & Cava, 2006).

The commercial breed beef meat pastes treated with BHT showed no significant change ($p \leq 0.05$) in lipid oxidation during storage. On the other hand, for wagyu beef pastes, both BHT and mānuka oil treatments were most effective in controlling lipid oxidation during storage. The sodium nitrate effectively reduced the lipid oxidation values throughout the storage period, but this effect was significantly lower than the mānuka oil treatment. Estévez et al. (2007) reported a higher antioxidant effect of sage and rosemary oils than the BHT in liver pâté samples. The difference in antioxidant activities of essential oils and chemical antioxidants in the current study could be attributed to the different affinities of the produced free radicals to scavenge the antioxidant compounds present in meat samples (Cantú-Valdéz et al., 2020).

5.3.3. Effects of the essential oils on changes in pH values of meat systems

In this study, the pH values of both meat pastes were significantly ($p \leq 0.05$) influenced by the essential oil addition and storage time (Table 5.1).

On the 0^{th} day, in the control commercial breed and wagyu beef paste, lower pH values were noticed for the former than for the latter. During the storage period, an increase in pH values of control wagyu beef pastes was observed during the storage period (Table 5.1). However, essential oil added-wagyu pastes had comparatively lower pH values throughout the storage than the control samples, which may be due to the antimicrobial action of the essential oil and thereby inhibiting changes in pH values. Similarly, in the case of essential oil-added commercial breed beef pastes, pH values were constant throughout the storage, indicating that essential oils were effective against inhibition of microbial growth, especially lactic acid bacteria, which produce lactic acid in their metabolism. The antimicrobial activity of mānuka and rosemary oils could be attributed to their naturally occurring bioactive compounds, β-

triketones and 1, 8-cineole, respectively (Porter & Wilkins, 1999). Owing to the lipophilic nature of these compounds, they cause the rupture of the bacterial cell membrane and affect the microbial cell's vital functions

The probable reason for a change in pH of both meat pastes could be more growth of lactic acid bacteria in commercial breed beef paste than in the wagyu beef paste. Due to the difference in chemical composition between the two types of meat, especially fat and moisture content, water activity may differ in both types of meat, which may result in further microbial growth (Barmpalia-Davis et al., 2009). The increase in pH of wagyu beef could be attributed to the enzymatic breakdown of proteins and microbial action, which can cause the accumulation of some basic compounds, including trimethylamine, ammonia, and other products of amino acid compounds (Barmpalia-Davis et al., 2009).

The initial difference in pH values of both meat pastes could be due to different chemical compositions (water and fat content) and intramuscular fat % between meats (Neath et al., 2007; Dixit et al., 2021). However, postmortem storage periods and conditions also influence the pH values of meat systems (Neath et al., 2007).

Table 5.1. The changes in TBARS and pH values for wagyu and commercial breed beef pastes with or without any added antioxidant agent during storage at 4 °C for 7 days.

Parameters	Meat system	Treatments					SEM	*p-value*			
		MO	RO	SN	BHT	C		MO×RO	MO×SN	MO×BHT	MO×C
0th day											
TBARS	Wagyu	0.10^{Bx}	0.14^{Ax}	0.14^{Ay}	0.13^{ABy}	0.13^{ABy}	0.04	*	*	ns	ns
	Commercial	0.11^{Cx}	0.07^{Dy}	0.22^{Bx}	0.29^{Ax}	0.24^{Bx}	0.01	**	**	**	**
pH	Wagyu	5.75^{Bx}	5.95^{Ax}	5.87^{ABy}	5.96^{A}	5.95^{Ax}	0.02	*	*	*	*
	Commercial	5.42^{Cy}	5.63^{By}	5.67^{Ax}	5.78^{B}	5.72^{ABy}	0.01	**	**	**	**
7th day											
TBARS	Wagyu	0.20^{By}	0.30^{BCx}	0.39^{Bx}	0.20^{Bx}	0.59^{Ax}	0.02	**	**	ns	**
	Commercial	0.49^{Ax}	0.30^{Bx}	0.47^{Ax}	0.33^{Bx}	0.51^{Ay}	0.19	**	ns	*	ns
pH	Wagyu	5.95^{Dx}	6.16^{Bx}	5.90^{Dx}	6.03^{Cx}	6.34^{Ax}	0.01	**	ns	ns	**
	Commercial	5.41^{By}	5.63^{Ay}	5.26^{Cy}	5.39^{By}	5.43^{By}	0.01	**	**	ns	ns
Storage effect (0th×7th day)											
TBARS	Wagyu	**	**	**	ns	**					
	Commercial	**	**	**	ns	**					

pH	Wagyu	*	**	ns	**	**
	Commercial	ns	ns	**	**	**

Treatments- **MO**-Mānuka oil, **RO**- Rosemary oil, **SN**- Sodium Nitrate, **BHT**- Butylated Hydroxytoluene, **C**- Control. **MO×RO**= comparison between mānuka oil and rosemary oil, **MO×SN**= comparison between mānuka oil and sodium nitrate, **MO×BHT**= comparison between mānuka oil and butylated hydroxytoluene, **MO×C**= comparison between mānuka oil and control, **Storage effect (0^{th}×7^{th} day)** = comparison between 0^{th} and 7^{th} day. **ns**= $p > 0.05$, ***= $p \leq 0.05$, **** =$p \leq 0.01$. **SEM**- Standard error mean.

[A-E] Means within a row with the same superscript letters are not significantly different ($p \leq 0.05$) between the treatments on the same storage day.

[xy] Means within a column with the same superscript letters are not significantly different ($p \leq 0.05$) between the meat systems.

Table 5.2. The changes in colour (*L**, *a** and *b**) values for wagyu beef and commercial breed beef paste with or without any added antioxidant agent during storage at 4 °C for 7 days.

Parameters	Meat system	Treatments					SEM
		MO	RO	SN	BHT	C	
0th day							
*L**	Wagyu	43.61^{By}	42.88^{By}	44.82^{Ax}	44.56^{Ax}	44.10^{Ax}	0.24
	Commercial	48.36^{Ax}	43.96^{Bx}	44.27^{Bx}	42.70^{Cy}	42.08^{Cy}	0.18
*a**	Wagyu	12.30^{Ax}	12.41^{Ax}	10.49^{Cx}	11.69^{ABx}	11.23^{Bx}	0.19
	Commercial	6.17^{Cy}	7.34^{By}	9.74^{Ax}	7.27^{By}	7.59^{By}	0.15
*b**	Wagyu	10.72^{Ay}	9.76^{Ay}	10.21^{Axy}	10.32^{Ax}	10.56^{Ax}	0.21
	Commercial	12.28^{Ax}	10.19^{Bx}	8.83^{BC}	8.68^{Cy}	8.87^{BCy}	0.24
7th day							
*L**	Wagyu	43.82^{Ay}	44.32^{Aay}	43.77^{Ay}	43.76^{Ay}	43.77^{Ax}	0.29
	Commercial	45.20^{Ax}	46.47^{Ax}	45.57^{Ax}	45.43^{Ax}	45.10^{Ax}	0.27
*a**	Wagyu	12.24^{ABx}	10.48^{Bx}	17.45^{Ax}	13.01^{ABx}	11.43^{ABx}	1.16
	Commercial	6.85^{Ay}	6.83^{Ay}	5.90^{By}	3.68^{By}	6.15^{Ay}	0.57
*b**	Wagyu	8.72^{ABy}	9.54^{Ay}	9.71^{Ax}	7.16^{Cy}	7.97^{BCy}	0.25

	Commercial	12.47^{Ax}	11.67^{ABx}	9.84^{Cx}	8.32^{Dx}	10.16^{BCx}	0.26
Storage effect (0^{th} × 7^{th} day)							
*L**	Wagyu	ns	*	ns	*	ns	
	Commercial	**	**	*	**	**	
*a**	Wagyu	ns	*	**	*	ns	
	Commercial	*	ns	**	ns	**	
*b**	Wagyu	**	ns	ns	**	**	
	Commercial	ns	**	ns	ns	**	

Treatments- **MO**-Mānuka oil, **RO**- Rosemary oil, **SN**- Sodium Nitrate, **BHT**- Butylated Hydroxytoluene, **C**- Control. **Storage effect (0^{th}×7^{th} day)** = comparison between 0^{th} and 7^{th} day. **ns**= $p > 0.05$, ***** = $p \leq 0.05$, ****** =$p \leq 0.01$. **SEM**- Standard error mean.

A-E Means within a row with the same superscript letters are not significantly different ($p \leq 0.05$) between the treatments on the same storage day.

xy Means within a column with the same superscript letters are not significantly different ($p \leq 0.05$) between the meat systems.

5.3.4. Effect of the essential oils on the colour of meat systems

The changes in colour values of beef paste samples with and without essential oil treatments are presented in Table 5.2. Depending on the addition of the antioxidants, both meat pastes exhibited different colour characteristics.

Comparing the redness values (a^*) of control commercial breed beef and wagyu beef paste on the 0^{th} day, the former was less red than the latter. At the end of the storage period, no significant difference in redness value (a^*) of wagyu beef treated with mānuka oil was observed (between the 0^{th} and 7^{th} day). The distinguishing effect of sodium nitrate on the redness (a^*) value of both meat pastes during storage (between the 0^{th} and 7^{th} day) was more noticeable than the other treatments. As expected, the addition of sodium nitrate increased the a^* values of both meat samples, which could be linked to their antioxidant effect and formation of stable red/pink colour pigment in sodium nitrate-added samples. Additionally, the initial difference in redness values of both meat could be linked to their pH, as it has been reported that meat colour gets darker with an increase in pH (Dixit et al., 2021).

Interestingly, different values of yellowness and blueness (b^*) for control commercial breed and wagyu beef pastes were observed in the initial phase of the storage period. It could be related to the concentration of β-carotene deposited in the fat of commercial breed beef carcass relative to other beef or breeds of cattle, including wagyu (Jaborek et al., 2019). With mānuka oils treatment, differences in b^* values of wagyu beef pastes were observed during the storage, but this effect was not statistically significantly different ($p \leq 0.05$) from rosemary oil and sodium nitrate treated samples and their control counterparts. The sodium nitrate did not show any effect against the b^* values of both types of meat, while BHT decreased these values during the storage of wagyu beef paste (between the 0^{th} and 7^{th} day). Similar to b^* values, the effect of mānuka oil against the lightness (L^*) values of wagyu beef paste was not significantly

different from all other treatments. However, in commercial breed beef, mānuka oil treatment resulted in significantly different lightness values than the other treated samples on the 0^{th} day and between the storage period (between the 0^{th} and 7^{th} day). The results of this study on colour changes agree with the previously reported study by (Ünal et al., 2014). They reported higher L^* values in beef treated with rosemary oil than in control. The effect of rosemary oil and BHT was less pronounced in wagyu beef ($p \leq 0.05$) than the commercial breed beef ($p \leq 0.05$).

Generally, meat's colour values, especially redness values, are associated with meat's protein and fat content. As Keokamnerd et al. (2008) reported, the reduced red colour of meat samples could be due to the interdependence of lipid and protein oxidation in meat. The free radicals produced during oxidative reactions may catalyse the oxidation of iron molecules or denture the protein molecules, thus affecting or changing the colour of meat (Keokamnerd et al., 2008).

5.3.5. Effects of the essential oils against microbial growth in meat pastes

The changes in microbial growth in wagyu and commercial breed meat paste with or without any added preservative agent during storage at 4 °C for 16 days are presented in Table 5.3.

In both types of meat pastes, mānuka oil treatment significantly inhibited the growth of Gram-positive microbes during the storage, followed by rosemary oil and sodium nitrate. The treatment of commercial breed pastes with mānuka oil resulted in *Listeria monocytogenes* and *Staphylococcus aureus* count lowered by 3 and 2.9 log cfu/g, respectively, than the control. However, in wagyu paste, the inhibition effect of mānuka oil showed reduced growth of *Staphylococcus aureus* (1.6 log cfu/g) and *Listeria monocytogenes* (2.5 log cfu/g) than their control counterparts. The addition of sodium nitrate to wagyu meat matrix samples resulted in 0.12 log cfu/g populations of *Escherichia coli* lower than their control samples.

On the other hand, rosemary oil was most effective against Gram-negative microbes and showed higher antimicrobial efficacy than the mānuka oil and sodium nitrate -treated meat pastes. In commercial breed meat pastes, treatment with rosemary oil resulted in 1.67 log cfu/g of *Escherichia coli,* whereas 0.77 log cfu/g with mānuka oil and 0.12 log cfu/g with sodium nitrate treatments, lower than the control. However, this inhibition was less pronounced in wagyu meat, showing 1.19 log cfu/g of *Escherichia coli* lowered than their control counterparts (Table 5.3).

The lower antimicrobial effect of the mānuka oil against Gram-negative bacteria has already been reported through *in vitro* studies (Klink et al., 2005). However, previous research studies on the antimicrobial potential of rosemary oil against *Salmonella* and *Escherichia coli* in meat models exhibited variable results. Ahn et al. (2004) showed approximately one log reduction in *Escherichia coli, Salmonella,* and *Listeria monocytogenes* counts with 1 % rosemary oleoresin in ground beef stored under refrigerated conditions (4 °C) for 9 days. Stojanović-Radić et al. (2018) reported that a shorter exposure (soaking) time (~ 15 min) with rosemary oil exhibited a higher antimicrobial effect against *Salmonella* Enteritidis in thermally processed chicken meat than the untreated control. However, Kahraman et al. (2015) did not observe any antimicrobial effect of the rosemary oil (0.2 % concentration) against *Listeria monocytogenes* and *Salmonella* Typhimurium in poultry fillets. Although in our study, the effect was similar to or higher than the previous studies, it possibly is due to various factors such as composition and concentration of the oils, different meat types (pork, beef or chicken), and inoculum size and sensitivity, which were influencing the experimental results.

Comparing the antimicrobial effect of rosemary and mānuka oils, a significant difference ($p \leq 0.05$) in their antimicrobial activity was found, which could be attributed to their chemical constituents 1, 8-cineole and β-triketones, respectively. Due to these compounds' hydrophobic

nature, they can rupture bacterial cell membranes and damage microbial cells' vital functions (Badia et al., 2020). However, the presence of an hydroxyl group in the structure of phenolic compounds, such as carvacrol, a bioactive compound in rosemary oil, makes them more strongly active than esters and hydrocarbons. It has been reported that -OH groups can easily form hydrogen bonds with the active site of enzymes (Gallucci et al., 2009).

Interestingly, the compositional difference of both meat pastes significantly affected the microbial growth in all treated samples, especially in control samples. The probable reasons could be a difference in fat content and fatty acid composition of both meat products (Huang & Frankel, 1997). Secondly, as both meat pastes contain different fat and moisture content, there could be differences in the water activity and, thereby, the growth of microbes (Barmpalia-Davis et al., 2009).

As per our results, the wagyu paste (high-fat meat matrix) treated with oils (either mānuka oil or rosemary oil) exhibited a lower inhibition of microbial growth than the commercial breed paste (low-fat meat matrix). It can be related to the influence of fat or octanol-water partition coefficient of the major constituents of the oils. The octanol-water partition coefficients of the main compounds in mānuka oil and rosemary oil were around 4 (Chapter 4), which directs towards their lipophilic nature. It exhibits that these compounds will be present in the fat part of the meat than in the aqueous phase. Their retention in non-aqueous food phases might not show the desired antimicrobial effect and direct action on the targeted microbe in the aqueous phase (Perricone et al., 2015). Owing to the absorption of carvacrol in the fat of regular ground beef, a decreased antimicrobial activity of carvacrol in regular beef (12 % fat) than in lean beef (5 % fat) has been reported by Wang et al. (2020). Similarly, in low and zero-fat hotdogs, thyme oil reduced the *Listeria monocytogenes* numbers but did not show any appropriate effect

in full-fat hotdogs (Singh et al., 2003). Our study's findings support that the fat content of meat and meat products may influence the antimicrobial efficacy of essential oils.

This study showed the antimicrobial potential of mānuka and rosemary oils against selected pathogenic microbes. As antimicrobial susceptibility may vary between strains in the same bacterial species, thus these oils need to be tested against several strains from each species to provide concrete evidence of their antimicrobial activity against targeted microbial species. It is important to mention that the sensory analysis of mānuka and rosemary oils-added beef pastes is essential for consumer acceptability; this analysis may be considered in future work. Additionally, studies on the safety and toxicity analysis of mānuka and kānuka oils will be needed to ensure their safety and acceptability for food applications.

Table 5.3. The changes in microbial growth in wagyu and commercial breed meat matrices with or without any added preservative agent during storage at 4 °C for 16 days.

Parameters	Meat system	Treatments[1]				SEM	*p- value*[2]		
		MO	RO	SN	C		MO×RO	MO×SN	MO×C
Staphylococcus aureus									
0	Wagyu	5.69^{Xa}	5.68^{Ya}	5.68^{Xa}	5.71^{Xa}	0.01	ns	ns	ns
	Commercial	5.67^{Xb}	5.83^{Xa}	5.77^{Xab}	5.88^{Xa}	0.02	*	ns	*
4	Wagyu	6.01^{Xb}	6.18^{Xb}	6.45^{Xb}	6.91^{Xa}	0.09	*	**	*
	Commercial	5.70^{Yb}	5.69^{Yb}	5.82^{Yb}	6.40^{Xa}	0.08	**	ns	*
10	Wagyu	6.53^{Xd}	6.63^{Xc}	6.84^{Xb}	7.47^{Xa}	0.01	**	**	**
	Commercial	5.11^{Yd}	5.37^{Yc}	6.32^{Yb}	7.27^{Xa}	0.01	**	**	**
16	Wagyu	5.93^{Xd}	6.23^{Xc}	6.86^{Xb}	7.46^{Ya}	0.02	**	**	**
	Commercial	5.08^{Yd}	5.54^{Yc}	6.32^{Yb}	7.90^{Xa}	0.04	**	**	**
Storage effect	Wagyu	**	**	**	**				
	Commercial	**	**	**	**				
Listeria monocytogenes									
0	Wagyu	5.83^{Xb}	5.85^{Xb}	5.84^{Xb}	5.96^{Xa}	0.10	ns	ns	**

	Commercial	5.66^{Yd}	5.75^{Yc}	5.83^{Xb}	5.92^{Ya}	0.01	*	**	**
4	Wagyu	6.69^{Xd}	6.89^{Xc}	7.45^{Xa}	7.12^{Yb}	0.02	*	**	**
	Commercial	6.67^{Yd}	6.87^{Xc}	7.34^{Yb}	7.53^{Xa}	0.80	**	**	**
10	Wagyu	6.47^{Xd}	6.70^{Xc}	7.57^{Yb}	7.89^{Ya}	0.02	**	**	**
	Commercial	6.41^{Yd}	6.67^{Xc}	7.63^{Xb}	8.18^{Xa}	0.01	**	**	**
16	Wagyu	6.04^{Xd}	6.45^{Xc}	7.71^{Yb}	8.57^{Ya}	0.02	**	**	**
	Commercial	5.98^{Xd}	6.45^{Xc}	7.88^{Xb}	8.94^{Xa}	0.03	**	**	**
Storage effect	Wagyu	**	**	**	**				
	Commercial	**	**	**	**				
Escherichia coli									
0	Wagyu	5.82^{Xab}	5.66^{Xc}	5.75^{Xb}	5.85^{Xa}	0.01	ns	*	*
	Commercial	5.58^{Yb}	5.62^{Xab}	5.70^{Xa}	5.70^{Ya}	0.01	ns	*	**
4	Wagyu	6.41^{Yc}	6.42^{Yc}	6.51^{Yb}	7.54^{Xa}	0.01	ns	**	**
	Commercial	7.02^{Xc}	5.25^{Xd}	7.40^{Xb}	7.49^{Ya}		**	**	**
10	Wagyu	7.41^{Xc}	6.41^{Yd}	8.15^{Xb}	8.68^{Xa}	0.00	**	**	**
	Commercial	7.41^{Xb}	6.61^{Xd}	7.33^{Yc}	7.71^{Ya}	0.00	**	**	**
16	Wagyu	8.15^{Xc}	6.54^{Yd}	8.64^{Yb}	9.34^{Xa}	0.08	**	ns	**

	Commercial	8.53^{Xb}	7.63^{Xc}	9.18^{Xab}	9.30^{Xa}	0.14	**	**	ns
Storage effect	Wagyu	**	**	**	**				
	Commercial	**	**	**	**				
***Salmonella* spp.**									
0	Wagyu	5.75^{Xb}	5.62^{Yc}	5.70^{Xb}	5.86^{Xa}	0.01	**	ns	**
	Commercial	5.73^{Xb}	5.70^{Xb}	5.75^{Xb}	5.81^{Xa}	0.03	ns	ns	**
4	Wagyu	6.81^{Yab}	5.71^{Xc}	6.62^{Yb}	7.25^{Xa}	0.1	**	**	ns
	Commercial	6.90^{Xa}	5.84^{Xb}	6.72^{Xa}	7.20^{Xa}	0.10	**	**	ns
10	Wagyu	7.65^{Yb}	6.42^{Xd}	7.45^{Xc}	8.40^{Xa}	0.09	**	**	**
	Commercial	8.20^{Xb}	5.95^{Yd}	7.59^{Yc}	8.58^{Xa}	0.11	**	**	**
16	Wagyu	8.12^{Xb}	6.24^{Xd}	7.68^{Xc}	8.69^{Xa}	0.01	**	**	**
	Commercial	7.40^{Yb}	5.16^{Yc}	7.50^{Yb}	8.46^{Xa}	0.15	*	**	**
Storage effect	Wagyu	**	**	**	**				
	Commercial	**	**	**	**				

Treatments- **MO**-Mānuka oil, **RO**- Rosemary oil, **SN**- Sodium Nitrate, **C**- Control. **MO×RO**= comparison between mānuka oil and rosemary oil, **MO×SN**= comparison between mānuka oil and sodium nitrate, **MO×C**= comparison between mānuka oil and control, **Storage effect (0^{th}×7^{th} day)** = comparison between 0^{th} and 7^{th} day. **[1]** represents the log cfu/g of the meat, [2]**ns**= $p > 0.05$, ***=** $p \leq 0.05$, ****** =$p \leq 0.01$. **SEM**- Standard error mean. **[a-e]** Means within a row with the same superscript letters are not significantly different ($p \leq 0.05$) between the treatments on the same storage day. **[XY]** Means within a column with the same superscript letters are not significantly different ($p \leq 0.05$) between the meat systems.

5.4. Conclusions

In conclusion, this study revealed that mānuka oils exerted better antimicrobial activities than kānuka oil against both Gram-negative and Gram-positive microbes, as exhibited by *in vitro* results. Indeed, as per disc diffusion and broth dilution assay results, Gram-positive microbes exhibited more sensitivity to mānuka oils than Gram-negative microbes. An increase in antimicrobial efficacies was observed with an increase in the triketone content of mānuka oils against Gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*). However, rosemary oil inhibited the Gram-negative microbes most effectively than the kānuka and mānuka oils.

In commercial breed (low-fat meat system) and wagyu (high-fat meat system) meat pastes, mānuka oil showed a significant inhibitory effect against Gram-positive bacteria than the rosemary and sodium nitrate treatments. However, rosemary oil inhibited the Gram-negative microbes more effectively than all other treated samples. Therefore, these oils have the potential to be used in food products as natural preservatives. Mānuka oil showed a better antimicrobial effect than synthetic preservatives (sodium nitrate), especially against Gram-positive microbes, indicating the potential of this as a natural preservative in meat products. Nevertheless, future studies on this oil's encapsulation and emulsification can improve their stability, and dispersibility, and mask undesirable flavour and odour to facilitate a wide range of food applications. The next chapter will discuss it.

Chapter 6 Synthesis and characterisation of mānuka and rosemary oil- based nanoemulsions and nanocapsules

Abstract

Mānuka and rosemary oils -containing nanoemulsions and nanocapsules made of sodium alginate and whey protein, were designed and compared for their antioxidant effect. Mānuka oil-nanoemulsions and nanocapsules had smaller particle sizes (343 and 330 nm), less negative zeta potential (-12 mV and -10 mV), higher phenolic content, and antiradical characteristics than rosemary oil -nano-entities. However, nano-entities of both oils showed more thermostability and sustained release than free oils. In Fourier transform Infrared spectroscopy (FT-IR) results, the appearance of peaks showed the molecular interactions between interactions essential oil constituents and carrier materials and confirmed the loading of essential oils in these carriers. Hence, nano-entities can be alternatives to chemical preservatives as natural antioxidants in meat preservation, along with improved thermal stability and release than free oils.

6.1. Introduction

Due to the overexposure of meat surfaces to oxygen and catalysts (metals) and the distribution of microbes during the grinding process, ground meat products are more prone to spoilage lipid oxidation reactions and microbial growth (Amiri et al., 2019). Other than the loss of colour, flavour, texture and nutritional values, these deteriorating reactions can produce free radicals and pathogenic microbes, which may be harmful and pathogenic to human health (Devatkal et al., 2010). The use of antioxidant agents is one of the most promising and practical approaches to reduce oxidative reactions and prolong the shelf of meat products. In recent years, natural antioxidants from plant sources have gained broad interest due to safety approvals, less regulatory requirements and consumer preferences over chemical preservatives like sodium nitrite (Pereira et al., 2017).

Several researchers have reported that the addition of plant essential oils to raw and processed meat products can be an additional hurdle to alleviate oxidative reactions (lipid and proteins) (Fratianni et al., 2010; Ghaderi-Ghahfarokhi et al., 2016). *Leptospermum scoparium* belonging to the Myrtaceae family, is an essential plant in Māori culture and grows in New Zealand and some parts of Australia. This plant, also known as mānuka, has been utilised in beverages and medicinal purposes since ancient times (Porter & Wilkins, 1999; Chen et al., 2016). Mānuka oil has been listed as a food flavouring ingredient in the Australia New Zealand Food Standards code; however, safety assessments are required at the proposed levels and patterns before its use (Australia New Zealand Food Standards, 2020). *Rosmarinus officinalis* is an aromatic plant, recognised as rosemary, belongs to the *Lamiaceae* family and comes from the Mediterranean region (Kaur et al., 2021). In the literature, rosemary is widely studied as a natural preservative to retard oxidative reactions and increase the shelf life of meat commodities (Vital et al., 2016; Kaur et al., 2021).

Nevertheless, facile degradation, low water solubility and undesirable taste and flavour, especially in the case of essential oils, pose a challenge for their use in food preservation. The application of nanotechnology is a promising and innovative approach to overcoming these challenges by improving stability and providing targeted delivery and controlled release characteristics to bioactives (Kaur & Kaur, 2020).

Extensive research has been conducted to devise various nano-entities, such as nanoemulsions, nanocapsules, nanospheres and nanoparticles, by employing several materials and techniques (Abbasi et al., 2019). Emulsion-based systems are mainly fabricated for this purpose because their composition and structures can be designed to alter the solubility and transformation of lipophilic bioactives. However, nanocapsules can be synthesised to provide targeted delivery, controlled release and protection against pH, light, oxygen and other environmental factors (Ghaderi-Ghahfarokhi et al., 2016).

Alginate is a biopolymer commonly utilised for the encapsulation of different bioactive compounds due to its easy availability, low cost, non-toxicity and ease of gelation (Rahnemoon et al., 2021). The use of alginate as a carrier material for delivery and protection of bioactive compounds, either in emulsions or capsules form, showed good efficiency and has the function of prolonging the shelf life of food products (Salvia-Trujillo et al., 2013; Zhang et al., 2022). However, the food matrix is an important factor that can influence the partitioning and release of bioactive compounds from the carrier material, and consequently, their influence may differ according to food (Wang et al., 2020). For instance, meat products produced from wagyu and other beef breeds are considerably different in composition due to the higher intramuscular fat content and high amounts of unsaturated fatty acids, thereby, more vulnerability to lipid oxidation for the latter one (Boylston et al., 1996; Bermingham et al., 2021). There is a knowledge gap in the effect of bioactive compounds encapsulated in nano entities in meat

products prepared from different breeds. The hypothesis of this research was whether nanoencapsulation of mānuka and rosemary oils in the form of nanoemulsions and nanocapsules would improve their stability, *in vitro* antioxidant activity, and release characteristics.

Hence, the objectives of this study were the synthesis and characterisation of mānuka and rosemary oils -containing nanoemulsions and nanocapsules and comparing their antioxidant effects against free oils. Encapsulated forms were characterised by laser diffraction, thermogravimetry, microscopy, Fourier-transform spectroscopy, and *in vitro* antioxidant assays.

6.2. Materials and Methods

6.2.1. Materials

The steam-distilled mānuka essential oil (triketone content around 25 %) was kindly supplied by Tairawhiti Pharmaceuticals Ltd. (Te Araroa, New Zealand). The rosemary oil was purchased from "Now Foods" (Auckland, New Zealand). The oil samples were stored in a freezer at -20 °C to avoid the effect of the light and temperature. Oils samples were removed from the freezer and kept at room temperature for 30-45 min just before the analysis. The vacuum-packed grain-fed Wagyu beef tenderloins were supplied by the Black origin (New Zealand Wagyu, Christchurch). The tenderloins from crossbred Angus/ Hereford were obtained from Gourmet Butchery, Napier (New Zealand). Meat samples were stored in the freezer at -20 °C and thawed overnight before the analysis. All chemicals used for this analysis were of analytical grade and purchased from Sigma-Aldrich (USA).

6.2.2. Preparation of essential oils- containing nanoemulsions

In this study, nanoemulsions and nanocapsules were synthesised using sodium alginate solution as an aqueous phase, mānuka/rosemary oil as the oil phase and whey protein as an emulsifier. The sodium alginate (1 %) was dissolved in distilled water under constant stirring for 24 hours to allow complete hydration. Separately, whey protein concentrate (WPC) was hydrated in distilled water under constant magnetic stirring for 12-24 hours. To prepare denatured WPC, the overnight hydrated protein solution was heated at 80 °C, and pH 8 was adjusted after cooling at room temperature. Both solutions were filtered through 0.4 µm membrane filters to remove any- aggregate materials. Finally, mānuka and rosemary oils were added to the whey protein solution, and then both solutions were mixed and homogenised at 12,000 rpm using an ultra-turrax homogeniser (Ultra-turrax, IKA) for 2-3 minutes. This coarse emulsion was prepared according to the method of Volić et al. (2022). The emulsion was subjected to a high-pressure homogenisation system (microfluidiser, Massachusetts, USA) to obtain nanoemulsions. The emulsions were passed through a microfluidiser working at 150 MPa for three cycles (Salvia-Trujillo et al., 2013).

6.2.3. Preparation of essential oil containing-nanocapsules

The calcium chloride (1 %) solution was added dropwise while magnetic stirring (at room temperature) the nanoemulsions to produce crosslinks and obtain nanocapsules. The nanocapsules were suspended in the same solution overnight and then recovered by centrifugation and washing twice with distilled water. Finally, obtained nanocapsules were re-suspended in distilled water and used for further characterisation.

6.2.4. Particle size and zeta potential analysis of essential oil containing nanocapsules and nanoemulsions

The particle size and zeta potential of the nanoemulsion and nanocapsules were measured using a Malvern Zetasizer Nano ZS instrument (Horiba, Japan). The samples were diluted with milli-

Q water, added to the electrophoretic mobility cell, and examined at a scattering angle of 173 ° utilising Malvern Zetasizer (Nano ZS).

6.2.5. Fourier-transform infrared spectroscopic (FTIR) analysis of essential oils and their nanoforms (nanoemulsions and nanocapsules)

Fourier-transform infrared spectroscopy (FTIR) analysis of oil, emulsion and nanocapsule samples was performed with an iDr 7 ATR-FTIR spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to confirm the presence of chemical constituents. Each emulsion and oil sample was put directly on the surface of platinum-diamond crystal using glass transfer pipettes, while the nanocapsule was dried in ambient air and then mixed with potassium bromide. The spectrum for each sample was obtained from 400 to 4000 cm^{-1}. The obtained data were examined using OMNIC^{TM} software (Thermo Scientific, Auckland, New Zealand).

6.2.6. Microscopic analysis of essential oil containing nanoemulsions and nanocapsules

Light microscopy was used to study the shape and distribution of nanoemulsions, while scanning electron microscope image was conducted to evaluate the shape and size of nanocapsules. Before SEM analysis, nanocapsules were mounted on stubs with double adhesive tape and sputter coating with gold (Baltec SCD 050, Balzers, Liechtenstein). The coated samples were observed under SEM (FEI Quanta 200, FEI Electron Optics, Eindhoven, the Netherlands) at 20 kV accelerating voltage.

6.2.7. Thermogravimetric analysis of essential oils and their nano-entities (nanoemulsions and nanocapsules)

To determine the thermal stability of mānuka oil, emulsion and nanocapsules, thermal gravimetric analysis was carried out using a thermal analyser (TGA, model STA 449 F5 Jupiter). Each sample of around 10 mg was heated from 30 to 300 °C with a heating rate of 10

°C. The curves were analysed using the NETZSCH ASC software (NETZSCH, Selb, Germany).

6.2.8. Total phenolic content determination of essential oils and their nano-entities (nanoemulsions and nanocapsules)

The total phenolic content of essential oil, nanoemulsion and nanocapsules was determined using the Folin-Ciocalteu method, according to Sridhar and Charles (2018). The 100 µL samples were mixed with 1000 µL Folin-Ciocalteu reagent (0.20 N), and after 6 minutes, 800 uL of sodium bicarbonate (7.5 %) was added, followed by vigorously mixing. The contents were incubated for 30 minutes in the dark, and absorbance was read at 765 nm using a spectrophotometer. The TPC content is expressed as µg of gallic acid equivalents per µL of the sample.

6.2.9. In vitro release profile of essential oils from their nano-entities (nanoemulsions and nanocapsules)

To determine the release profile of mānuka oil from nanoentities, model food systems were prepared to mimic real aqueous, alcohol-containing, and fatty foods using distilled water (with 0.02 % tween 80), 10 % and 50 % ethanol-containing solutions (Amani et al., 2021). The exact quantities of free, emulsified, and encapsulated mānuka oil were added and sealed into a dialysis membrane cut off between 12 and 14 kDa (Snakeskin dialysis tubing, Thermo Fisher, USA) 25 mL of food simulants and stirred at 150 rpm at 4 °C. At preselected time intervals of 0, 1, 2, 4, 5, 7, 9, 24, 48 and 96 hours, 2 mL of samples were removed from the food stimulants and replaced with fresh simulant to maintain a constant volume. The absorbance of samples was noted at 260 nm using a Multiskan GO microplate spectrophotometer equipped with Skanlt software version 3.2 (Thermo Scientific, USA).

6.2.10. Mathematical modeling

In order to determine the release mechanism of encapsulated essential oils, mathematical modelling was performed by fitting the release data to some models available in the literature, as described by Rochín-Wong et al. (2018). All these models are based on quantification of the amount of drug released as a function of time: $M_t = M_t(t)$, and the release rate is presented by the time derivative of M_t: $r = \{dMt(t)\}/dt$

6.2.10.1. Zero-order kinetic model

The equation used to calculate zero-order drug delivery is as follows:

$$\frac{M_t}{M\infty} = k_0 t$$

Here M_t / M_∞ = fractional amount of the drug released at time t (hours), k_0 = zero order release constant.

6.2.10.2. First-order kinetic model

This model accurately describes the release profiles when the main mechanism is related to anomalous diffusion. The equation used to calculate first-order drug delivery is as follows:

$$\frac{M_t}{M\infty} = 1 - e^{-k_1 t}$$

Here M_t / M_∞ = fractional amount of the drug released at time t (hours), k_1 = first order release constant

6.2.10.3. Higuchi Model

This model shows a good correlation, where release profiles have simple Fickian diffusion as the main mechanism. The equation used to calculate first-order drug delivery is as follows:

$$\frac{M_t}{M\infty} = k_H t^{0.5}$$

Here M_t / M_∞ = fractional amount of the drug released at time t (hours), k_H = Higuchi diffusion constant

6.2.10.4.Hixson-Crowell model

The equation used to calculate Hixson-Crowell drug delivery is as follows:

$$M_{\infty}{}^{1/3} - M_t{}^{1/3} = k_2 t$$

Here M_∞ = initial amount of the drug present in solution, M_t drug released at time t (hours), k_2 = constant showing surface-volume relation

6.2.10.5.Korsmeyer-Peppas model

This model explains the general solute release from different polymeric devices (Rochín-Wong et al., (2018). The equation used to calculate the Korsmeyer-Peppas model drug delivery is as follows:

$$\frac{M_t}{M\infty} = k_{KP} t^n$$

Here M_t / M_∞ = fractional amount of the drug released at time t (hours), k_{KP} = Characteristic constant of the system

n is a diffusion exponent used to describe the release mechanism. The values of n= 0.5 indicate that the release mechanism is Fickian diffusion, n= 0.5-1.0 release mechanism is non-Fickian diffusion and values above 1 show that Case II transport (zero order).

6.2.11. *Determination of in vitro antioxidant activities of essential oils and their nano-entities (nanoemulsions and nanocapsules)*

6.2.11.1.2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The electron-donating ability of mānuka-free oil, emulsion and nanocapsules was measured with 1, 1-diphenyl-2, picrylhydrazyl (DPPH) as a free radical, according to the method of Li et al. (2022) with slight modifications. For this purpose, the nanoemulsion and nanocapsule

samples were diluted in ethanol and vortexed, followed by filtration using 0.45 µm filter paper. To determine the antiradical activity, 100 µL of the prepared sample was mixed with 2 mL of DPPH solution (0.5 mmol/L). The mixture was shaken vigorously and left in the dark for 30 minutes for incubation at room temperature. The absorbance was measured at 515 nm using a UV-Vis spectrophotometer (Evolution 201) equipped with INSIGHT ™ software (Thermo Scientific™, United States). The following equation was used to calculate the free radical scavenging activity:

$$Radical\ scavenging\ activity = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad \text{..... Equation 1}$$

Here, A_{sample} is the absorbance of the DPPH+ essential oil sample, and $A_{control}$ is the absorbance of the DPPH methanol.

6.2.11.2.2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The ABTS radical scavenging activity of the oil, emulsion and nanocapsule samples was determined according to the method of Sridhar and Charles (2018). Firstly, $ABTS^{\bullet+}$ radical was generated by dissolving equal amounts of 7 mM of ABTS aqueous solution and 2.45 mM aqueous potassium persulfate solution, allowing it to react at room temperature for 12-16 hours in the dark. The stock solution of $ABTS^{\bullet+}$ radical was prepared by diluting it with ethanol and adjusting its absorbance value in the range of 0.90 ± 0.02 at 734 nm. Then, 0.2 mL of the sample/ standard was mixed with the 1.8 mL of the generated $ABTS^{\bullet+}$ radical. The control sample was prepared by adding the exact amounts of radicals with double distilled water. Lastly, the absorbance reading was noted at 734 nm, and the percentage inhibition was determined using Equation 1.

6.2.11.3.Ferric radical absorbing power (FRAP) assay

The free radical absorbing power (FRAP) assay of essential oil, nanoemulsions and nanocapsules samples was conducted using a method of Sridhar and Charles (2018) with few modifications. Firstly, FRAP reagent was produced through the mixing of sodium acetate buffer (300 mM, pH 3.6), $FeCl_3$.6H2O (20 mM) and tripyridyl triazine (10 mM in 40 mM HCl) in a ratio of 10:1:1 in volume. Samples of essential oil, nanoemulsions and nanocapsules were mixed with FRAP reagent and incubated for 30 min at 37 °C. The increase in absorbance of the ferrous tripyridyl triazine-coloured complex was noted at 593 nm against the blank containing all the reagents except the sample. In this assay, $FeSO_4$ was used as a standard to make a calibration curve at different concentrations. The antioxidant activity of samples was calculated from the obtained curve and expressed as µM of $FeSO_4$ /µL of the sample.

6.2.12. Statistical analysis

Each experiment was carried out on three different replicates. To determine the effects of different treatments on meat, a one-way analysis of variance (ANOVA), followed by the *Tukey* method analysis (at a 95 % confidence interval), was conducted using Minitab Version 19.2020.2.0 (Minitab Inc., State College, PA, USA).

6.3. Results and Discussions

6.3.1. Characterisation of nanoemulsions and nanocapsules

The results of particle size and zeta potential (PDI) of nanoemulsions and nanocapsules expressed by intensity (%) are presented in Figure 6.1 (a and b) and Table 6.1. The average particle size analysis showed that rosemary nanoemulsion (369 ± 18.38 nm) and nanocapsules (550 ± 65.06 nm) had significantly larger ($p \leq 0.05$) particles size than the mānuka nanoentities. The nanocapsules and nanoemulsions containing mānuka oil had particle sizes around 343.8 ± 15.12 and 330.6 ± 17.61 nm, respectively. The dynamic laser scattering showed that all nanoemulsions and nanoparticles were uniformly scattered between 220 and 800 nm (Figure

6.1 (a)). The size of essential oils containing nanoemulsions was within the size range reported by other studies. Abbasi et al. (2019) also reported that particle size of ultrasound assisted-water in oil nanoemulsion stabilised by alginate and whey protein and designed as carrier delivery of α-linolenic acid of flaxseed oil was uniformly distributed in less than 1000 nm. Similarly, Yilmaz et al. (2019) reported the particle size of oregano oil nanoparticles between 290 and 483 nm. A schematic representation of nanoemulsions and nanocapsules is presented in supplementary data (Figure 6.3). The essential oil stability and retention inside a core material depend on various factors such as the chemical nature of essential oil (active groups and chemical functionality), molecular weight, polarity and wall material characteristics (Assadpour & Jafari, 2019; Ju et al., 2019). The hydrophobicity-hydrophilicity of an essential oil affects the encapsulation process, and surfactants/emulsifiers allow it to balance this character and ameliorate the encapsulation efficiency (Pavoni et al., 2020).

Zeta potential represents the surface charge of nanoentities in solutions and is an essential factor influencing these nanodispersions' stability. In this study, the mānuka nanoemulsions and nanocapsules were moderately negatively charged, varying from – 12 mV to -10 mV. The zeta potential of rosemary nanoemulsions was -14 mV, while rosemary nanocapsules were highly negative (-25.31 mV) (Figure 6.1 (b)). The negative zeta potential values of nanoemulsions and nanocapsules indicate the adsorption of sodium alginate onto whey protein to form a bilayer around the droplets. The results agree with previous reports describing the negative zeta potential of essential oil-loaded nanoforms. Abbasi et al. (2019) also reported the formation of negatively charged nanoemulsions using sodium alginate and whey protein. However, Yilmaz et al. (2019) showed positively charged nanoparticles, and their zeta potential values increased (from +25.2 to 47.7 mV) with an increase in essential oil. Zeta potential values usually vary from -100 to 100 mV, and higher values (positive to negative) result in a stable nanosystem (Jafari, 2017; Abbasi et al., 2019). These values depend on the charge of actual

particles and cationic and anionic ions, which move with particles in the electric field. Thereby, the negative charge seems to be attributed to the anionic constituents in whey protein and sodium alginate nanodispersions (Surh et al., 2006; Yilmaz et al., 2019).

Table 6.1. Physico-chemical and *in vitro* antioxidant properties of nanoemulsions, nanocapsules and free essential oils (mānuka and rosemary oils).

		Mānuka oil	Mānuka nanoemulsion	Mānuka nanocapsules	Rosemary oil	Rosemary nanoemulsion	Rosemary nanocapsules
Total phenolic compounds (µg GA /µL sample)		1104 ± 37.65^{a}	389 ± 52.14^{c}	316 ± 34.32^{cd}	704 ± 54.27^{b}	246 ± 18.60^{d}	268 ± 44.79^{d}
Radical scavenging activity	**DPPH•** (%)	85 ± 2.28^{a}	48 ±2.66^{b}	47 ± 1.73^{bc}	39 ± 1.34^{bcd}	37 ± 4.66 ^{cd}	34 ± 6.11^{d}
	ABTS• (%)	65 ± 4.15^{a}	38 ± 0.23^{b}	24 ± 3.05^{c}	49 ± 2.37^{b}	41 ± 10.38^{b}	17 ± 0.46^{c}
	$Fe^{2+\bullet}$	2050 ± 147.2^{a}	691 ± 20.59^{c}	636 ± 13.01^{c}	1448 ± 155.6^{b}	574 ± 1.31^{c}	524 ±17.09^{c}
Average Particle size (nm)		-	343.8 ± 15.12^{c}	330.6 ± 17.61^{c}	-	369 ± 18.38^{b}	550 ± 65.06^{a}
Zeta potential (mV)		-	-12.64 ± 3.58	-10.07 ± 0.28	-	-25.31 ± 0.68	-14.12 ± 1.138

FRAP units: (µg of $FesO_4$/µL of the sample), Total phenolic compounds (TPC) units: (µg GA/µL sample).

Different superscripts within a row represent a statistically significant difference ($p \leq 0.05$).

6.3.2. FTIR analysis

FT-IR spectroscopic analysis of essential oils, nanoemulsions, and nanocapsules was performed to determine the molecular interactions between the components in nanoemulsions and nanocapsules (Figure 6.1 (c)). The absorption peaks in alginate spectra near 1418 and 1616 cm^{-1} are due to a carboxyl group's symmetric and asymmetric vibration (COO-), respectively. These peaks were also observed in mānuka and rosemary nanoentities, either nanocapsules or nanoemulsions; however, the absorption was weakened. For the sodium alginate, whey protein, nanoemulsions and nanocapsules, it is observed that characteristics peak in the range between 3200-3600, which is due to the stretching vibration of O-H bonds of these molecules. Other peaks attributed to the stretching vibration of C-H aliphatic chains are observed near 2900 cm^{-1} in the spectrum of all compounds. In the whey protein, peaks between 3000 and 3500 cm-1 correspond to the stretching vibration of free hydroxyl and N-H bonds in the amino groups. These peaks in the mānuka and rosemary nanoentities spectrum indicate that essential oils were successfully encapsulated in the sodium alginate and whey protein because these peaks were absent in native oils. Absorption bands appearing near the 2750 and 3000 cm^{-1} could be due to the stretching vibration of the C-H bond in the CH_2 and CH_3 groups, respectively. Two absorption bands at 1403 and 1541 cm^{-1} associated with the O-H bending are less discernible in the nanoemulsions and nanocapsules containing rosemary oil than in the neat rosemary oil. It may be due to the hydrogen bonding between the O-H group of rosemary compounds and the N-H and O-H group of carrier materials such as whey protein and sodium alginate.

Observing Figure 6.1 (c), an absorption peak between 1690-1720 cm^{-1} in the infrared spectrum of mānuka oil, ME and MC is due to strong C=O stretching and attributed to the presence of β-triketones, including leptospermone, isoleptospermone, flavesone and grandiflorone (Liu et al., 2021). These peaks were stronger in neat mānuka oil compared to the mānuka

nanoemulsions and nanocapsules. Characteristics peak of the keto group of camphor in rosemary oil and its nanoentities displayed at 1746 cm^{-1}, whereas peaks around 1375 cm^{-1} and 1450 cm^{-1} could be due to the ether group from the epoxy region of 1,8 cineole. The major compounds in rosemary are 1, 8 cineole and camphor, which constitute more than 50 % of its total content (confirmed by Gas Chromatography-Mass Spectroscopy (GCMS)). All these compounds contribute to C-H stretching bands between 1375 and 1442 cm^{-1} in the fingerprint region and between 2873 and 2967 cm^{-1} in the functional group region. In spectra of rosemary oil and rosemary oil containing nanoentities, an intense peak was observed at 885 cm due to the carbonyl (CH and CH_2) groups bending, which seems to be due to the presence of α-pinene. Mānuka oil and its nanoemulsions and nanocapsules also exhibited this peak but were sustainably lower than the rosemary oil because alpha-pinene is present in lower quantities in the mānuka oil (Kinninmonth et al., 2013). In studies by Liu et al. (2021) and Kim et al. (2022), the presence of aliphatic and conjugated triketones bands between 1724 and 1674 cm^{-1} in the FTIR spectrum of mānuka oil was also noticed. The changes observed in the absorption of the FTIR spectrum indicate the molecular interactions between essential oils and carrier agents, i.e., sodium alginate and whey protein.

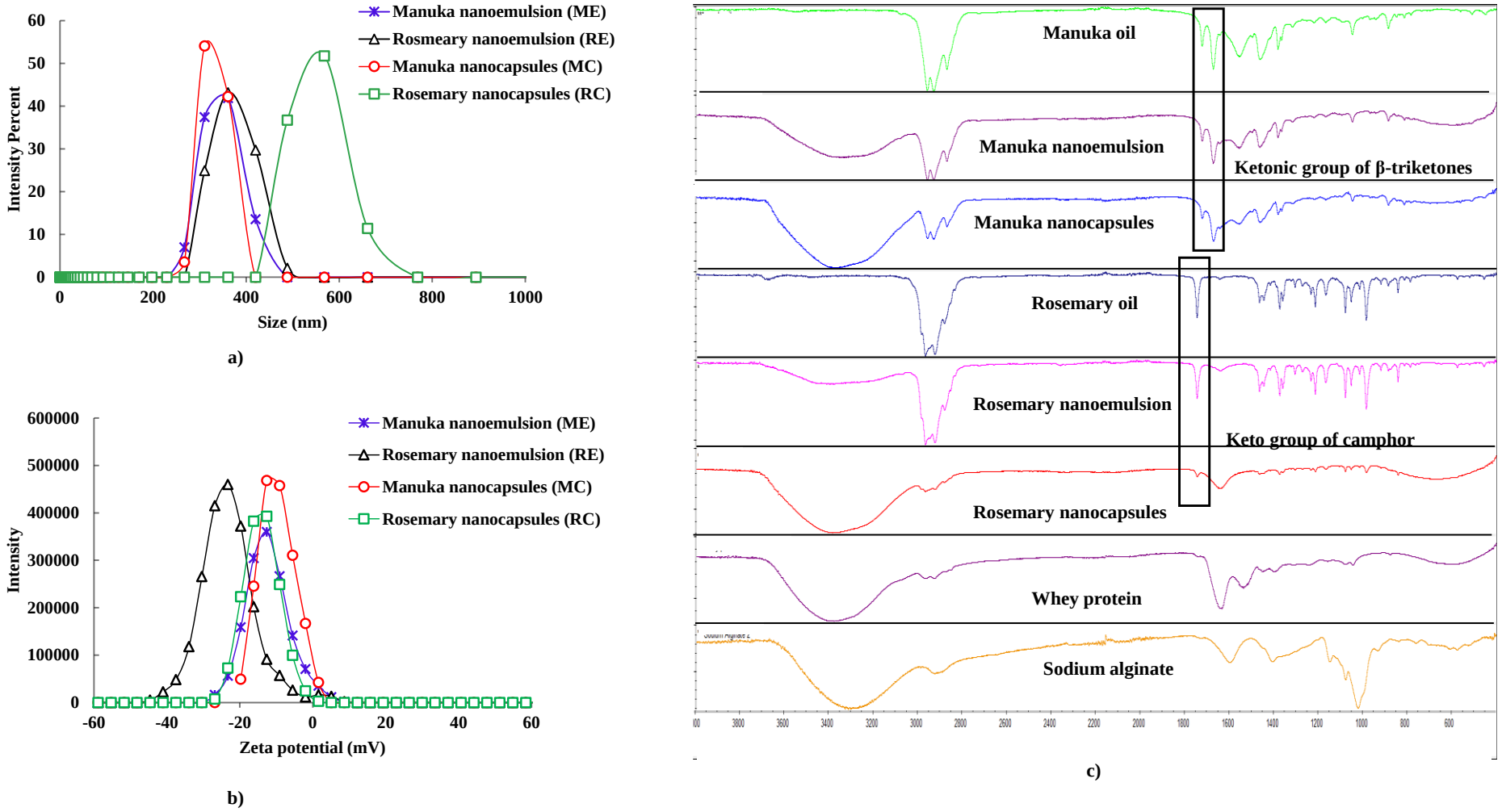


Figure 6.1. Particle size distribution (a), zeta potential (b), and Fourier transform infrared spectroscopic (c) analysis of nanoentities.

6.3.3. Thermogravimetric analysis

The thermograms of essential oil, nanoemulsions and nanocapsules are presented in Figure 6.2 (a and b). This analysis was conducted to determine the effect of nano-emulsification and nanoencapsulation on the thermal stability of essential oils. The mānuka and rosemary oil presented a single thermal event associated with its evaporation, beginning at 50 °C and having maximum evaporation at 160 °C. When the maximum temperature was reached, mānuka oil was completely degraded, while rosemary oil showed some remaining ashes. Essential oils are usually made up of volatile constituents, which sometimes account for more than 95 % of the compounds of the total oil constituents.

In the TGA thermogram of mānuka oil nanoemulsions and nanocapsules, the first weight reduction was under 100 °C, which may be due to the evaporation of internal water. The second weight loss was observed between 100 and 175 °C related to the mānuka oil decomposition. For rosemary oil thermograms, instant weight loss was observed up to 130 °C, and then 28 % of the rosemary oil compounds were maintained at 250 °C. Nanocapsules loaded with rosemary oil lost a very small amount of weight below 100 °C (internal water evaporation) and then between 100 and 150 °C (rosemary oil compounds evaporation). Rosemary nanoemulsions and nanocapsules had around 42 and 65 % of the remaining compounds, which were not decomposed even at 275 °C.

Nonetheless, nanoemulsions and nanocapsules loaded with essential oils presented a similar degradation profile to their respective essential oils; however, a slighter shift towards higher temperatures may be due to alginate coating and crosslinking reaction. Among all forms (free oil, nanoemulsions and nanocapsules), nanocapsules of both oils showed the highest thermal stability.

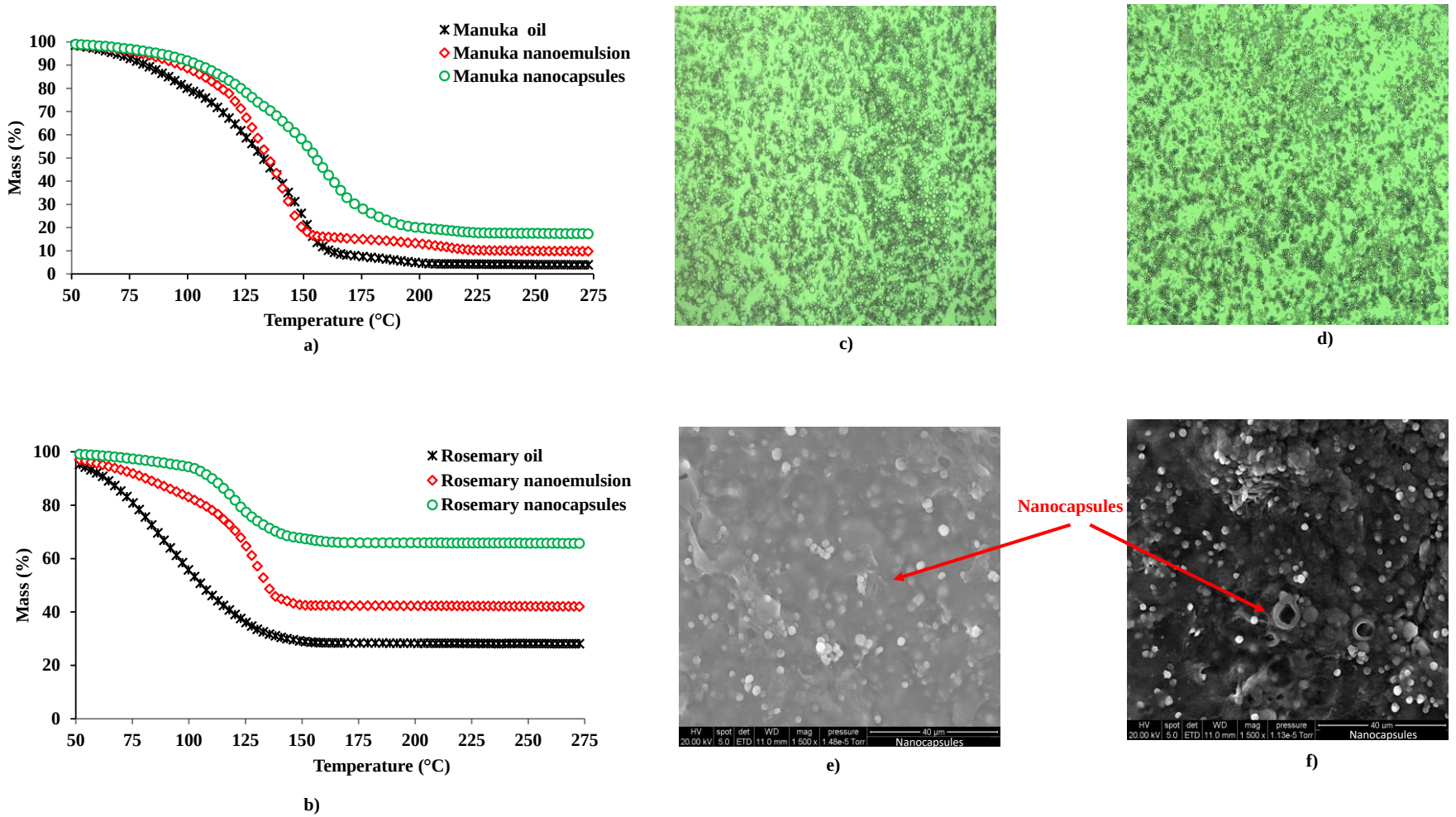


Figure 6.2. Thermogravimetric analysis of mānuka (a) and rosemary oil (b) and their nanoentities. Optical microscopic images of mānuka (c) and rosemary nanoemulsions (d). Scanning electron microscope images of mānuka (e) and rosemary nanocapsules (f).

This thermal behaviour confirms the high volatility of free essential oils and justifies ameliorated thermal stability of nanocapsules. Amani et al. (2021) also reported improvement in the thermal stability of rosemary essential oil after encapsulation in high amylose corn starch nanoparticles through inclusion complexation.

6.3.4. Microscopic analysis

The structure and dispersion of nanoemulsions were observed through an optical microscope (Figures 6.2 (c and d)). These images confirm the presence of essential oils as dispersed phases in an aqueous phase of sodium alginate and whey protein. It suggests that oil droplets have consistent shapes and were uniformly distributed in the aqueous phase, thus confirming the oil in water-type nanoemulsion formation. In the microscope images, since the freshly prepared nanoemulsions were observed, it can be seen that a significantly less degree of aggregation occurred. A scanning electron microscope was used to observe the structure of nanocapsules after coating these with gold. The nanocapsule's core-shell type structure can be clearly observed from the markings in Figure 6.2 (f and e). The synthesised nanocapsules showed a spherical structure and, up to some extent, aggregation. This aggregation could be due to the higher concentration of alginate and emulsifier (Yang et al., 2021). A difference in the droplet size of mānuka and rosemary nanoemulsions was observed, also confirmed by the Zetasizer analysis, which might be due to the difference in viscosities of both oils (Wooster et al., 2008). In general, particle disruption during homogenisation could be hindered when the viscosity of the lipid phase increases, thereby leading to a larger particle size of droplets (Wooster et al., 2008).

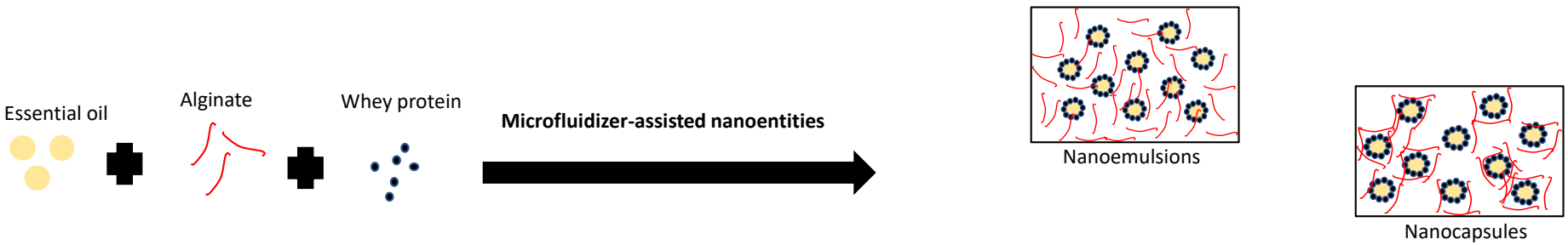


Figure 6.3. Schematic representation of essential oil-added alginate and whey protein nanoemulsions and nanocapsules.

6.3.5. Total phenolic content and in vitro antioxidant activity

The mānuka essential oil had a higher total phenolic content (1104 µg GA /µL sample) than the rosemary oil (704 µg GA/µL sample), as shown in Table 6.1. Among the free mānuka oil, nanoemulsions and nanocapsules containing mānuka oil, free mānuka oil showed a higher total phenolics than the nanoemulsions and nanocapsules (Table 6.1). However, the total phenolic content of rosemary nanocapsules and nanoemulsions were not significantly different. Rosemary oil possessed significantly lower ($p \leq 0.05$) phenolic content than mānuka oil. The phenolic content and composition of rosemary oil have been widely studied (Kaur et al., 2021). In a previous study, the total phenolic content of rosemary oil was 225 ± 6 mg/L, which was significantly lower than the clove, thyme and oregano (Viuda-Martos et al., 2010).

The antiradical activities of free oils, nanoemulsions, and nanocapsules were evaluated by the DPPH, ABTS and FRAP assay. Both essential oils exhibited concentration-dependent antioxidant effects. As shown in Figure 6.4 (a, b, c, and d), mānuka and rosemary oils exhibited strong antioxidant activities against DPPH, ABTS and Fe^{2+} radicals. The highest DPPH•, ABTS• and $Fe^{2+\bullet}$ scavenging activity was shown by mānuka oil (85 %, 64 % and 2050 µg of $FesO_4$/µL of the sample, respectively)) followed by rosemary oil (39 %, 49 % and 1448 µg of $FesO_4$/µL of the sample). Nanoemulsions and nanocapsules of essential oils showed lower free radical (DPPH•, Fe^{2+}•, and ABTS•) scavenging activities than the free form of both oils. The reason may be the limited release of essential oils/bioactive compounds from the nanocapsules and nanoemulsions into the antioxidant measuring medium. It has been reported that adding bioactive droplets to the whey protein matrix reduces their mobility and diminishes the release from the matrix and antioxidant efficacy. In addition, the low solubility of whey protein in organic solvents like ethanol, which was used to prepare antioxidant reagent solution DPPH and ABTS, has been noticed (Daniel et al., 2004).

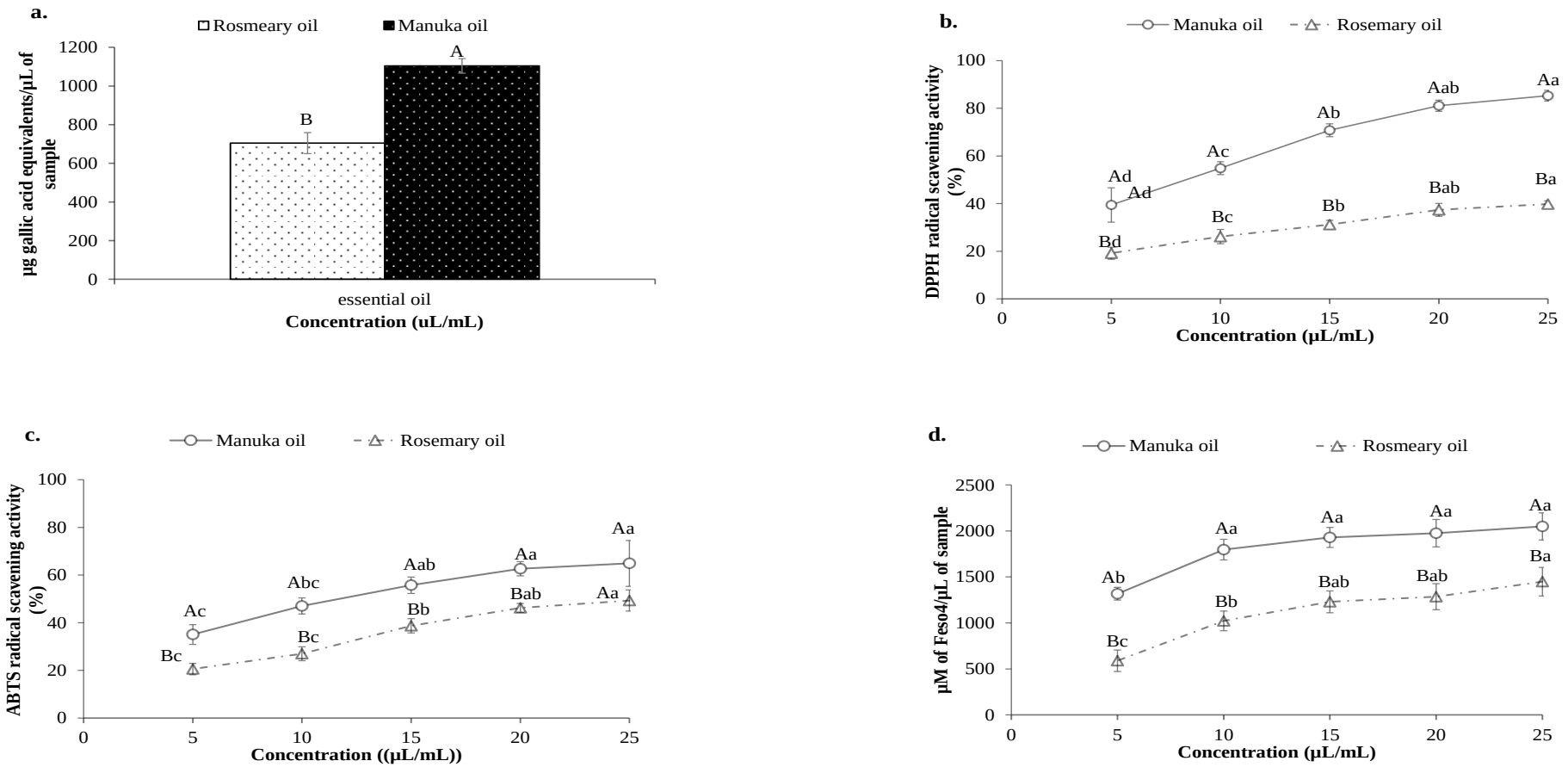


Figure 6.4. Total phenolic contents and antioxidant effects of mānuka and rosemary oils. a) Total phenolic content, b) DPPH radical scavenging activity, c) ABTS radical scavenging activity and d) FRAP radical scavenging activity.

Capital letters (A, B, C) show a significant difference between different treatments under the same concentration in each graph. Lowercase letters (a, b, c) show a significant difference ($p \leq 0.05$) between different concentrations under the same treatment at a in each graph.

Nikolaidis and Moschakis (2018) reported that ethanol induces reversible denaturation-related structural changes in the structure of whey proteins, which may interfere with the essential oil retention or release in nanoemulsions and nanocapsules. The antioxidant activity of rosemary oil is due to the presence of carnosol and carnosic acid, while alpha-terpinene and terpineol are responsible for the antioxidant characteristics of mānuka oil. Previous studies have reported the strong antioxidant efficacy of rosemary oil (Kaur et al., 2021).

6.3.6. In vitro release profile of mānuka oil from nanoemulsions and nanocapsules

The effect of different incubation times on the release profile of mānuka oil in three food-simulating materials is presented in Figure 6.5. Incubation time significantly influenced ($p \leq 0.05$) the release of essential oils from nanocapsules and nanoemulsions.

In general, a rapid release of mānuka oil was observed, followed by a slowed release at longer intervals until a plateau value was achieved. In distilled water, 45 % of mānuka oil was released from the free form before reaching its plateau value of full release, while nanoemulsions and nanocapsules released 72 and 85 % of oil, respectively. After 24 hours, nanoemulsions and nanocapsules continuously released the oil, indicating their continuous release than the free oil, which showed its burst release for 24 hours. A similar pattern was observed for the mānuka oil suspended in 10 % ethanol either in free, emulsified or encapsulated form. However, all forms released their maximum amount during the first 24 hours in 50 % ethanol. The difference in release profile of encapsulated and emulsified mānuka oil in different food-simulating media could be related to their solubility in the aqueous medium, as well as the stability of the essential oil compounds. For example, free mānuka oil suspended in distilled water and 10 % ethanol solution showed a slower release than its nanoforms. However, the higher release of mānuka oil in a 50 % ethanol solution could be linked to its increased solubility in ethanol. The increased release of mānuka oil from nanoentities could be attributed to the smaller droplet size

and increased surface areas for oils to come in contact with a simulating solvent. The mathematical modelling to understand the release mechanisms of essential oils from nanoemulsions and nanocapsules was shown in supplementary data (Figures 6.6, 6.7, and 6.8).

However, the higher release rate of oil from the nanoemulsions than nanocapsules could be attributed to the absence of crosslinks in the former. We hypothesise that the reduced release of essential oils from the nanocapsules was due to crosslinks, which served as a barrier to the mobility and partitioning of essential oils through the why protein particles. A similar study by Karimi-Khorrami et al. (2022) reported a lower release of thymol from the calcium alginate films containing thymol-loaded nanostructured lipid carriers (NLC) than the films containing nanoemulsions due to the presence of triglycerides with crystalline domains acting as a barrier to release of thymol. Similar to our results, Amani et al. (2021) also reported a slow release of rosemary essential oil in distilled water and 10 % ethanol from high amylose corn starch nanoparticles prepared through inclusion complexation than the 50 % ethanol solution (Amani et al., 2021).

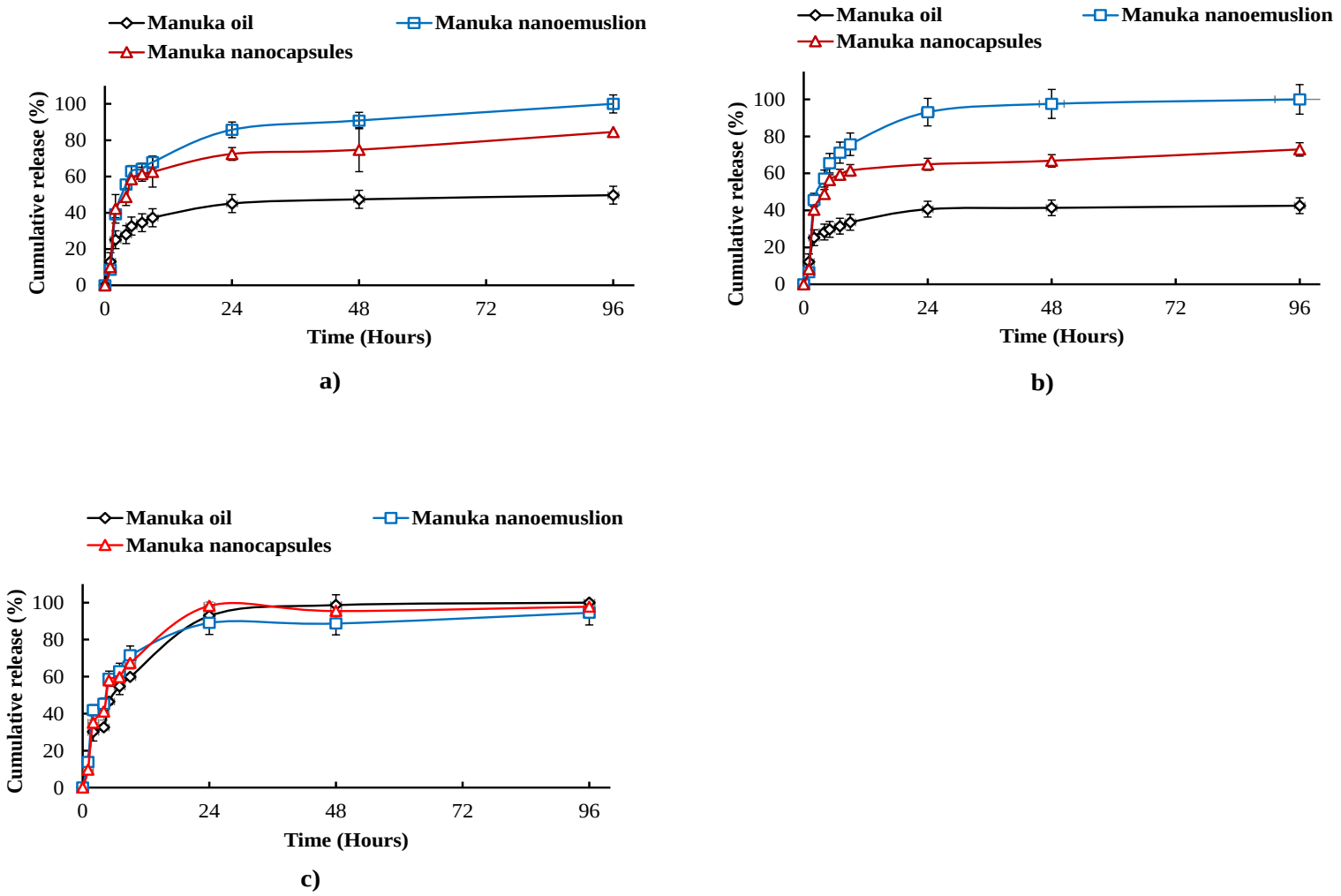


Figure 6.5. Release profile of mānuka oil from nanocapsules and nanoemulsions in a) distilled water containing 0.01 % tween 80, b) 10 % ethanol solution, and c) 50 % ethanol solution.

The data obtained from *in vitro* release of essential oil from nanoentities in different solvents were fitted into several kinetic models such as Higuchi, first order, zero order, Hixson-Crowell and Korsmeyer-Peppas models (shown in Figures 6.6, 6.7 and 6.8). The linearity and correlation of each graph were evaluated by the closeness of its regression coefficient to unity, while Korsmeyer-Peppas was applied to understand the drug release mechanism. In the case of distilled water (with 0.02 % tween 80), the best-fit model was the Higuchi model, in which the highest correlation values, i.e., R^2 = 0.9551, 0.9139 and 9076 for mānuka oil, nanoemulsion and nanocapsules was observed. Similarly, the release of essential oil from free nanoemulsion and nanocapsules showed the best fit for the Higuchi model. The best-fit model for data on the release of essential oil from free, nanoemulsion and nanocapsules in 50 % ethanol was the Higuchi model with R^2 values around 0.95, while first-order and Hixson-Crowell models also showed values above 0.90. As per Higuchi's law, the solvent penetrates the matrix and dissolves the embedded essential oil, and thus the release seems to be a process mainly controlled by diffusion (Xu et al., 2016). In distilled water, 10 % ethanol and 50 % ethanol, essential oils were released from nanoentities through this mechanism. In addition, the values obtained from the Keresmaeyer model for diffusion exponent (n) varied between 0.5 and 1, indicating a non-Fickian diffusion mechanism for essential oil release. Non-Fickian diffusion, also known as anomalous diffusion, describes both diffusion and swelling-controlled drug release. Like our study, the release kinetics of drugs from nanoparticles and micelles was reported by Gandhi et al. (2014) and Li et al. (2014). In these studies, the Higuchi model showed the best fit and release was related to non-Fickian diffusion (Gandhi et al., 2014; Li et al., 2014); however, Fickian diffusion was also observed for some core-shell micelle by Li et al. (2014).

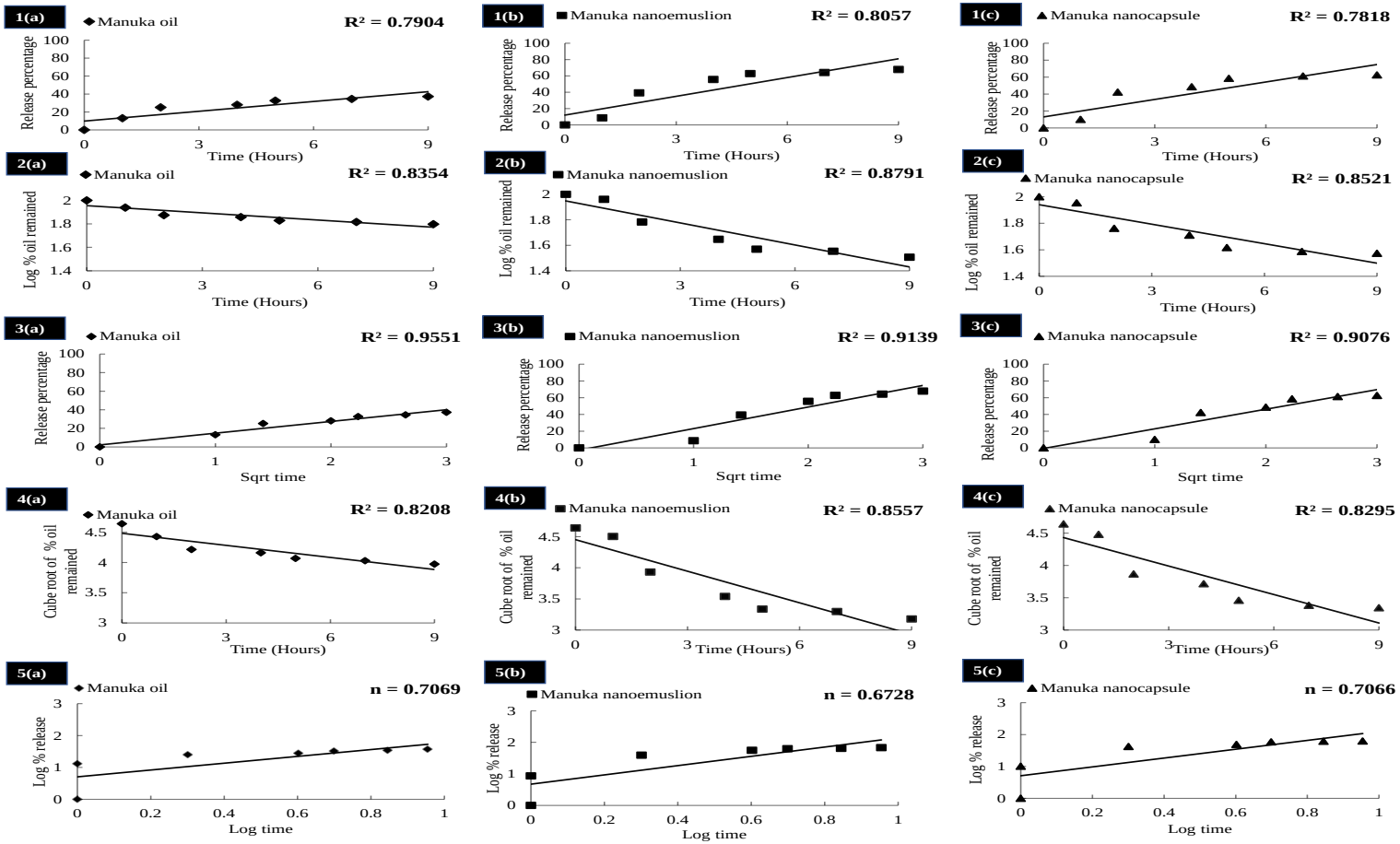


Figure 6.6. *In vitro* release characteristics of essential oil in distilled water fitted to various models 1) zero order, 2) first order, 3) Higuchi, 3) Hixson Crowell and Korsmeyer-Peppas model.

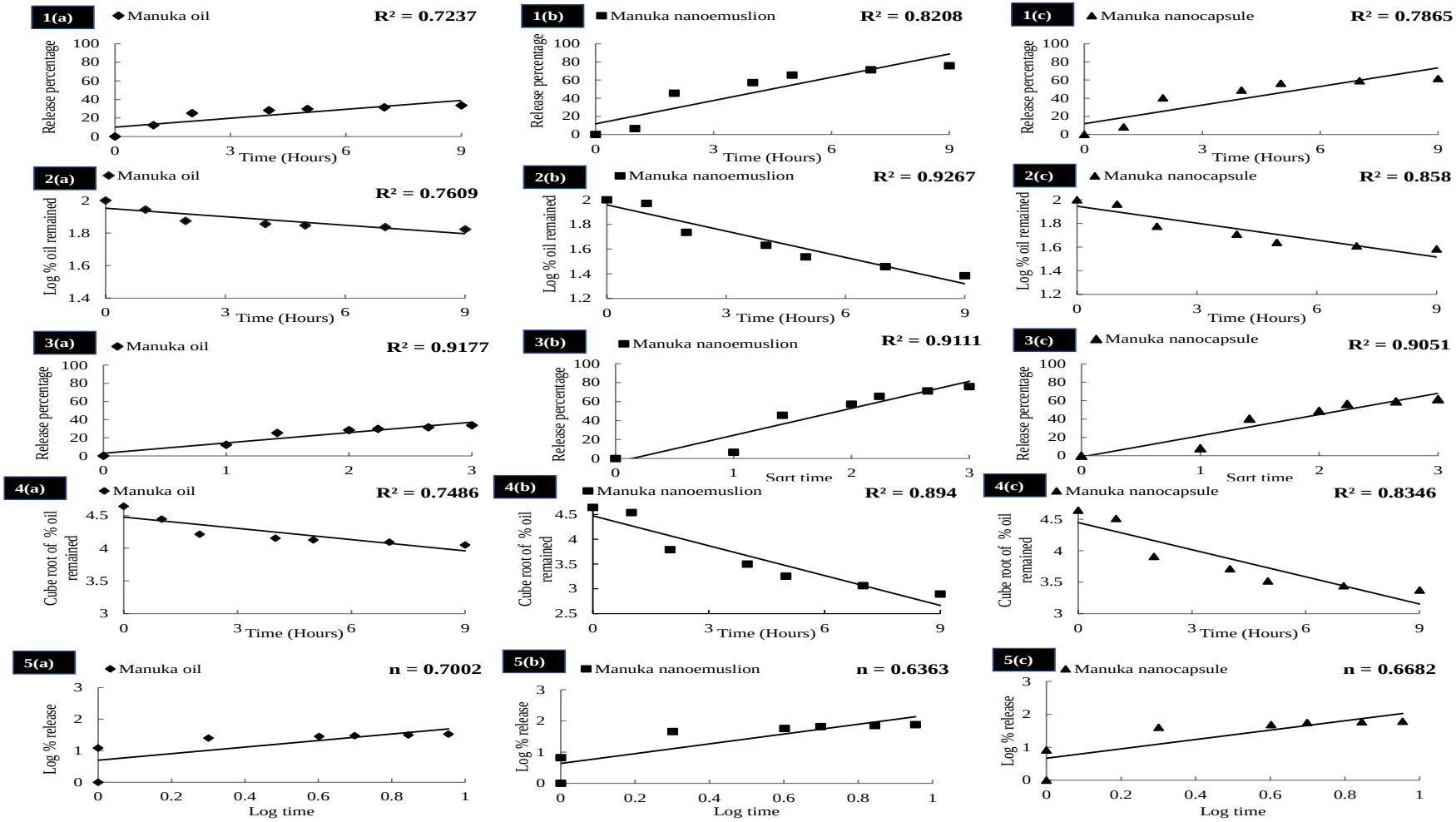


Figure 6.7. *In vitro* release characteristics of essential oil in 10 % ethanol fitted to various models 1) zero order, 2) first order, 3) Higuchi, 4) Hixson Crowell and 5) Korsmeyer-Peppas model.

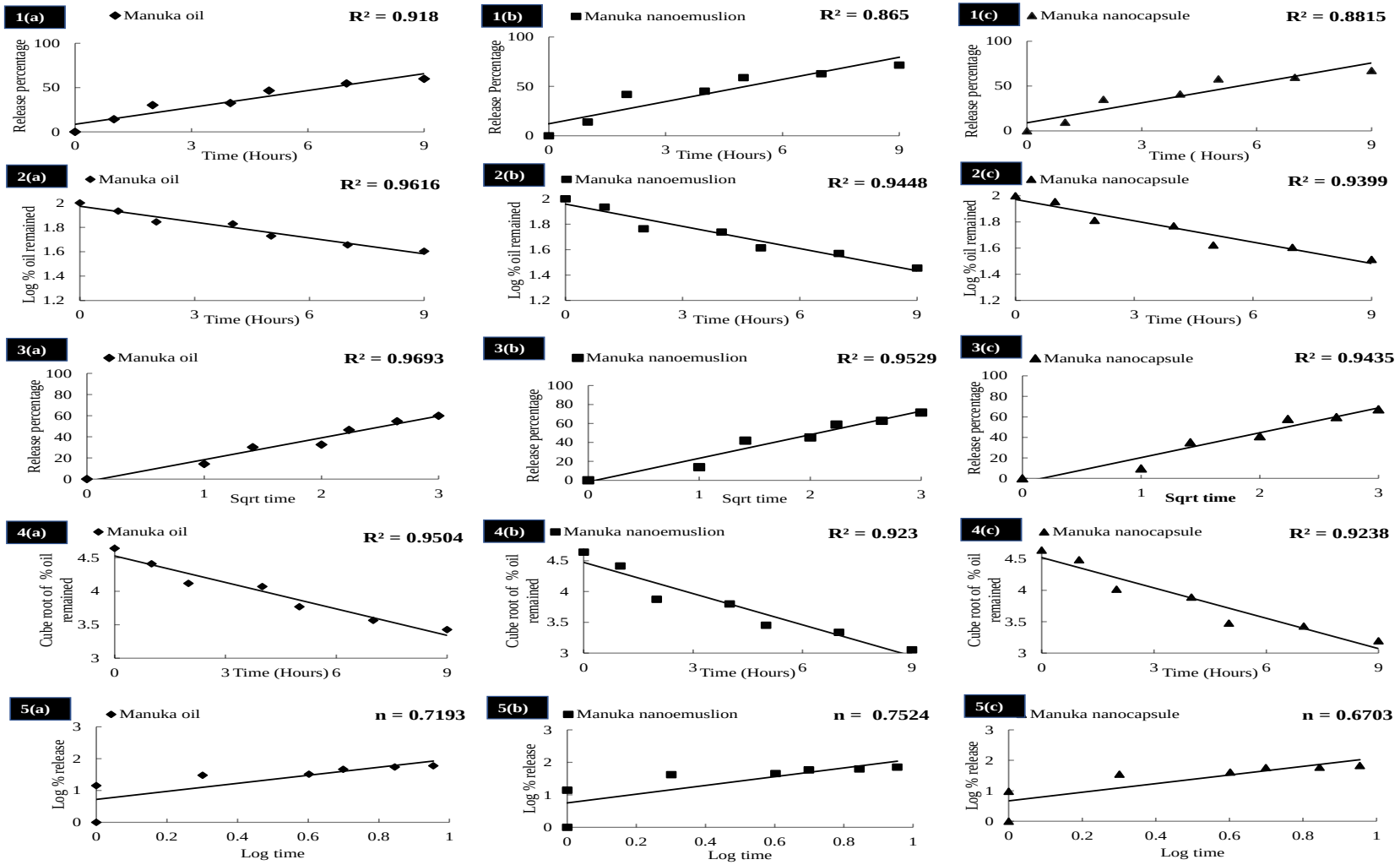


Figure 6.8. *In vitro* release characteristics of essential oil in 50 % ethanol fitted to various models 1) zero order, 2) first order, 3) Higuchi, 3) Hixson Crowell and Korsmeyer-Peppas model.

6.4. Conclusions

This study successfully fabricated essential oil-loaded nanoemulsions and nanocapsules using sodium alginate as an aqueous phase and whey protein as an emulsifier. Nanoemulsions and nanocapsules exhibited more thermostability and sustained release characteristics; however, their antioxidant activity was lower than the free oils. Molecular interactions between essential oil constituents and carrier materials in FTIR spectrums confirmed the successful loading of essential oils in these carriers. The findings of this study showed that mānuka or rosemary oils containing nanoemulsions and nanocapsules could be used as a natural antioxidant agent for meat preservation, along with improved stability. Improved thermal stability of nanoemulsions and nanocapsules than free oils would allow the use of these bioactives in cooked food products. Encapsulation of volatile and unstabilised bioactive in food-grade carriers like sodium alginate and whey protein confer thermostable systems than their free forms. In addition, the present method may be used for encapsulating other food-grade essential oils.

Chapter 7 Antimicrobial and antioxidant characteristics of nanoentities containing mānuka and rosemary oils: Application in wagyu (high-fat) and crossbred (low-fat) beef preservation

Abstract

In recent years, encapsulated essential oils have gained widespread attention as natural antimicrobial agents and alternatives to chemical preservatives for food applications. This study determined the antimicrobial efficacies of mānuka and rosemary oil containing nanoemulsions and nanocapsules against pathogenic Gram-positive and Gram-negative microbes through *in vitro* assays (broth dilution and disc diffusion assays). Further, the antimicrobial and antioxidant effect of both essential oils and their nano-entities was compared against sodium nitrite-added and without any antimicrobial agents-added (controls) wagyu (23 %- fat) and crossbred (2.3 %) beef pastes (15 days refrigerated storage). Free mānuka and rosemary oils showed better *in vitro* antimicrobial effects against all tested microbes than their nanoforms; however, rosemary oil was more effective against Gram-negative bacteria than mānuka oil. The mānuka oil showed more inhibition effect against Gram-positive microbes, decreased cell viability, disrupted cell wall permeability, and released intracellular materials and proteins, as confirmed through spectrophotometric assays and electron micrographs. In wagyu and crossbred beef pastes, emulsions of both oils showed the lowest microbial growth than free oils, nanocapsules, sodium nitrite and controls, and this effect was significantly different in both meat pastes. Rosemary oil and its nanoentities showed the lowest growth of Gram-negative microbes than the other treatments, while mānuka oil and its nanoentities were effective against Gram-positive bacteria. No significant difference ($p \leq 0.05$) among mānuka oil, rosemary oil and their nano-entities was noticed in lipid oxidation values of crossbred pastes, while in wagyu, nanoemulsions showed the lowest oxidation values than controls and

sodium nitrate-added pastes. The results suggest the complete replacement of sodium nitrite with mānuka oil and its nanoentities, particularly against Gram-positive microbes.

7.1. Introduction

In recent years, foodborne illness and the biodeterioration of food products by pathogenic and spoilage microbes have remained a significant concern for the food industries (Lee et al., 2019; Amani et al., 2021). Chemical preservatives like sodium nitrite in processed (cured) meat products have long been used to retard microbial spoilage and other deteriorative reactions. However, due to increased consumer interest in natural products and awareness of the side effects of chemical preservatives (especially sodium nitrite), there is a demand for natural or plant-based preservatives for meat products (Thales et al., 2011; Kaur & Kaur, 2020; Fraqueza et al., 2021). An attractive alternative to chemical preservatives is the usage of essential oils as a natural antimicrobial agent to prevent the growth of harmful and pathogenic food spoilage microbes. Several research studies have reported the potential antimicrobial effect of essential oils such as tea tree, thyme, oregano, clove, cinnamon, basil and citronella in various meat commodities (Burt, 2004; Ghaderi-Ghahfarokhi et al., 2016). *Rosmarinus officinalis*, also known as rosemary, is an aromatic plant; its essential oil possesses antimicrobial characteristics against various microbes that trigger meat spoilage (Kaur et al., 2021). Similarly, essential oils obtained from a plant native to New Zealand and some parts of Australia, i.e., *Leptospermum scoparium* (renowned as mānuka), possess a wide range of antimicrobial activities against oral, skin and food bacteria. These antimicrobial efficacies are due to the presence of beta-triketones in their chemical composition (Perry et al., 1997; Porter & Wilkins, 1999; Chen et al., 2016). These oils have great potential to be used in food formulations to prolong their shelf life and inhibit the growth of spoilage and pathogenic microbes.

Nevertheless, essential oils are seldom utilized as food preservatives because of their interactions with food constituents, high concentrations required, ability to change the organoleptic characteristics of food products (strong odour and flavour), low stability and hydrophobic nature (less solubility), which affects their bioavailability and homogeneity on the applied food surfaces (Tisserand & Young, 2013; Baser & Buchbauer, 2015). Encapsulation is an alluring and promising approach to ameliorate stability, and solubility, mask the undesirable characteristics (odour and flavour) and maintain the sustained release characteristics (Ghaderi-Ghahfarokhi et al., 2016; Bora et al., 2018). In this context, several nano and micro-scale approaches have been developed and applied to produce appropriate encapsulating material for essential oils to be added to food commodities, which can overcome the limitation of their application in pure form (Delshadi et al., 2020; Kaur & Kaur, 2020). Due to the wonderful characteristics of alginate, such as safety, non-toxic nature, biodegradability, biocompatibility, gel-forming capability, and low cost, it is one of the most commonly used carrier materials (Natrajan et al., 2015; Salvia-Trujillo et al., 2015). Natrajan et al. (2015) reported that 0.3 mg/mL of sodium alginate and 0.6 mg/mL of chitosan produced minimum size (below 300 nm) turmeric and lemon grass oil containing nanoparticles with 71 % and 86.9 % encapsulation efficiency, respectively (Natrajan et al., 2015). Compared with the coarse emulsion, nanoemulsions of lemongrass and clove oils prepared using microfluidization application showed improved antimicrobial activity through faster and increased inactivation kinetics of *Escherichia coli* (Salvia-Trujillo et al., 2015).

However, the food matrix is an essential factor affecting bioactive compounds' separation (partitioning and release) from the encapsulating material (Wang et al., 2020). For instance, meat products from beef breeds, such as wagyu, are considerably different in fatty acid composition and fat contents than the other breeds like crossbred (Bermingham et al., 2021), thereby may show a difference in microbial growth. There is a knowledge gap in the

antimicrobial effect of bioactive compounds encapsulated in nano entities in meat products prepared from different fat content, i.e., low and high-fat. In addition, there is a lack of scientific evidence about the improved functionality of essential oil containing alginate nanoemulsions and nanocapsules to neat oils (non-encapsulated ones). The application of alginate nanoemulsions containing essential oils in the form of an edible film has been reported. At the same time, there is a literature gap on the direct addition of essential oils containing nanoentities (nanoemulsions and nanocapsules) in food products, especially meat products.

The main objective of this research was to determine the antimicrobial effectiveness of mānuka and rosemary oil containing nanoemulsions and nanocapsules against pathogenic Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative (*Salmonella* spp. and *Escherichia coli*) microbes through *in vitro* assays (broth dilution and disc diffusion assays). In addition, the mechanisms of antimicrobial activity of mānuka and rosemary oils against tested microbes were also determined. Further, the prepared nanoentities containing essential oils were tested in wagyu and crossbred beef pastes to evaluate paste preservation from inoculated Gram-positive and Gram-negative microbes during the refrigerated shelf-life of beef pastes for 15 days.

7.2. Materials and Methods

7.2.1. Materials

The oil samples of mānuka oil and ROs were provided by Tairawhiti Pharmaceuticals Ltd. (Te Araroa, New Zealand) and "Now Foods" (Auckland, New Zealand), respectively. The vacuum-packed grass-fed wagyu tenderloins were purchased from Black origin (New Zealand Wagyu, Christchurch). The crossbred beef (Angus/Hereford) tenderloins were from Gourmet Butchery, Napier (New Zealand). Both samples were stored in a -20 °C freezer and thawed overnight before the analysis. All chemicals used in this study were of analytical grade.

7.2.2. *Chemical composition analysis of mānuka and rosemary oils using GC-MS*

Gas-chromatography-mass spectrometry (7890A, Agilent Technologies, USA) equipped with a VL MSD triple-axis detector was used to analyze the chemical composition of mānuka and rosemary oils (Van Vuuren et al., 2014). TG-5MS (Thermo Fisher) column with dimensions of 30 m length, 0.25 mm diameter and 0.1 um film thickness were used in this analysis. The essential oil samples were injected using a split ratio of 200:1 and an oven temperature of 220 °C. The initial temperature used was 60 °C for 10 min and then increasing to 220 °C at a rate of 4 °C/min, and finally rising to 240 °C at a rate of 1 °C min. The detector conditions, such as temperature and ionization mode, were set at 250 °C and electron impact, respectively. Three beta triketones (Leptospermone, isoleptospermone and flavesone), alpha-pinene and γ-terpinene were used as internal standards. The chemical components were identified by comparing the obtained peaks with the mass spectra library (NIST 05). The structure and properties of the identified chemical compounds were determined using the EPI suite.

7.2.3. *Preparation of essential oils containing-nanoemulsions*

Alginate-whey protein nanoemulsions containing mānuka oil/rosemary oil were prepared according to the method of Salvia-Trujillo et al. (2013). Firstly, whey protein and sodium alginate were dissolved in water separately and hydrated overnight. Whey protein was denatured by heating it at 80 °C for 30 minutes and adjusting pH to 8 using NaOH (at room temperature). Solutions of sodium alginate and whey protein were filtered through 0.4 µm membrane filters to remove any- aggregate materials. Essential oils were dissolved in whey protein under constant magnetic stirring for 2 minutes, then mixed with sodium alginate solutions. Contents were homogenized at 12000 rpm using an ultra-turrax homogenizer (Ultra-turrax, IKA) for 2-3 minutes to prepare coarse emulsions. After this, the prepared emulsion

was subjected to high-pressure homogenization using microfluidics (Massachusetts, USA) at 150 MPa for 3 cycles.

7.2.4. Preparation of essential oils containing-nanocapsules

The dropwise addition of calcium chloride was used to prepare nanocapsules while magnetic stirring the nanoemulsions (Ghayempour & Mortazavi, 2015). Nanocapsules were left in the same solution for 12-24 hours and then recovered by washing with distilled water and centrifugation. Finally, obtained nanocapsules were resuspended in distilled water and used for further analysis.

7.2.5. Determination of in vitro antimicrobial activity of essential oils and their nanoentities

7.2.5.1. Disc diffusion assay

The antimicrobial activity of essential oils, nanoemulsions and nanocapsules was determined by Kirby and Bauer's agar disc diffusion assay using the method of Jeong et al. (2018). The overnight grown bacterial cultures were adjusted to 0.5 Mc Farland, followed by swabbing of this suspension on Mueller Hinton agar plates. The paper discs were placed in the centre of the prepared agar plates. 40 µL of essential oil (pure, 5 and 2.5 % diluted in sterile water containing 0.02 % tween 80) /nanoemulsions/nanocapsules were added to the discs. In positive controls, antibiotics paper discs of streptomycin were placed in the centre of inoculated agar plates, whereas negative control contained only sterile water without any active agent. All prepared agar plates were incubated at 37 °C for 24-36 hours. After incubation, the diameter of the zone inhibition around discs was measured using a vernier calliper, and results were expressed in millimetres. In the case of the non-circular zone, measurements were taken horizontally and vertically at four different places.

7.2.5.2. Determination of minimum inhibitory concentrations

The minimum inhibitory and minimum bactericidal concentrations of essential oils, nanoemulsions and nanocapsules were determined according to the National Committee for Clinical Laboratory Standards (NCCLS) method with few modifications (Wayne, 2003). Firstly, a two-fold serial dilution of essential oil was performed in MHB using 0.02 % tween. 100 µL of prepared dilutions were transferred to 96 well plates and mixed with equal volumes of bacterial suspensions. Positive control containing only bacterial suspension with active agents and negative control without added bacterial suspension was prepared. The microwell plates were incubated for 24 hours at 37 °C, and absorbance reading was taken every 1-hour using Varioskan ^{TM} LUX multimode microplate reader (Thermo Fisher, USA) at 600 nm. In the case of nanoemulsions and nanocapsules, 40 µL of 0.4 mg/mL of p-iodonitrotetrazolium violet dye was added to the well plates after incubation and further incubated for 1 hour at 37 °C. Wells contents were examined for colour change; pink colour development indicates microbial growth. The lowest concentration of essential oils or emulsions at which there is no dye colour change or development of pink colour was considered as MIC value for that nanoemulsion/nanocapsules. To determine the MBC value, around 100 µL of samples were taken from the well, spread on MHB agar plates, and incubated at 37 for 24 hours. The concentration of essential oils at which 99.9 % of the inoculated microbes were killed was considered a bactericidal concentration.

7.2.6. Mechanisms of action of essential oils

7.2.6.1. Cell viability assay

This assay was carried out using the method of Pahalagedara et al. (2022). Overnight-grown bacterial cultures of *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Staphylococcus aureus* were adjusted to a cell density of about 10^7 cfu (colony-forming units)/mL. Essential

oils or sterile water (in case of controls) were added to the cultures and incubated at 37 °C for 24 hours. A BacTiter-Glo assay kit was used to determine the bacterial cell viability at different concentrations. BacTiter reagent was prepared at room temperature by following the manufacturer's instructions and combining lyophilized BacTiter-Glo enzymes/substrate mixture with the buffer. One hundred microliters of prepared BacTiter-Glo reagent were transferred to an opaque walled 96-well plate and combined with the essential oil-treated bacterial cultures. The contents were mixed in the dark, and luminescence intensity was noted using Varioskan ™ LUX multimode microplate reader (Thermo Fisher, USA). The results were expressed as a percentage of untreated cells at different concentrations.

7.2.6.2. Cell membrane integrity

To determine the cell membrane integrity of *Salmonella*, *Escherichia coli*, *Listeria monocytogenes,* and *Staphylococcus aureus*, a CellTox™ Green cytotoxicity kit (Promega, UAS) was used, following the manufacturer's instructions (Pahalagedara et al., 2022). In brief, overnight-grown microbial cultures were centrifuged at 10000× g for around 5 minutes and adjusted to 10^7 cfu/mL by resuspending in MHB. Essential oils/sterile water was added to the microbial suspensions and incubated for 24 hours at 37 °C. 100 µL of samples were removed in opaque walled 96-well plates at 0, 2, 4 and 24 hours and mixed with CellTox™ Green regent (2X). The 30 µL of CellTox™ Green dye was combined with assay buffer to prepare 2X green cell tox reagent. At last, samples were incubated for 15 minutes (in the dark), and fluorescence readings (490 nm excitation, 520 nm emission) were noted using Varioskan ™ LUX multimode microplate reader (Thermo Fisher, USA).

7.2.6.3. Outer membrane permeability

Outer membrane permeability of *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* was determined using a method according to the method of

Pahalagedara et al. (2022). For this analysis, non-polar fluorescent probe 1, N-phenylnapthylamine (NPN), was used, as discussed by Pahalagedara et al. (2022).

7.2.6.4. Cell wall damage using alkaline phosphatase (AKP)

An alkaline phosphatase leakage assay was performed to determine the effect of essential oils on *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella* cell walls. This assay was carried out according to the method of Bai et al. (2023) with few modifications. Overnight-grown bacterial cultures were treated with different concentrations (5 and 2.5 %) of mānuka and rosemary essential oils and incubated at 37 °C for 24 hours. After specified incubation time, samples were centrifuged at 5000 rpm for 10 minutes, supernatants were collected, and extracellular alkaline phosphatase was measured using an AKP kit (Sigma, USA). Each untreated microbial culture was used as a control.

7.2.6.5. Release of extracellular material and proteins

The release of 260 nm absorbing materials from the microbial cells was determined according to the method discussed by Zhang (2017) with few modifications. The overgrown microbial cells were centrifuged at 4500 rpm for 10 minutes and washed with the physiological saline thrice. Cells were resuspended in a buffer and incubated in the presence of essential oils or sterile water (in the case of controls) at 37 °C (24 hours). After incubation, the cells were centrifuged at 4500 rpm for 10 minutes, and the supernatant was diluted with saline. The absorbance of supernatants was read at 260 nm using Varioskan ^{TM} LUX multimode microplate reader (Thermo Fisher, USA). The concentration of proteins in the supernatant was determined using the Bradford assay kit, according to the protocol described in the manufacturer's instructions. Bovine serum albumin was used as a standard, and the amount of released proteins was calculated from the calibration curve.

7.2.6.6. Scanning electron microscopy (SEM)

Scanning electron microscopy was conducted to determine the morphological changes in microbial cells treated with essential oils. This analysis was performed according to the method of Zhang (2017). Overnight-grown microbial cells were centrifuged and adjusted to 0.5 McFarland using fresh MHB. Essential oils were added into microbial suspensions and incubated at 37 °C for 24 hours. After incubation, cells were centrifuged at 4000 rpm for 10 minutes and washed with phosphate-buffered saline thrice. The washed cells were fixed in 5 % glutaraldehyde at 4 °C for around 8 hours and then dehydrated using different ethanol concentrations, 10, 30, 50, 80 and 100 %, for 10 minutes each. After dehydration, cells were fixed on an aluminium stub using double adhesive tape and sputter coated with gold for 200 sec. Eventually, cells were observed under SEM (FEI Quanta 200, FEI Electron Optics, Eidhoven, the Netherlands) at 20 kV accelerating voltage. Images were captured under different magnifications (10, 000 and 30, 000X).

7.2.7. Meat pastes preparation and storage conditions

Traces of excess fat were removed from wagyu and crossbred tenderloins using a knife and were chopped into small cubes. Both types of meat were minced through a meat mincer separately (Mainca, PM-98, Barcelona, Spain) with a plate of 8 mm diameter holes. The specific mixture was transferred into Hobart meat bowl Chopper (Ohio, USA) and mixed for about 15 minutes to obtain a homogeneous paste. The prepared paste was further used for the different treatments of essential oils, nanoemulsions and nanocapsules.

7.2.7.1. Proximate composition analysis of meat pastes

The proximate composition analysis of meat pastes, including fat, protein, moisture, and ash content, was determined using an AOAC-approved method (AOAC, 1990). Protein content

was evaluated with the Dumas method (AOAC, 1995) and multiplied with the factor of 6.25 to calculate crude protein content. For the determination of fat content, the Soxhlet method was used (AOAC, 2005).

7.2.8. Preparation of essential oils and nanoentities added meat- systems

The prepared pastes were divided into eight different lots for treatments with 2.5 % mānuka oil, 2.5 % rosemary oil, 2.5 % mānuka oil nanoemulsion (ME), 2.5 % mānuka oil nanocapsules (MC), 2.5 % rosemary oil nanoemulsions (RE), 2.5 % rosemary oil nanocapsules (RC), and 150 mg/kg sodium nitrite (SN), followed by mixing in a mixer (Kogan, 1600 W, New Zealand) for about 15 min at room temperature. A control sample without any preservative/treatment was also prepared from crossbred and wagyu meat pastes. All prepared samples were packed in zip-lock bags (100 mm × 155 mm) and stored at 4 °C in a dark room. Samples were removed at different time intervals of 0, 7 and 14 days and checked for colour and lipid oxidation.

7.2.8.1. Lipid oxidation analysis

The 2-Thiobarbituric acid (TBA) values were evaluated with modifications to the method of Botsoglou et al. (1994). To plot the calibration curve, 1, 1, 3, 3- tetraethoxypropane (TEP) was used as a standard. The results were noted as mg of malonaldehyde per kg of meat sample.

7.2.8.2. Colour

The colour of the meat pastes was evaluated by using the Minolta colourimeter (Chroma meter, CR 400, Hong Kong, China). Firstly, equipment was calibrated using a white tile, and each meat paste was scanned for colour values (L^*, a^* and b^*) in a petri dish. All readings were taken in triplicates.

7.2.9. Essential oils and nanoentities- treatment of meat pastes for microbiological analysis

The prepared meat paste was divided into four different lots and mixed with an overnight grown bacterial culture of *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* adjusted to 10^4-10^5 cfu/g. Each bacterial suspension was mixed separately using a bench mixer (Kogan, 1600 W, New Zealand) in a sterile container and on separate days to avoid cross-contamination. After mixing bacterial suspensions, pastes were left at room temperature for 10-15 minutes to ensure complete adhesion of microbes to the meat surface. Each microbe-inoculated paste was further divided into eight different batches for treatments with mānuka oil (MO), rosemary oil (RO), mānuka nanoemulsion (ME), mānuka nanocapsules (MC), rosemary oil (RO), rosemary nanoemulsions (RE), rosemary nanocapsules (RC), and 150 mg/kg sodium nitrite (SN) respectively. Control samples for each paste (wagyu and crossbred) and each microbe were prepared separately, containing bacterial suspensions without added preservatives. A higher concentration (2.5 %) of essential oil was tested against Gram-negative microbes than the Gram-positive ones (1.25 %). Samples were transferred in vacuum pouches, sealed, and stored at refrigerator temperature (- 4 °C). Samples were taken out at specified time intervals and analyzed for microbial growth.

7.2.9.1. Microbial growth analysis in meat

At preplanned storage intervals, paste samples were aseptically removed and transferred to a stomacher bag, followed by the addition of 45 mL sterile peptone water. The samples were homogenized in a lab blender at 200 rpm for 2 min. Serial dilution was prepared using the same diluent, and 0.1 mL samples of each dilution were spread on the selected agar plates. *Salmonella* was enumerated on brilliant green modified agar, and *Escherichia coli* was evaluated on Eosin-Methylene blue (EMB) agar plates. *Listeria monocytogenes* and

Staphylococcus aureus were enumerated on Oxford's and Baird Parker's agar plates. All plates were incubated at 37 °C for 24-36 hours and then observed for the number of colonies. The microbiological results were transformed into a logarithmic scale and expressed as log cfu/g of meat paste.

7.2.10. Statistical analysis

Each experiment was carried out on three different replicates. Statistical evaluation was performed using IBM SPSS to determine the significant differences between the treated and untreated samples ($p \leq 0.05$).

7.3. Results and Discussions

7.3.1. Chemical composition of essential oils

The complete GCMS profile and chromatograms of mānuka and rosemary oils are shown in Table 7.1. Twenty-nine compounds representing about 95 % of mānuka oil and 20 constituents comprising about 99 % of the rosemary oil, respectively, were identified. The rosemary oil possessed a typical *Rosmarinus officinalis* profile (Daferera et al., 2000) with elevated amounts of 1, 8 cineole (50 %) followed by α-pinene (12 %), camphor (11 %), β-pinene (6 %) and camphene (5 %). All other compounds were below 16 %, in which cymene, borneol, and caryophyllene were also present. Regarding the chemical class, oxygenated monoterpenes were the top class constituting about 65 % of rosemary oil. The remaining compounds belonged to monoterpenes and sesquiterpene hydrocarbons. However, in mānuka oil, sesquiterpenes were the main class, followed by β-triketones and monoterpene hydrocarbons. Mānuka oil was characterized by a high content of calamenene (12 %) and leptospermone (15 %). Beta triketones, such as flavesone and isoleptospermone, were also present in mānuka oil. In addition, some other compounds, like cubebene, copaene, caryophyllene, and selinene, were also found in mānuka oil. In comparing our results with the findings of Yang et al. (2020) and

Daferera et al. (2000), we observed that oxygenated terpene, especially 1,8 cineole, was the most abundant chemical compound present in rosemary oil. The major component of mānuka oil was leptospermone and calamene, which was consistent with previous results of Porter and Wilkins (1999) and Fratini et al. (2019).

7.3.2. Disc diffusion assay

The antimicrobial efficacy of pure oils (mānuka oil and rosemary oil), nanoemulsions and nanocapsules at various concentrations was measured by paper disk diffusion assay, and the inhibition zones observed in this study are shown in Table 7.2.

The results exhibited that *Listeria monocytogenes* and *Staphylococcus aureus* were the most sensitive to mānuka oil, with an inhibition zone of 15 mm and 12 mm, respectively, at 5 % concentrations. The nanoemulsions and nanocapsules of this oil against the same microbes did not exhibit significant differences ($p \leq 0.05$) in inhibition zones. However, *Salmonella* and *Escherichia coli* were most resistant to mānuka oil, and their nanoentities also showed no inhibition zones at 2.5 % concentrations.

There were no statistically significant differences ($p \leq 0.05$) between Gram-positive and Gram-negative bacteria regarding their sensitivity to rosemary oil (pure and 5 % concentration). On the contrary, rosemary nanocapsules showed no antibacterial effect against all tested bacteria (*Escherichia coli, Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus*) at 2.5 % concentration. The rosemary nanoemulsion showed some antibacterial activity but was only at high concentrations (5 %) and was equal for all microbes. In this experiment, Streptomycin discs were used as a positive control, which showed similar antimicrobial effects against Gram-positive and Gram-negative microbes with an inhibition zone of 25 mm for all bacteria.

The non-significant differences in inhibition zones of oils, nanoemulsions and nanocapsules indicated that emulsification and encapsulation of oils using sodium alginate and whey protein did not adversely affect the antimicrobial activity of essential oils. The results on the antimicrobial potential of mānuka oil using disc diffusion assay are consistent with the previously reported data.

The research study on the antibacterial activity of various essential oils such as mānuka, kānuka, cajuput and niaouli oils and Australian tea tree oil demonstrated the antimicrobial efficacy of mānuka oil against antibiotic-resistant *Staphylococcus aureus*, while no effect against *Pseudomonas aeruginosa* (Harkenthal et al., 1999). Several studies have reported the higher antimicrobial activity of pure essential oils than their nanoforms. Hassanzadazar et al. (2019) also observed no significant differences in the antimicrobial activity of rosemary nanoemulsion on the studied microbes, i.e., *Salmonella* Enteritidis, *Listeria monocytogenes*, *Staphylococcus aureus Shewanella* spp., *Escherichia coli* and *Pseudomonas aeruginosa* than its pure oil (Hassanzadazar et al., 2019). The results of this study are inconsistent with the study of Moghimi et al. (2016a), who reported a four-fold increase in antimicrobial activity of sage oil nanoemulsions prepared using non-ionic surfactants such as tween 80 and span 80 than its bulk oil against *Escherichia coli* and *Salmonella Typhimurium* (Moghimi et al., 2016a). These differences could be attributed to the active binding sites of bioactive compounds of essentials oils with the used surfactant or emulsifier and the prevention of bringing the essential oil constituents into the proximity of the bacterial cell membrane (Swathy et al., 2018; Hassanzadazar et al., 2019).

7.3.3. MIC and MBC values

Table 7.1. Chemical composition analysis of mānuka and rosemary oil using gas chromatography-mass spectrometry (GCMS).

Mānuka oil				Rosemary oil		
Sr No	**Compound name**	**Area %**	**Class**	**Compound name**	**Area %**	**Class**
1.	α-Pinene	1.34	Monoterpene Hydrocarbon	Tricyclene	0.10	Monoterpene Hydrocarbon
2.	β-Pinene	0.11	Monoterpene Hydrocarbon	α-Pinene	12.3	Monoterpene Hydrocarbon
3.	β-Pinene	0.18	Monoterpene Hydrocarbon	Camphene	5.20	Monoterpene Hydrocarbon
4.	m-Cymene	0.13	Monoterpene Hydrocarbon	Bicyclo (3, 1, 1) heptane	0.02	Monoterpene Hydrocarbon
5.	Limonene	0.06	Monoterpene Hydrocarbon	α-pinene	5.07	Monoterpene Hydrocarbon
6.	Cineole	0.13	Oxygenated Monoterpene	β-pinene	0.95	Monoterpene Hydrocarbon
7.	γ -Terpinene	0.09	Monoterpene Hydrocarbon	α-Phellandrene	0.13	Monoterpene Hydrocarbon
8.	iso-Amyl N-valerate	0.03	Monoterpene Hydrocarbon	3-Carene	0.13	Monoterpene Hydrocarbon
9.	α-Cubebene	4.07	Others	α-Terpinene	0.33	Monoterpene Hydrocarbon
10.	Copaene	5.20	Sesquiterpene	o-Cymene	1.89	Monoterpene Hydrocarbon
11.	β-Elemene	0.62	Sesquiterpene	1,8 Cineole	50.0	Oxygenated Monoterpene
12.	α-Gurjunene	0.94	Sesquiterpene	γ-Terpinene	0.51	Monoterpene Hydrocarbon
13.	Caryophyllene	2.34	Sesquiterpene	Terpinolene	0.24	Monoterpene Hydrocarbon
14.	Allo-Aromadendrene	1.60	Sesquiterpene	β-Linalool	0.52	Oxygenated Monoterpene

15.	β-Cubebene	7.24	Sesquiterpene	Camphanone	11.0	Oxygenated Monoterpene
16.	Allo-Aromadendrene	0.62	Sesquiterpene	Borneol	2.50	Oxygenated Monoterpene
17.	Cadina-1(10),4-diene	3.44	Sesquiterpene	1-Terpinen-4-ol	0.60	Oxygenated Monoterpene
18.	Eudesma-4(14),11-diene	4.51	Sesquiterpene	p-Menth-1-en-8-ol	1.92	Oxygenated Monoterpene
19.	α-Selinene	5.10	Sesquiterpene	Bornyl acetate	0.85	Others
20.	γ-Murolene	1.30	Sesquiterpene	β-Caryophyllene	3.95	Sesquiterpene
21.	1.Cadina-1,3,5-triene	6.20	Sesquiterpene	α-Caryophyllene	0.61	Sesquiterpene
22.	Trans-calamenene	18.4	Sesquiterpene			
23.	α-amorphene	4.96	Sesquiterpene			
24.	Flavesone	4.75	Sesquiterpene			
25.	Isoleptospermone	4.32	β-triketones			
26.	Leptospermone	15.75	β-triketones			
27.	Cubenol	1.17	β-triketones			
28.	β-Eudesmol	0.59	Sesquiterpene			
29.	α-Eudesmol	0.849	Sesquiterpene			

Table 7.2. The inhibition zone values observed for mānuka and rosemary oils and their nanoentities against different microorganisms.

Concentration (%)	Treatment	*Listeria monocytogenes*	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella* spp.
Pure	**Mānuka oil**	24.8 ± 4.9^{a}	23.6 ± 1.6^{a}	13.6 ± 1.7^{b}	11.4 ± 0.9^{b}
	Rosemary oil	27.3 ± 2.5^{a}	27.6 ± 2.7^{a}	28.2 ± 2.0^{a}	25.7 ± 6.0^{a}
5 %	**Mānuka oil**	15.5 ± 2.2^{a}	12.5 ± 2.5^{a}	7.3 ± 0.5^{b}	6.3 ± 0.2
	Mānuka nanoemulsion	14.1 ± 0.9^{ab}	12.6 ± 0.7^{a}	6.8 ± 0.7^{b}	6.4 ± 0.4^{bc}
	Mānuka nanocapsules	13.4 ± 1.5^{ab}	10.8 ± 0.8^{ab}	6.7 ± 0.4^{b}	6.5 ± 0.3^{bc}
	Rosemary oil	11.2 ± 1.3^{b}	12.4 ± 2.5^{a}	10.7 ± 1.4^{a}	8.3 ± 1.5^{ab}
	Rosemary nanoemulsion	11.2 ± 1.2^{b}	10.3 ± 1.2^{ab}	11.0 ± 0.0^{a}	9.9 ± 0.2^{a}
	Rosemary nanocapsules	6.5 ± 0.5^{c}	6.6 ± 0.7^{b}	6.6 ± 0.8^{b}	7.0 ± 0.6^{bc}
2.5 %	**Mānuka oil**	13.2 ± 2.0^{a}	11.6 ± 2.5^{ab}	Nd	Nd
	Mānuka nanoemulsion	10.1 ± 1.0^{b}	13.7 ± 1.5^{a}	Nd	Nd
	Mānuka nanocapsules	10.3 ± 0.4^{b}	10.0 ± 0.0^{b}	Nd	Nd
	Rosemary oil	Nd	Nd	6.0 ± 0.0^{b}	Nd
	Rosemary nanoemulsion	Nd	Nd	9.2 ± 0.8^{a}	6.8 ± 0.6^{bc}
	Rosemary nanocapsules	Nd	Nd	Nd	Nd

The results of the antibacterial activity of mānuka oil and rosemary oils against *Salmonella, Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus* are shown in Figures 7.1 and 7.2. Both essential oils inhibited the growth of tested microbes in a dose-dependent manner.

As shown in Figures 7.1 and 7.2, mānuka oil inhibited Gram-negative microbes, *Salmonella* and *Escherichia coli,* at 2.5 % concentration only. On the contrary, this oil was most effective against *Listeria monocytogenes* and *Staphylococcus aureus*, showing MIC of 0.08 % for *Listeria monocytogenes* and 0.04 % for *Staphylococcus aureus*. The lowest MIC value of mānuka oil was found against *Staphylococcus aureus* than the rosemary oil. The antimicrobial activity of rosemary oil against Gram-negative and Gram-positive microbes used in this research study was similar, exhibiting a MIC value of 2.5 % for all microbes (*Salmonella, Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*). Regarding the minimum bactericidal concentration, all microbes were susceptible to rosemary oil at the first concentration and showed no growth on MHB agar plates. The MBC values observed for rosemary oil against *Salmonella* and *Escherichia coli* was 2.5 %, while *Listeria monocytogenes* and *Staphylococcus aureus* were 0.31 %.

The mānuka and rosemary nanoemulsions exhibited variable results against the tested microbes. For instance, as shown in Figure 7.3, mānuka nanoemulsion and nanocapsules inhibited *Salmonella* at only 5 and 2.5 % concentration, while *Escherichia coli* at 5 %. Similar to mānuka oil, mānuka nanoemulsion showed no growth of *Listeria monocytogenes* and *Staphylococcus aureus* at 0.3 % concentration. However, Mānuka nanoemulsion was not very effective against these microbes and inhibited their growth at 0.6 and 2.5 %, respectively. Like rosemary oil, its nanoemulsions inhibited all tested microbes at only the first two concentrations (5 and 2.5 %). Compared with rosemary oil and rosemary nanoemulsion, rosemary nanocapsules were ineffective against tested microbes at tested concentrations,

possibly due to the insufficient release of rosemary oil from its larger nanoforms. The particle size results showed a larger size of rosemary nanocapsules, which may interfere with its release from the nanocapsules.

The antimicrobial efficacies of mānuka oil against *Staphylococcus aureus* (Chen et al., 2016; Jeong et al., 2018; Fratini et al., 2019; Pedonese et al., 2022), *Escherichia coli* (Chen et al., 2016), *Listeria monocytogenes* (Chen et al., 2016; Pedonese et al., 2022), and *Salmonella* (Jeong et al., 2018) have been evaluated by various studies. About Gram-negative bacteria, examining the effect of mānuka seed oil against *Escherichia coli* and *Salmonella*, Prosser et al. (2014) reported their inhibition effect at high doses (EC_{50} =27.8 %). On the contrary, Jeong et al. (2009) reported the dose-dependent effect of mānuka oil (4 % diluted in tween 80) against *Escherichia coli* (K12C600), and oil was not as effective as *Melaleuca alternifolia* oil (also known as Australian tea tree oil).

Different studies have reported the impressive antibacterial effects of mānuka oil against different strains of *Staphylococcus aureus*. For instance, *Staphylococcus sobrinus and Staphylococcus mutans* inhibition at MIC = 480 µg/mL of mānuka oil were shown by Fratini et al. (2017), while Takarada et al. (2004) reported 0.13-0.25 % concentration of this oil to inhibit same strains.

About the antimicrobial potential of rosemary oil, MIC and MBC values of 0.125 % and 0.25 % against *Staphylococcus aureus* and 0.25 and 0.50 % against *Escherichia coli* have been reported by Fu et al. (2007). However, Lara et al. (2016) showed the ineffectiveness of rosemary against *Escherichia coli* (isolated from the *Alouatta* spp. faeces) at a concentration equal to or less than 6.4 mg/mL. The difference in MIC and MBC values could be attributed to the difference in methodologies, microbial strains, and composition of rosemary oil used in these studies.

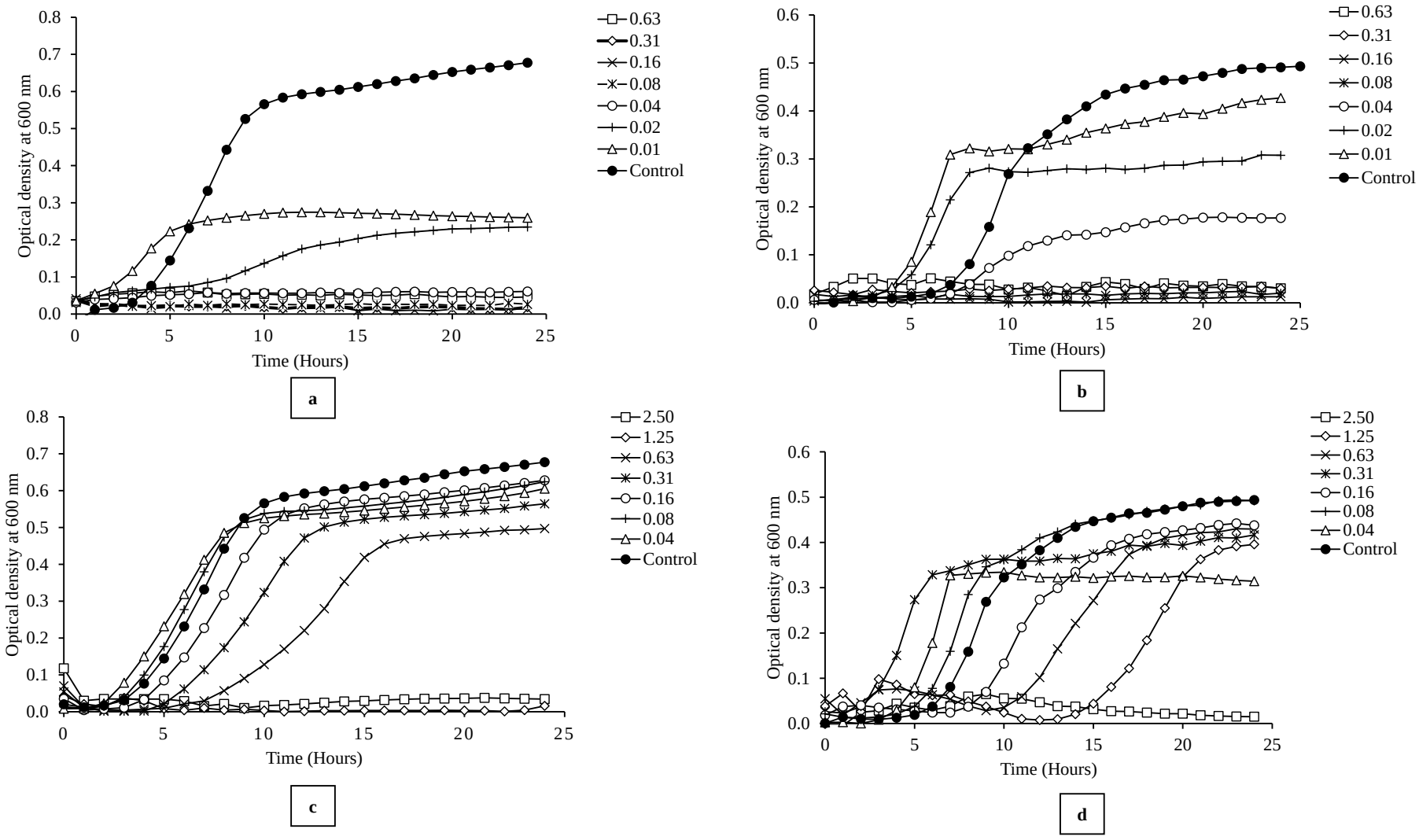


Figure 7.1. Growth curves of *Staphylococcus aureus* (a and c) and *Listeria monocytogenes* (b and d) after treatment with mānuka oil (a and b) and rosemary oil (c and d) at different concentrations. (The control sample contained no preservatives, but the test microbes).

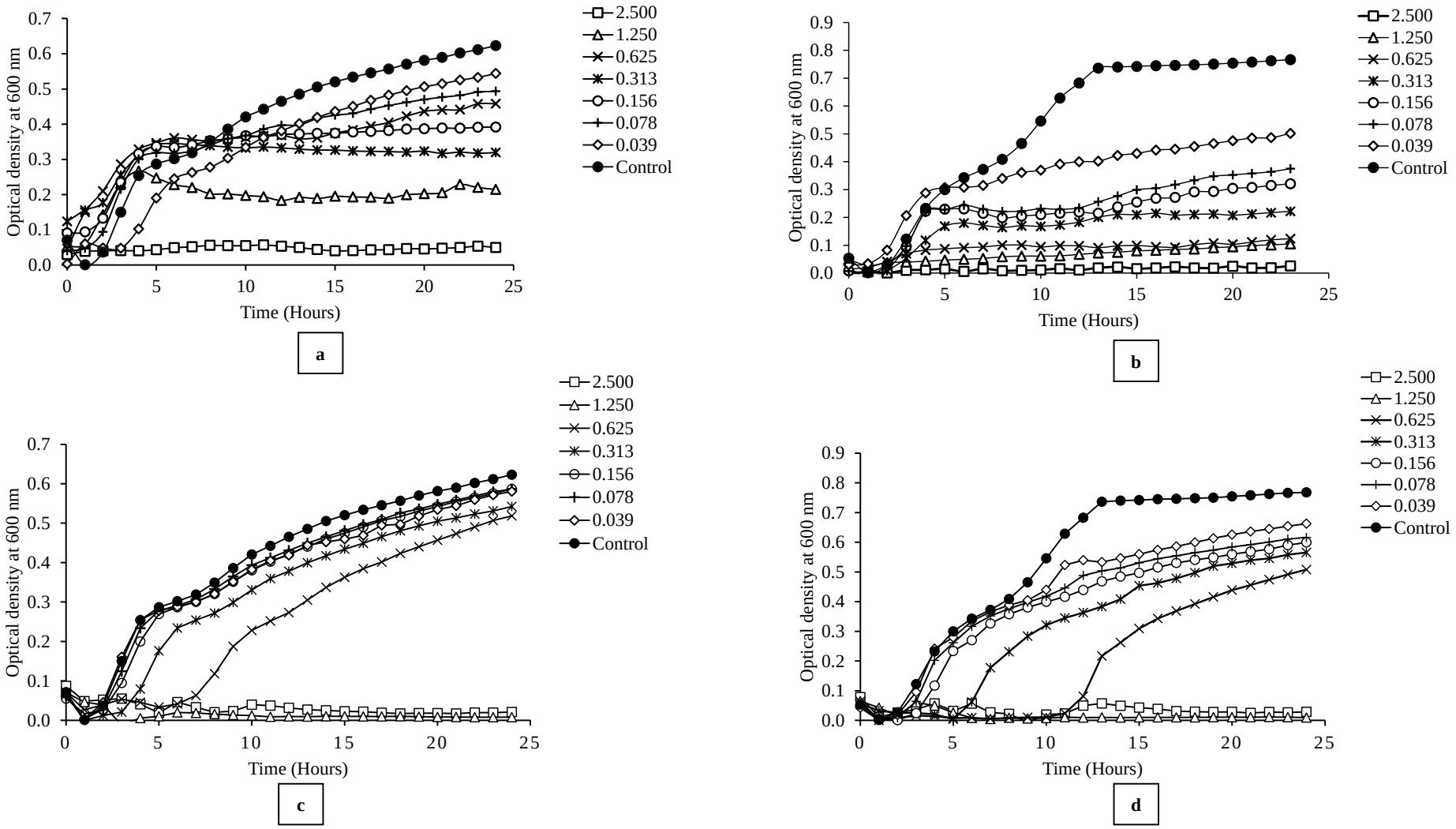


Figure 7.2. Growth curves of *Salmonella* spp. (a and c) and *Escherichia coli* (b and d) after treatment with mānuka oil (a and b) and rosemary oil (c and d) at different concentrations. (The control sample contained no preservatives, but the test microbes).

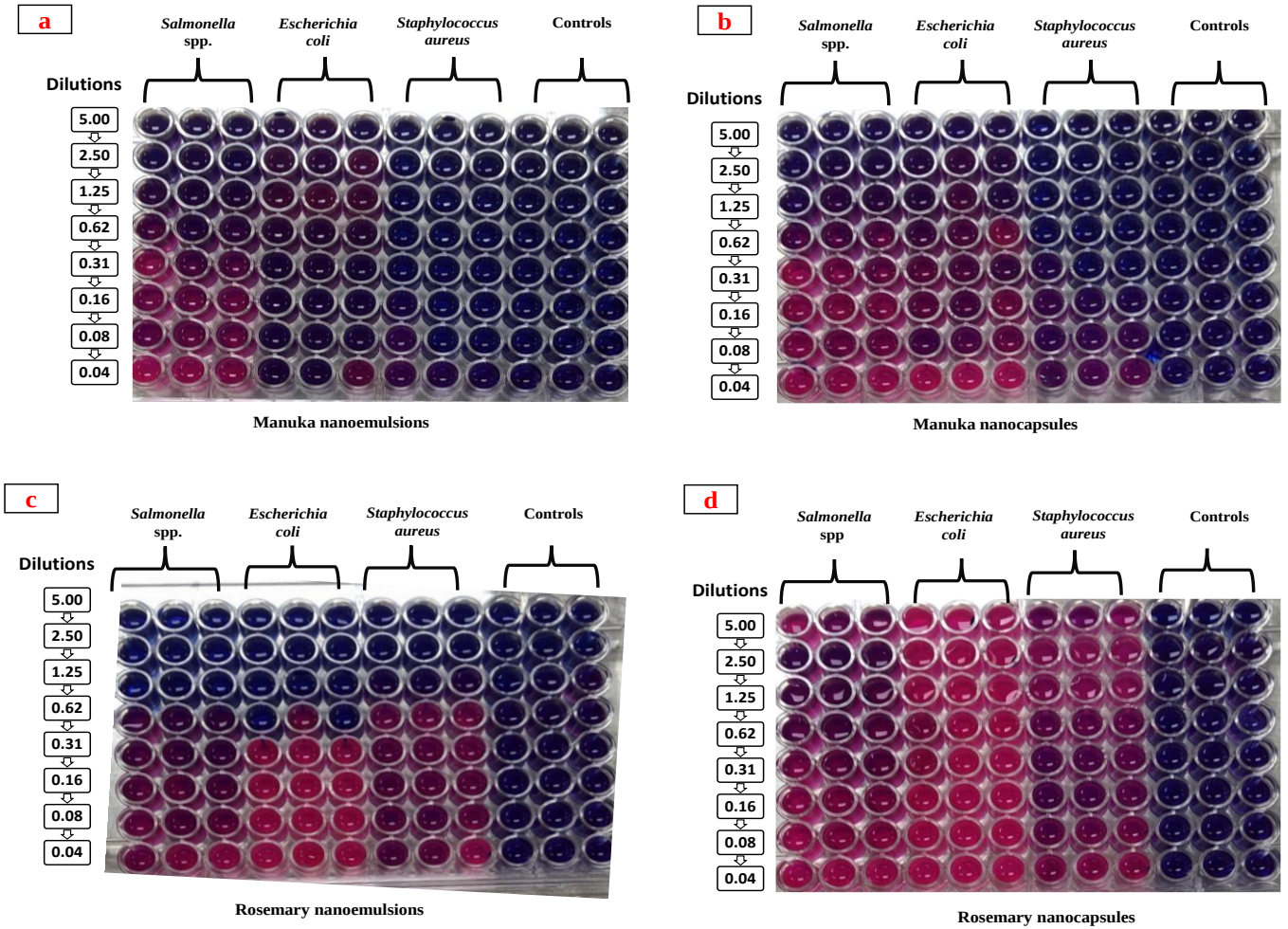


Figure 7.3. 96-well plates showing the growth of *Salmonella* spp., *Escherichia coli* and *Staphylococcus aureus* in the presence of mānuka nanoemulsion (a), mānuka nanocapsules (b), rosemary nanoemulsion (c) and rosemary nanocapsules (d).

(The control sample contained no microbes, but the essential oils were diluted at different concentrations. The p-iodonitrotetrazolium violet dye was used as a growth indicator and the pink colour development in wells indicates microbial growth)

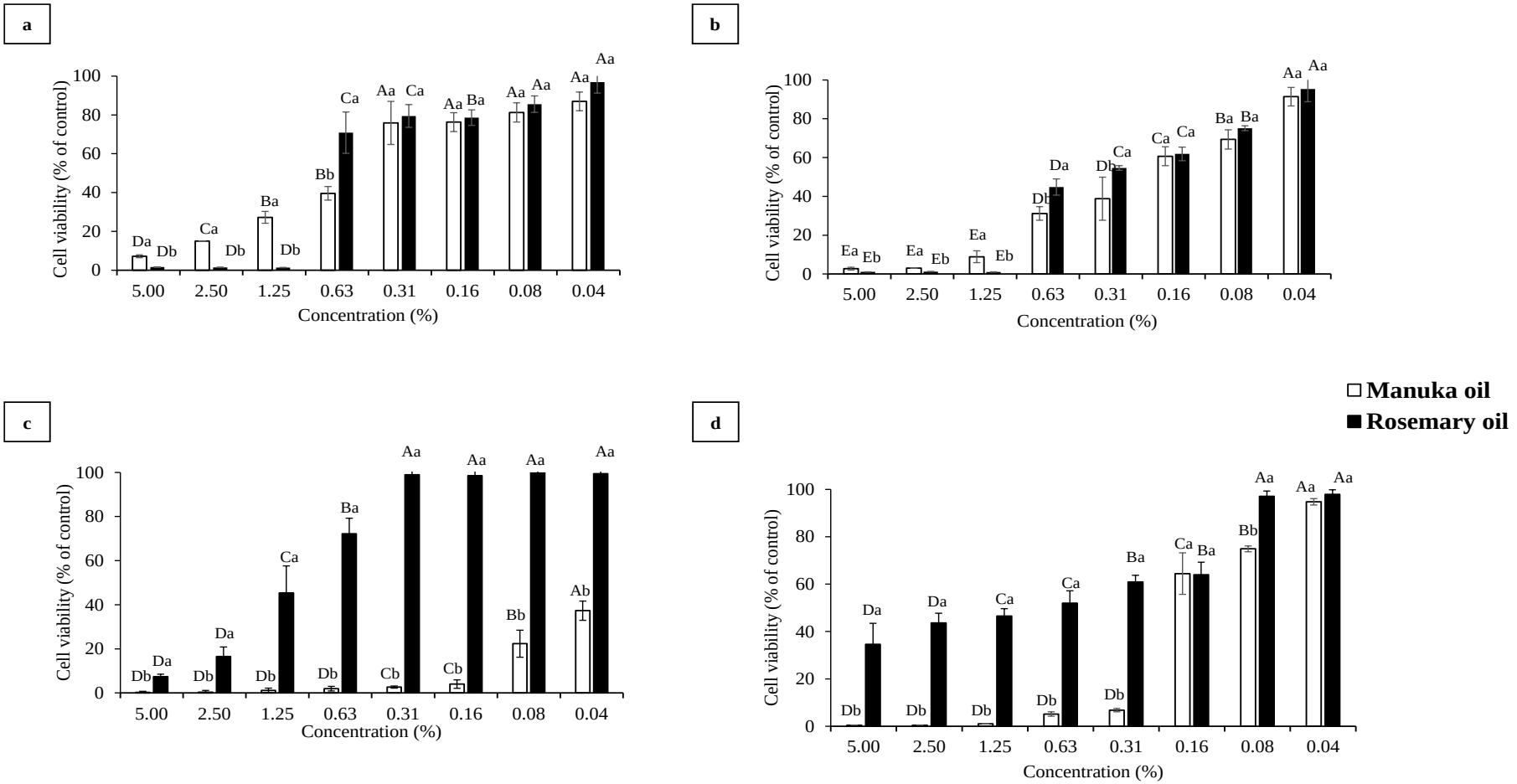


Figure 7.4. Antimicrobial effect of mānuka and rosemary oils on cell viability of *Salmonella* spp. (a), *Escherichia coli* (b), *Staphylococcus aureus* (c), and *Listeria monocytogenes* (d).

7.3.4. Cell viability

The effect of the essential oils against bacterial cell viability was determined using a luciferase bioluminescence-based assay, and the results are presented in Figure 7.4. Both essential oil treatments reduced the cell viability of all tested bacteria in a concentration-dependent manner, despite the differences in the percentage of viable cells for every bacteria group at different concentrations. In the rosemary oil treatment, a significant reduction of *Salmonella* and *Escherichia coli* was noticed until the first three concentrations (5, 2.5 and 1.25 %); however, mānuka oils showed the presence of some viable cells at these concentrations. On the contrary, mānuka oil showed reduced cell viability of Gram-positive microbes, i.e., *Listeria monocytogenes* and *Staphylococcus aureus*. The rosemary showed the presence of some viable cells, especially *Listeria monocytogenes*, even at the highest concentration, indicating a less antimicrobial effect of this oil than the mānuka oil. The assay results correlate with the microbial growth curves, showing the antimicrobial effect of rosemary against Gram-negative microbes at the first three concentrations and less effect against Gram-positive microbes.

In this assay, the luminescent signal is directly proportional to the ATP quantities, indicating the number of metabolically active intact cells in the culture (Pahalagedara et al., 2022). ATP production is vital for the tricarboxylic cycle, and essential oil compounds have been found to cause a change in bacterial metabolism, dysregulation of the citrate metabolic pathway, and inhibition of ATP formation. Similarly, the *Amomum villosum* Lour essential oil treatment reduced the ATP levels of methyllin-resistant *Staphylococcus aureus* from 1082.62 to 297.70 nmol and downregulated it by 72.50 % than the control (Mousavi et al., 2016; Tang et al., 2021). The findings indicate that essential oil caused cell death at the highest concentration; thus, low luminescence values were observed. It is well known that there may be strain variations under the same bacterial species to the susceptibility of the oils. Both oils must be

tested against various microbes and their strains from different species to provide concrete evidence of their antimicrobial efficacies. As the bacterial cell viability is determined as the percentage of viable cells in the control group, the limitation of this method is that the actual cell numbers are not presented in cfu/mL from each targeted species.

7.3.5. Loss of cell membrane integrity

The cell membrane integrity of *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* was evaluated using fluorescent green cell tox dye, and results are presented in Figures 7.5.

The results showed that when microbes were treated with mānuka and rosemary oils at 5 % (Figure 7.5) and 2.5 % concentrations, the fluorescence values increased directly with increased exposure time and concentration of essential oils. In the absence of essential oils, the fluorescence intensity of all microbes was very low, suggesting their intact cell membranes. After mānuka and rosemary oil treatments, there was a significant increase in the fluorescence intensity of *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* than the control, indicating their loss of cell membrane integrity. Against Gram-negative bacteria, higher fluorescence values were observed in rosemary oil -treated *Salmonella* and *Escherichia coli* than in the mānuka oil and control. On the contrary, mānuka oil showed higher fluorescence values against gram-positive microbes. Consistent with cell viability results, rosemary oil caused more cell death of Gram-negative microbe than mānuka oil. For each bacterium, a difference in maximum fluorescence intensity was observed. It could be due to the outer membrane, especially in Gram-negative bacteria, which provides extra layer protection against antimicrobial agents by reducing or not allowing access to inner cellular targets like cytoplasmic membranes and other intracellular structures (Pahalagedara et al., 2022).

Even minor damage to the structure of the cell membrane can affect cell metabolism and has been reported as a potential mechanism for cell death (Hartmann et al., 2010). The cytoplasmic membrane of bacteria provides a permeability barrier to access small ions such as Na^+, K^+, and H^+, which are imperative to maintain normal metabolism and enzyme activity and enable cell membrane functions (Diao et al., 2014). This membrane's chemical composition and structure manage and sustain the impermeability of small ions. Essential oils destroy cell membrane integrity by releasing proteins and intracellular compounds, lowering their viability. It has been reported that essential oil creates channels through the membrane by pushing apart the fatty acid's chains of phospholipids, facilitating ions to leave the cytoplasm (Burt, 2004; Zhang et al., 2017). This assay uses proprietary asymmetric cyanin dye, excluded from the intact cells while preferentially staining the dead cells' DNA. When the dye binds to the compromised cell's DNA, its fluorescent characteristics are substantially increased, and intact cells produce no increase in fluorescence (Armitage, 2008).

7.3.6. Cell membrane permeability

The influence of mānuka and rosemary essential oils on NPN uptake has been presented in Figure 7.6. The higher concentration of essential oils (5 %) showed higher fluorescence intensities than the lower concentration (2.5 and 1.25 %), indicating that the degree of damage to the microbe's cell membrane was dose-dependent. Compared with the control, adding essential oils to microbial suspension caused a sharp increase in fluorescence values. The fluorescence intensity of *Salmonella* and *Escherichia coli* treated with 5 % rosemary oil was significantly higher ($p \leq 0.05$) than the mānuka and control samples. On the contrary, 5 % mānuka oil -treated *Listeria monocytogenes* and *Staphylococcus aureus* had higher fluorescence intensities than the (same concentration) rosemary and control-treated samples.

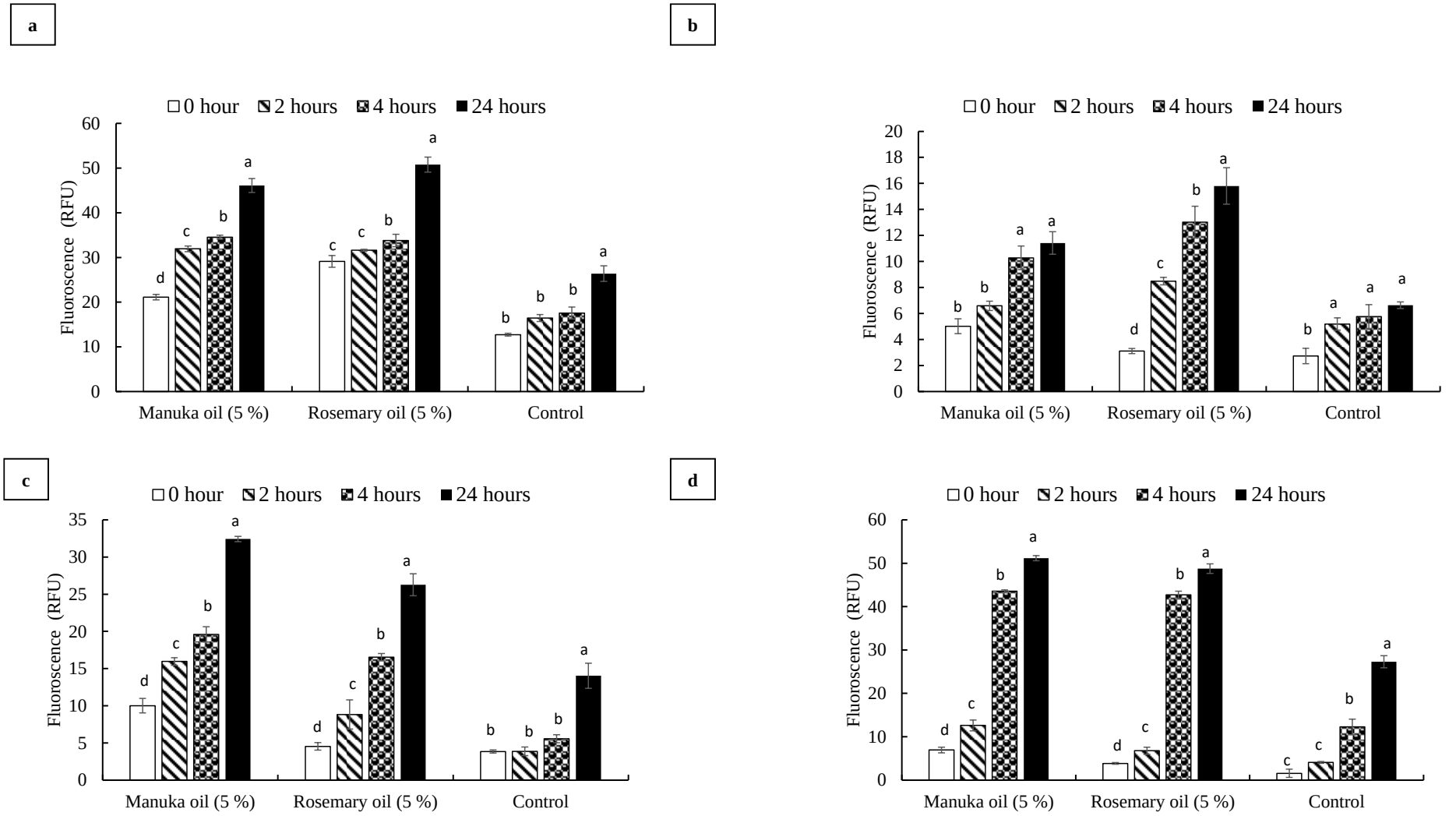


Figure 7.5. Antimicrobial effect of mānuka and rosemary oils on cell membrane integrity of *Salmonella* spp. (a), *Escherichia coli* (b), *Staphylococcus aureus* (c), and *Listeria monocytogenes* (d) at 5 % concentration
Lowercase letters (a, b, c) on bars indicate a significant difference ($p \leq 0.05$) between hours under the same treatment.

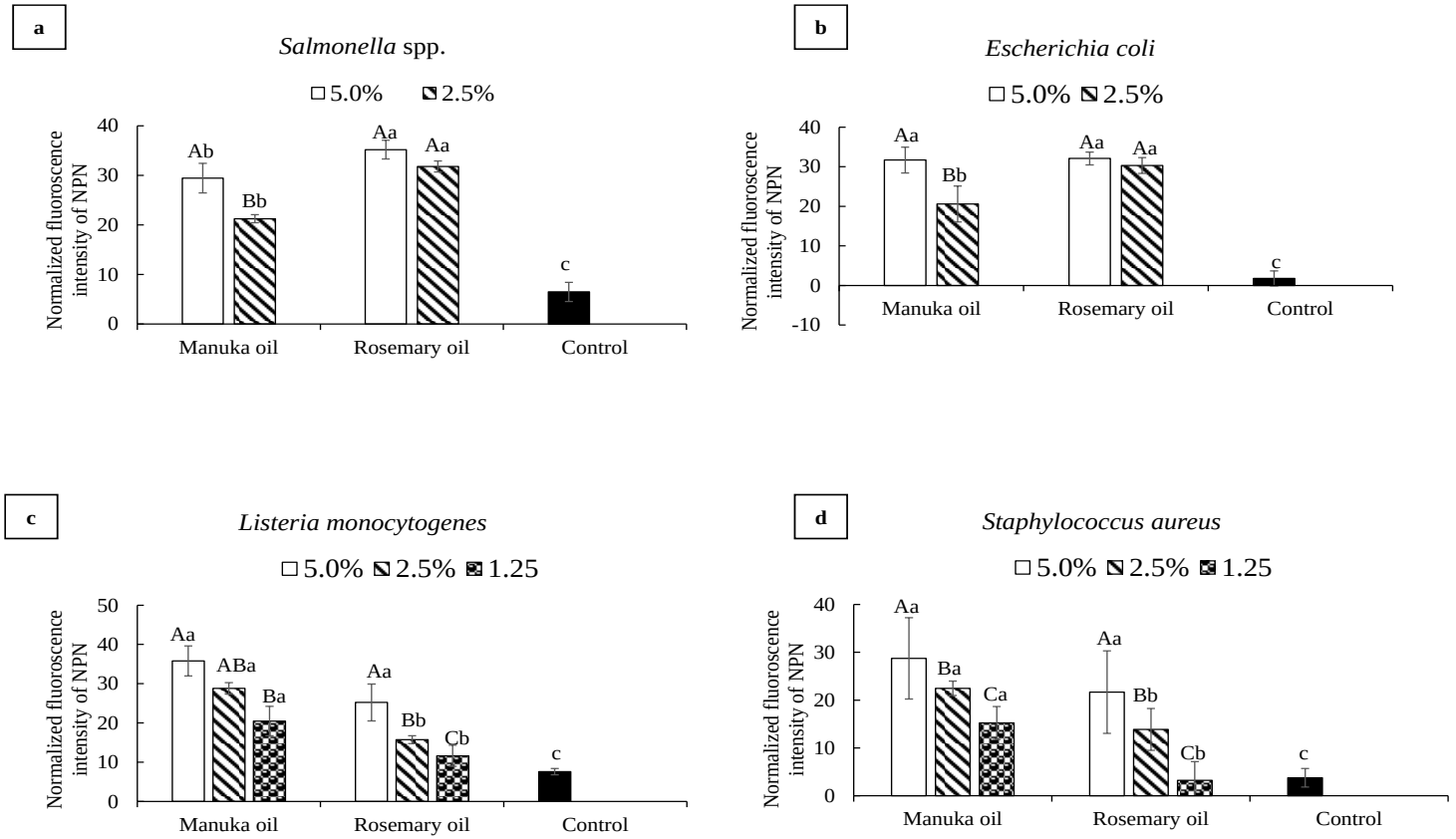


Figure 7.6. Antimicrobial effect of mānuka and rosemary oils on outer membrane permeability of *Salmonella* spp. (a), *Escherichia coli* (b), *Staphylococcus aureus* (c), and *Listeria monocytogenes* (d) at different concentrations.

Note: NPN stands for 1, N-phenylnapthylamine. Capital letters (A, B, C) show a significant difference between different concentrations under the same treatment in each microbe. Lowercase letters (a, b, c) show a significant difference ($p \leq 0.05$) between treatments under the same concentration at a difference in each microbe.

The NPN uptake assay determined the capability of mānuka oil to permeabilize the outer membrane of *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Staphylococcus aureus*. NPN is generally excluded by the outer membrane of intact cells and shows very low fluorescence intensities in these cells. However, in dead cells with damaged outer membranes, NPN can be taken by these cells and show prominent fluorescence in the phospholipid layer. The outer membrane of bacteria, especially Gram-negative, is an asymmetric bilayer composed of phospholipids and lipopolysaccharides and is vital for cell viability by preventing the penetration of toxic substances (Murínová & Dercová, 2014). The results showed that essential oils showed irreversible damage to the cell membrane of *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*, which might be linked to the presence of hydrophobic components in both essential oils. It has been reported that hydrophobic compounds may easily penetrate bacterial cells and then act within the cytoplasm and on the cell wall, which may lead to irreversible changes in their structure and functionality and subsequently result in cell death (Chouhan et al., 2017).

Several studies have shown increased fluorescence intensity of cell suspensions after essential oil treatment at different concentrations in NPN assay. For instance, Helander et al. (1998) exhibited increased NPN uptake of *Salmonella* and *Escherichia coli* after thymol and carvacrol treatment, while carvone and trans-cinnamaldehyde showed no effect on NPN uptake for both microbes. The essential oil from *Cyperus rotundus* rhizomes increased the NPN uptake of *Staphylococcus aureus* by 31.8, 79.2 and 216.6 % by treating with 1/2, MIC, MIC, and 2 MIC levels (Zhang et al., 2017).

7.3.7. Release of proteins and intracellular compounds

The release of proteins and nucleic acids from the microbial cells with and without essential oil treatment is presented in Figures 7.7 and 7.8. After mānuka and rosemary oil treatment, a

significant increase ($p \leq 0.05$) in absorbance values was observed. The absorbance values of *Salmonella* and *Escherichia coli* treated with rosemary oil increased to 0.2 from 0.05 (at 5 % concentration), which was significantly higher ($p \leq 0.05$) than the control and mānuka oil. However, rosemary oil had a lower effect at 2.5 % concentration against both microbes. After the application of 5 % mānuka oil, the absorbance value increased to 0.1 from 0.25 for *Listeria monocytogenes* and *Staphylococcus aureus* from 0.05 to 0.25, indicating a higher antibacterial effect of this oil against Gram-positive than the Gram-negative microbes. The rosemary oil at the same concentration showed OD values around 0.25 for both microbes.

Similarly, an increase in protein values of essential oil-treated samples at different concentrations was observed. For *Salmonella* and *Escherichia coli*, 5 % rosemary oil caused higher protein release than 5 % mānuka oil. This release was less at lower concertation (2.5 %). On the other hand, mānuka oil showed higher protein release values of Gram-positive microbes, i.e., *Listeria monocytogenes* and *Staphylococcus aureus*, than rosemary oil. The 5 % mānuka oil treatment showed 0.324 and 0.28 protein values for *Listeria monocytogenes* and 0.31 and 0.25 for *Staphylococcus aureus* at 5 and 2.5 % concentrations, respectively. Upon treating bacteria with essential oils at 5 %, the amount of leaked proteins is higher compared with the bacteria treated with 2.5 %. The assay results are consistent with those obtained from the release of 260 nm absorbing materials and cell membrane permeability.

The absorbance values are a measurement of the release of UV-absorbing intracellular materials as an index of cell lysis (Zhang et al., 2017). The results suggested that essential oils treatment was causing damage to the cytoplasmic membrane and, subsequently, leakage of intracellular compounds, which was also supported by cell membrane permeability and scanning electron microscopy results.

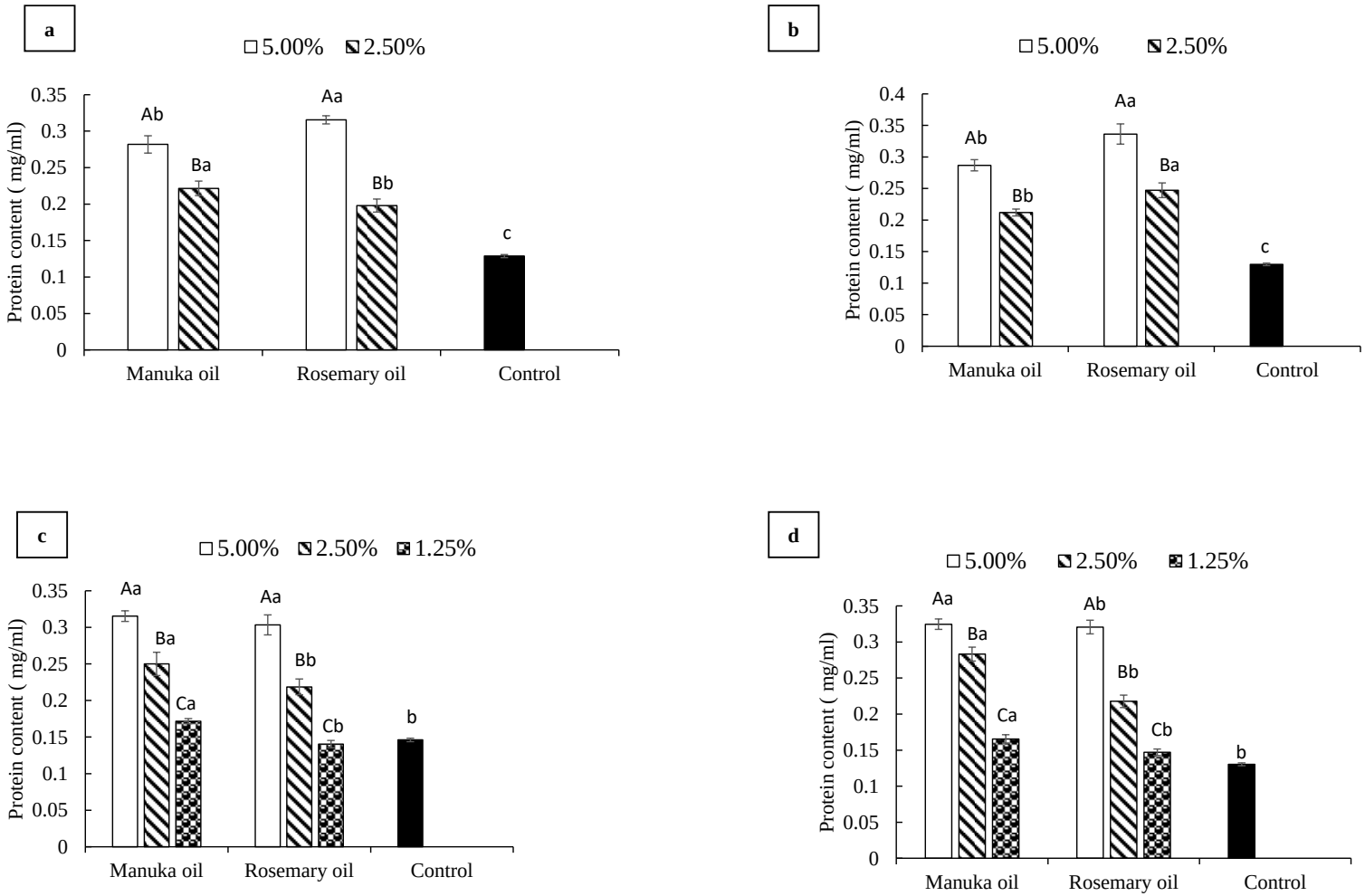


Figure 7.7. The protein content released from *Salmonella* spp. (a), *Escherichia coli* (b), *Staphylococcus aureus* (c), and *Listeria monocytogenes* (d) cells after treatment with mānuka and rosemary oils.

Capital letters (A, B, C) show a significant difference between different concentrations under the same treatment in each microbe. Lowercase letters (a, b, c) show a significant difference ($p \leq 0.05$) between treatments under the same concentration at a difference in each microbe.

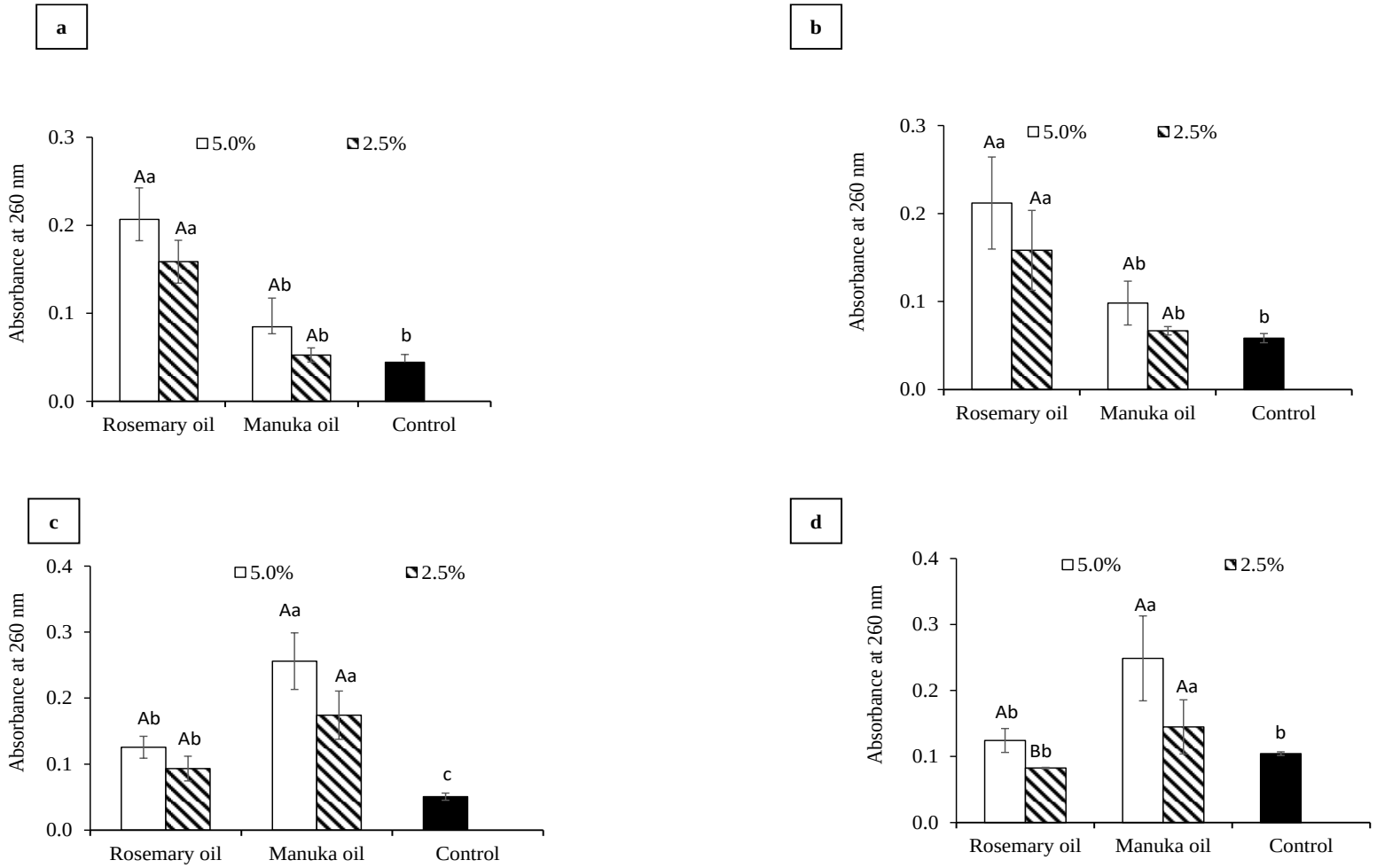


Figure 7.8. The release of 260 nm absorbing materials from *Salmonella* spp. (a), *Escherichia coli* (b), *Staphylococcus aureus* (c), and d) *Listeria monocytogenes* (d) cells after treatment with mānuka and rosemary oils.

Capital letters (A, B, C) show a significant difference between different concentrations under the same treatment in each microbe. Lowercase letters (a, b, c) show a significant difference ($p \leq 0.05$) between treatments under the same concentration at a difference in each microbe.

The macromolecules, such as nucleic acids and proteins, reside inside the bacterial cell and cytoplasm and are key structural components. The loss of these macromolecules could cause a function disorder in synthesizing vital proteins and DNA materials and further bacterial growth inhibition (Zhang et al., 2017). The results on leakage of compounds after essential oil treatment in this study are consistent with the previously reported studies. Devi et al. (2010) reported that after treatment of *Salmonella typhi* with 1 and 5 % eugenol, the absorbance values were increased from 0.01 to 0.4, and less effect was observed at 0.0125 % concentration (Devi et al., 2010). The black pepper essential oil at 2×MIC and 1×MIC increased OD values than the control as an indicator of loss of nucleic acids from *Escherichia coli* (Zhang et al., 2017). Other antibacterial agents (essential oils) such as mustard and clove oil have also been shown to increase the loss of intracellular 260 nm absorbing materials from *Escherichia coli* cells (Cui et al., 2015). The amount of leaked protein was higher in 2×MIC of *Ocimum gratissimum* oil-treated microbes (*Staphylococcus aureus*, *Escherichia* and *Salmonella* Typhimurium and *Shigella flexneri)* than 1×MIC treated microbes (Chimnoi et al., 2018). A similar effect on *Escherichia coli* has also been reported for other antibacterial agents like thymus oil, sage oil and their nanoemulsion (Moghimi et al., 2016a; Moghimi et al., 2016b).

From the obtained results, it can be concluded that mānuka and rosemary can damage the cell membrane, leak the intracellular constituents and proteins and eventually cause cell death, and this destruction becomes more evident with increasing concentration of essential oils.

7.3.8. Cell wall damage using alkaline phosphatase (AKP)

This study noted a significant increase ($p \leq 0.05$) in alkaline phosphate quantities after essential oils treatment at different concentrations, as represented in Figure 7.9. After treatment with 5 % rosemary oil, the levels of AKP in *Salmonella* and *Escherichia coli* supernatants were around 450 and 491 U/L, significantly higher ($p \leq 0.05$) than the mānuka oil (321 and 447 U/L) and

control (129 and 192 U/L). On the other hand, the extracellular AKP activity of Gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) with mānuka oil (5 %) treatment increased from 138 to 829 U/L for *Staphylococcus aureus* and up to 497 from 175 U/L for *Listeria monocytogenes*. As shown in Figure 6.9, the extracellular AKP activity of the rosemary group for both Gram-positive microbes was around 200 U/L less than the mānuka oil treatment. With a decrease in essential oil concentration (from 5 to 2.5 %) of mānuka and rosemary oils, a significant drop ($p \leq 0.05$) in the release of extracellular AKP of all microbes was observed.

A protease, also known as alkaline phosphate, is located between the cell wall and cell membrane of bacteria, which is not detected extracellularly unless the cell wall has been destroyed by antimicrobial agents (Wang et al., 2017). Thus, the concentration of AKP in an extracellular medium or cell suspension could reflect the integrity of the bacterial cell wall. Several studies have reported an increase in the concentration of AKP after different essential oil treatments. Wang et al. (2017) reported that the MBC of *Dodartia Orientalis* was more effective in increasing the quantities of AKP in the cell suspension of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Enteritidis than the MIC values (Wang et al., 2017). In a similar way, *Litsea cubeba* oil treatment at MBC values showed increased AKP content in cultures of *Escherichia coli* O157 and *Staphylococcus aureus*, and these values were higher than the MIC-treated cultures (Yang et al., 2020). Clove oil caused damage to the cell wall of *Staphylococcus aureus* and resulted in leakages of AKP in cell suspensions, as shown by studies by Bai et al. (2023).

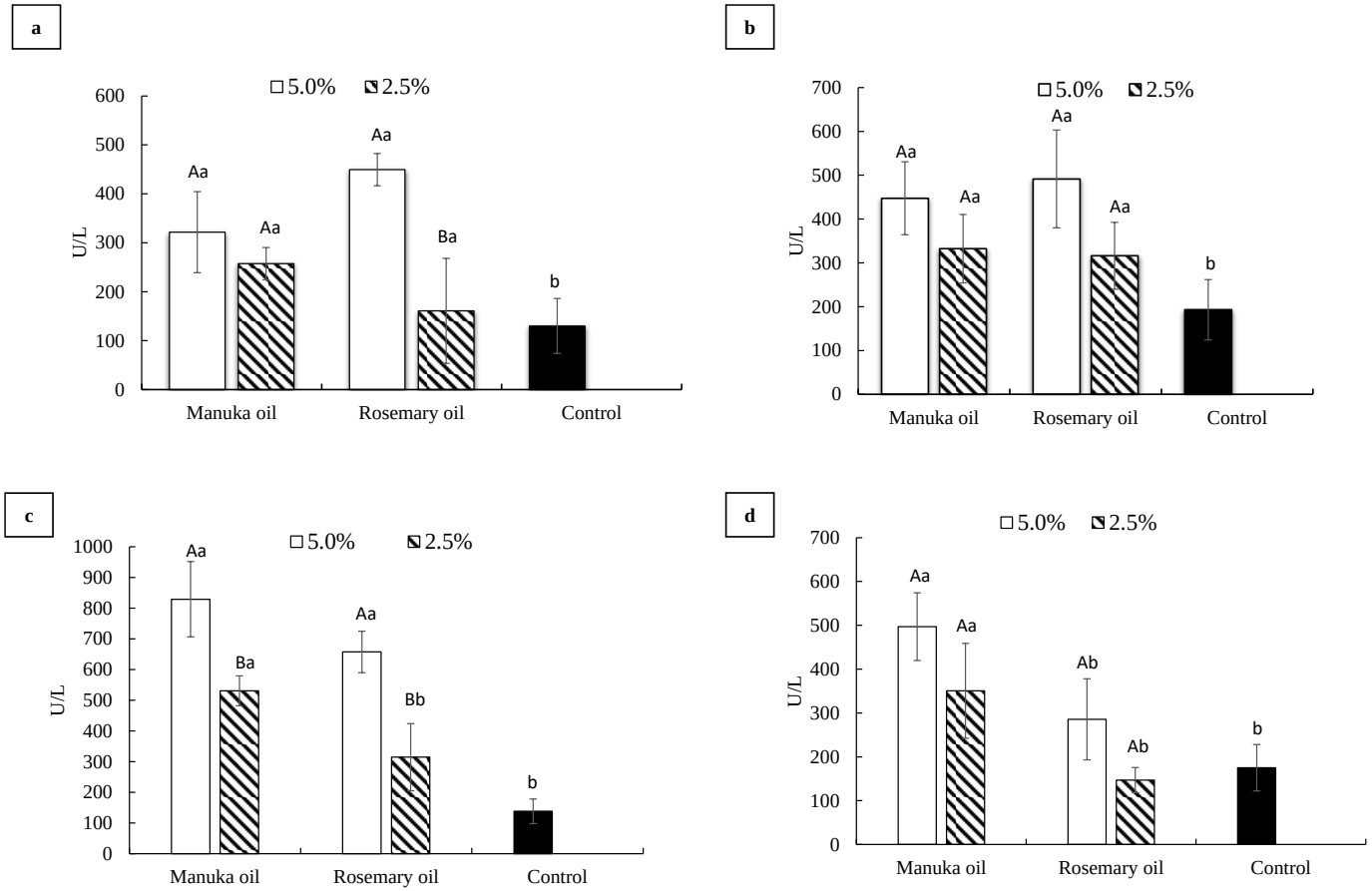


Figure 7.9. Antimicrobial effect of mānuka and rosemary oils on alkaline phosphatase activity of *Salmonella* spp. (a), *Escherichia coli* (b), *Staphylococcus aureus* (c), and d) *Listeria monocytogenes* (d) at different concentrations.

Capital letters (A, B, C) show a significant difference between different concentrations under the same treatment in each microbe. Lowercase letters (a, b, c) show a significant difference ($p \leq 0.05$). between treatments under the same concentration at a difference in each microbe.

7.3.9. Cell structure using SEM

The influence of mānuka and rosemary oil treatment on the *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* morphology was visualized through a scanning electron microscope, as presented in Figures 7.10 and 7.11 at different magnifications. The differences in cell shape and structure of control and essential oil-treated samples are visible in electron micrographs (Figures 7.10 and 7.11).

As observed from the figures, cells of *Salmonella*, and *Escherichia coli*, in exponential phase, had perfectly rod and striated membranes, whereas *Staphylococcus aureus* was oval or spherical when microbes were untreated. However, *Listeria monocytogenes* and *Staphylococcus aureus* cells treated with mānuka oil exhibited more damage and breakdown to cell structure, possibly due to the release of cellular components and proteins. The main mechanisms, including impaired cell walls, distinct cell boundaries, collapsing cells, and release of cellular components, were noticed after the essential oil treatment of bacterial cells. Clear images of single cells of *Escherichia coli* treated with rosemary and mānuka oils exhibited this bacteria's incomplete and deformed shape compared to the control. The incomplete structure may be due to the release of inside cellular components, such as proteins and metabolites from the cells, which leave ovoid spaces. For instance, Zou et al. (2015) reported that black pepper essential oil altered the cellular membrane permeability, induced low-molecular-weight metabolites and other constituents' leakage, and consequently triggered the cell death of *Escherichia coli* and *Staphylococcus aureus* (Zou et al., 2015).

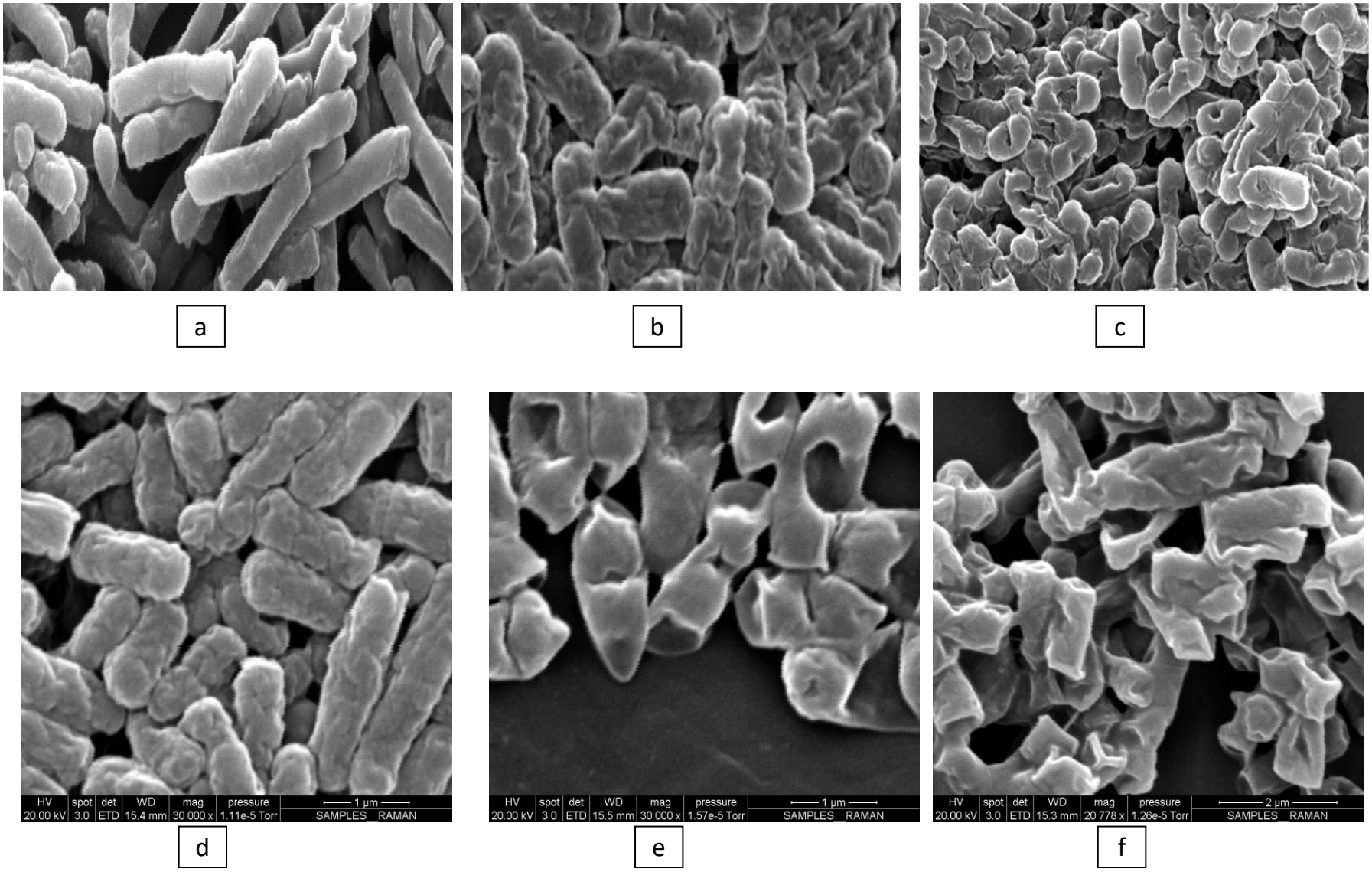


Figure 7.10. Scanning electron microscope images of *Salmonella* spp. (a, b, and c) and *Escherichia coli* (d, e and f) cells treated with mānuka oil (b and e), rosemary oil (c and f) and without any antimicrobial agent (a and d).

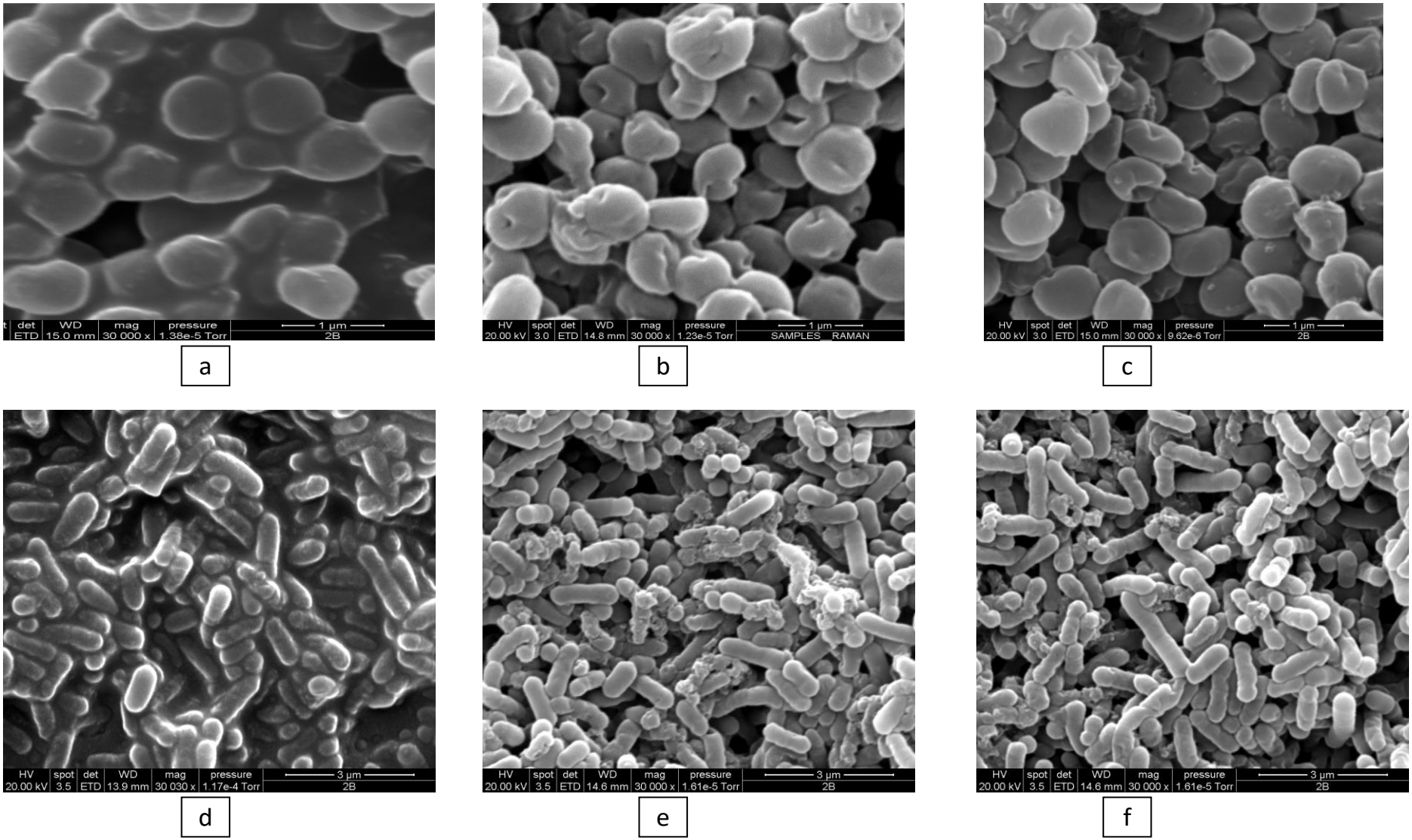


Figure 7.11. Scanning electron microscope images of *Staphylococcus aureus* (a, b, and c) and *Listeria monocytogenes* (d, e and f) treated mānuka oil (b and e), rosemary oil (c and f) and without any antimicrobial agent (a and d).

7.3.10. Analysis of beef pastes

7.3.10.1. Proximate composition analysis

The data for proximate composition analysis of wagyu and crossbred beef are presented in Table 7.3. A significant difference ($p \leq 0.05$) in fat and moisture contents of both meat pastes was observed. Crossbred beef's initial ash, protein, and moisture contents were 1.2 %, 21.5 % and 71.9 %, which was higher than the wagyu beef paste (0.8 %, 17.9 % and 58.1 %). However, the wagyu beef paste exhibited higher fat content (23 %) than the crossbred beef (2.3 %), possibly due to the higher intramuscular fat content in former beef than the other beef breeds. Consistent with our results, Bermingham et al. (2021) reported a higher fat content in grain-fed wagyu than the pasture fed-Angus. Comparing the moisture, protein and fat levels and content of both beef pastes, it can be noted that fat content showed an inverse relationship with moisture and protein content. Like our results, Corbin et al. (2015) documented that raw beef strip loin steaks from several fat levels and quality treatments showed that moisture and protein content was inversely related to the fat content.

Table 7.3. Proximate composition analysis of meat pastes from wagyu and crossbred tenderloins.

	Moisture (%)	**Fat (%)**	**Protein (%)**	**Ash (%)**
Wagyu	58.1^{b}	23.0^{a}	17.9^{b}	1.2^{a}
Crossbred	71.9^{a}	2.3^{b}	21.5^{a}	0.8^{a}

7.3.10.2. Microbiological analysis

The influence of free, emulsified and encapsulated essential oil (mānuka and rosemary oils) on *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* in beef pastes stored at 4 °C are shown in Table 7.4. Compared with the control, paste samples treated

with essential oil, either free oil, nanoemulsions or nanocapsules, led to a significant inhibition in microbial growth during storage.

At the beginning of storage, no significant differences were observed between the with and without preservatives-treated samples. During the storage period, *Salmonella* and *Escherichia coli* numbers significantly increased ($p \leq 0.05$) in wagyu and crossbred pastes, while the rising rate was higher in untreated meat, control emulsions and nanocapsules-than the sodium nitrite, essential oils and their nanoentities- treated samples. The rosemary nanoemulsions resulted in the lowest counts of *Salmonella,* followed by rosemary oil/nanocapsules, sodium nitrite, mānuka nanoemulsions, mānuka oil/nanocapsules and controls in both types of meat pastes. Similarly, against *Escherichia coli*, rosemary oil and its nanoentities showed the lowest microbial growth compared to mānuka oil, its nonentities, and sodium nitrite-containing samples.

On the other hand, mānuka oil, mānuka nanoemulsion and nanocapsules exhibited significantly higher ($p \leq 0.05$) antimicrobial efficacies against *Listeria monocytogenes* and *Staphylococcus aureus* than the rosemary oil, sodium nitrite treated, and control wagyu and crossbred samples. At the end of storage, mānuka oil and its nanoentities had significantly lowered *Staphylococcus aureus*, and *Listeria monocytogenes* counts by around 2 log cfu/g and 3 log cfu/g, respectively, than the crossbred control samples. In wagyu pastes (high-fat beef), the antimicrobial effect of these preservatives was also noticed, but it was less pronounced than in the crossbred pastes (low-fat beef). Against Gram-positive microbes, rosemary oil had significantly lower antimicrobial activity than mānuka oil in both meat pastes. In this study, sodium nitrite exhibited an antimicrobial effect against all tested microbes; for some microbes (*Salmonella* and *Escherichia coli*), it was similar to mānuka oil, while for *Listeria monocytogenes* and *Staphylococcus aureus,* it was lower than this oil.

Table 7.4. The changes in microbial growth in wagyu and crossbred meat paste with or without added preservative agent during storage at 4 °C for 15 days.

Meat	Crossbred				Wagyu			
	0th day	**5th day**	**10th day**	**15th day**	**0th day**	**5th day**	**10th day**	**15th day**
***Salmonella* spp.**								
MO	5.58 ± 0.26^{a}	6.72 ± 0.22^{abc}	7.07 ± 0.21^{c}	7.80 ± 0.20^{bc}	5.93 ± 0.04^{a}	6.50 ± 0.22^{ab}	6.94 ± 0.17^{b}	7.72 ± 0.12^{abc}
ME	5.79 ± 0.17^{a}	6.33 ± 0.27^{bcd}	7.00 ± 0.05^{c}	7.32 ± 0.34^{cd}	5.86 ± 0.10^{a}	6.04 ± 0.08^{bc}	7.02 ± 0.05^{b}	7.49 ± 0.20^{bcd}
MC	5.72 ± 0.27^{a}	6.85 ± 0.16^{abc}	7.15 ± 0.13^{bc}	7.63 ± 0.25^{c}	5.96 ± 0.06^{a}	6.04 ± 0.08^{bc}	6.76 ± 0.17^{b}	7.73 ± 0.10^{ab}
RO	5.55 ± 0.24^{a}	5.61 ± 0.22^{ef}	5.78 ± 0.13^{d}	5.96 ± 0.10^{e}	5.86 ± 0.13^{a}	5.88 ± 0.12^{c}	5.71 ± 0.20^{c}	6.91 ± 0.11^{d}
RE	5.60 ± 0.24^{a}	5.38 ± 0.36^{f}	5.54 ± 0.22^{d}	5.60 ± 0.27^{e}	5.87 ± 0.12^{a}	5.63 ± 0.16^{c}	5.62 ± 0.28^{c}	6.83 ± 0.21^{d}
RC	5.71 ± 0.24^{a}	5.75 ± 0.22^{def}	5.81 ± 0.11^{d}	5.97 ± 0.05^{e}	5.94 ± 0.12^{a}	5.78 ± 0.11^{c}	5.80 ± 0.18^{c}	7.05 ± 0.02^{cd}
CE	5.87 ± 0.15^{a}	6.94 ± 0.07^{ab}	7.75 ± 0.13^{a}	8.55 ± 0.24^{ab}	5.92 ± 0.16^{a}	5.90 ± 0.14^{c}	7.59 ± 0.17^{a}	8.19 ± 0.23^{a}
CC	5.85 ± 0.16^{a}	7.00 ± 0.06^{a}	7.62 ± 0.31^{ab}	8.48 ± 0.44^{a}	5.97 ± 0.12^{a}	6.44 ± 0.18^{ab}	7.72 ± 0.16^{a}	8.17 ± 0.31^{a}
C	6.03 ± 0.04^{a}	6.82 ± 0.14^{abc}	7.98 ± 0.05^{a}	8.95 ± 0.08^{a}	5.96 ± 0.11^{a}	6.58 ± 0.20^{a}	7.91 ± 0.08^{a}	8.28 ± 0.28^{a}
SN	5.61 ± 0.26^{a}	6.25 ± 0.31^{cde}	6.96 ± 0.10^{c}	6.69 ± 0.14^{d}	5.88 ± 0.13^{a}	6.76 ± 0.21^{a}	6.66 ± 0.17^{b}	7.25 ± 0.42^{bcd}
Escherichia coli								
MO	5.75 ± 0.12^{a}	6.12 ± 0.19^{b}	6.69 ± 0.18^{bcd}	7.08 ± 0.22^{c}	5.51 ± 0.41^{a}	6.17 ± 0.26^{abc}	6.63 ± 0.28^{cd}	7.63 ± 0.24^{b}

ME	5.62 ± 0.14^{a}	5.84 ± 0.19^{b}	6.37 ± 0.39^{cde}	6.96 ± 0.10^{c}	5.74 ± 0.16^{a}	6.30 ± 0.76^{abc}	7.06 ± 0.36^{bc}	7.36 ± 0.26^{bc}
MC	5.47 ± 0.41^{a}	5.93 ± 0.07^{b}	6.16 ± 0.28^{de}	6.55 ± 0.21^{c}	5.53 ± 0.19^{a}	6.16 ± 0.24^{abc}	6.73 ± 0.21^{c}	7.86 ± 0.19^{b}
RO	5.66 ± 0.18^{a}	5.91 ± 0.12^{b}	5.96 ± 0.13^{e}	5.60 ± 0.25^{d}	5.64 ± 0.17^{a}	5.93 ± 0.09^{bc}	6.33 ± 0.29^{cd}	6.80 ± 0.13^{cd}
RE	5.63 ± 0.29^{a}	5.88 ± 0.19^{b}	5.80 ± 0.29^{e}	5.10 ± 0.19^{d}	5.54 ± 0.13^{a}	5.64 ± 0.21^{c}	5.89 ± 0.19^{d}	6.33 ± 0.30^{d}
RC	5.55 ± 0.23^{a}	6.93 ± 0.13^{a}	5.88 ± 0.15^{e}	5.11 ± 0.19^{d}	5.63 ± 0.28^{a}	5.93 ± 0.11^{bc}	6.35 ± 0.28^{cd}	6.86 ± 0.07^{cd}
CE	5.87 ± 0.11^{a}	6.75 ± 0.32^{a}	7.66 ± 0.33^{a}	8.68 ± 0.35^{ab}	5.90 ± 0.10^{a}	6.98 ± 0.06^{a}	7.61 ± 0.23^{ab}	8.64 ± 0.23^{a}
CC	5.73 ± 0.38^{a}	6.85 ± 0.13^{a}	7.36 ± 0.31^{ab}	8.10 ± 0.19^{b}	5.76 ± 0.15^{a}	6.68 ± 0.10^{ab}	7.62 ± 0.18^{ab}	8.87 ± 0.12^{a}
C	5.63 ± 0.28^{a}	6.78 ± 0.25^{a}	7.92 ± 0.04^{a}	9.13 ± 0.20^{a}	5.59 ± 0.19^{a}	6.73 ± 0.16^{ab}	7.88 ± 0.19^{a}	8.70 ± 0.17^{a}
SN	5.56 ± 0.48^{a}	5.77 ± 0.24^{b}	6.91 ± 0.16^{bc}	6.98 ± 0.08^{c}	5.65 ± 0.21^{a}	5.75 ± 0.20^{c}	6.40 ± 0.31^{cd}	7.62 ± 0.17^{b}
Listeria monocytogenes								
MO	5.81 ± 0.07^{a}	5.41 ± 0.10^{c}	5.13 ± 0.05^{f}	4.73 ± 0.12^{h}	5.88 ± 0.07^{a}	5.80 ± 0.09^{cde}	5.70 ± 0.08^{de}	5.26 ± 0.04^{f}
ME	5.92 ± 0.11^{a}	5.45 ± 0.10^{c}	5.18 ± 0.07^{f}	4.80 ± 0.12^{gh}	5.85 ± 0.08^{a}	5.62 ± 0.06^{e}	5.45 ± 0.18^{e}	4.79 ± 0.07^{g}
MC	5.85 ± 0.11^{a}	5.65 ± 0.10^{bc}	5.41 ± 0.02^{e}	5.10 ± 0.07^{fg}	5.93 ± 0.05^{a}	5.75 ± 0.09^{de}	5.89 ± 0.07^{cd}	5.49 ± 0.17^{e}
RO	5.79 ± 0.20^{a}	5.54 ± 0.10^{bc}	6.06 ± 0.04^{cd}	5.02 ± 0.04^{fg}	5.88 ± 0.12^{a}	5.98 ± 0.04^{abc}	6.04 ± 0.17^{c}	6.14 ± 0.03^{c}
RE	5.70 ± 0.19^{a}	5.64 ± 0.10^{bc}	6.03 ± 0.07^{d}	5.12 ± 0.03^{f}	5.87 ± 0.11^{a}	5.87 ± 0.07^{bcd}	6.03 ± 0.05^{c}	5.74 ± 0.07^{d}
RC	5.77 ± 0.15^{a}	5.63 ± 0.12^{bc}	6.12 ± 0.02^{cd}	5.50 ± 0.09^{e}	5.80 ± 0.16^{a}	5.95 ± 0.07^{abc}	6.13 ± 0.04^{c}	6.22 ± 0.03^{c}
CE	5.97 ± 0.08^{a}	6.07 ± 0.04^{a}	7.18 ± 0.03^{a}	8.54 ± 0.12^{b}	5.99 ± 0.06^{a}	6.11 ± 0.05^{a}	7.45 ± 0.06^{a}	8.12 ± 0.02^{a}

CC	5.95 ± 0.08^{a}	6.14 ± 0.02^{a}	7.08 ± 0.03^{a}	8.18 ± 0.09^{c}	5.90 ± 0.10^{a}	6.12 ± 0.04^{a}	7.52 ± 0.11^{a}	8.09 ± 0.01^{a}
C	5.92 ± 0.13^{a}	6.26 ± 0.03^{a}	7.58 ± 0.20^{a}	8.86 ± 0.14^{a}	5.87 ± 0.05^{a}	6.12 ± 0.06^{a}	7.46 ± 0.15^{a}	8.10 ± 0.03^{a}
SN	5.91 ± 0.10^{a}	5.80 ± 0.10^{b}	6.26 ± 0.03^{b}	6.23 ± 0.05^{d}	5.97 ± 0.03^{a}	6.03 ± 0.07^{ab}	6.75 ± 0.13^{b}	7.21 ± 0.04^{b}
Staphylococcus aureus								
MO	5.66 ± 0.22^{a}	5.61 ± 0.28^{cd}	5.83 ± 0.18^{d}	5.85 ± 0.14^{ef}	5.49 ± 0.17^{a}	5.69 ± 0.16^{e}	6.73 ± 0.15^{bc}	6.86 ± 0.13^{c}
ME	5.56 ± 0.22^{a}	5.35 ± 0.33^{d}	5.55 ± 0.42^{d}	5.06 ± 0.24^{f}	5.87 ± 0.13^{a}	5.74 ± 0.11^{de}	5.91 ± 0.11^{d}	6.16 ± 0.14^{d}
MC	5.50 ± 0.38^{a}	5.92 ± 0.09^{cd}	5.85 ± 0.14^{d}	6.03 ± 0.07^{de}	5.50 ± 0.18^{a}	5.84 ± 0.15^{cde}	6.52 ± 0.53^{cd}	6.20 ± 0.35^{d}
RO	5.92 ± 0.43^{a}	5.67 ± 0.31^{cd}	6.68 ± 0.29^{bc}	7.58 ± 0.27^{bc}	5.80 ± 0.17^{a}	6.41 ± 0.35^{bc}	6.99 ± 0.11 ^{abc}	7.58 ± 0.51^{ab}
RE	5.62 ± 0.18^{a}	6.09 ± 0.20^{bcd}	6.58 ± 0.2^{c}	7.02 ± 0.29^{cd}	5.83 ± 0.13^{a}	6.15 ± 0.30^{bcde}	6.62 ± 0.30^{bcd}	7.03 ± 0.08^{bc}
RC	5.67 ± 0.29^{a}	6.11 ± 0.46^{bc}	6.35 ± 0.32^{c}	7.14 ± 0.44^{c}	5.85 ± 0.23^{a}	5.91 ± 0.21^{cde}	6.89 ± 0.13^{abc}	7.29 ± 0.21^{bc}
CE	5.56 ± 0.47^{a}	6.82 ± 0.25^{a}	7.60 ± 0.21^{ab}	8.01 ± 0.08^{ab}	5.88 ± 0.17^{a}	6.59 ± 0.25^{ab}	7.33 ± 0.28^{ab}	7.97 ± 0.11^{a}
CC	5.84 ± 0.21^{a}	6.69 ± 0.25^{a}	7.73 ± 0.37^{a}	7.97 ± 0.16^{ab}	5.83 ± 0.21^{a}	7.08 ± 0.19^{a}	7.54 ± 0.22^{a}	7.97 ± 0.05^{a}
C	5.63 ± 0.28^{a}	6.76 ± 0.30^{a}	7.96 ± 0.1^{a}	8.75 ± 0.28^{a}	5.71 ± 0.19^{a}	6.38 ± 0.28^{bcd}	7.05 ± 0.35^{abc}	7.99 ± 0.07^{a}
SN	5.67 ± 0.24	6.17 ± 0.32^{bc}	6.46 ± 0.36^{c}	6.99 ± 0.08^{cd}	5.66 ± 0.19^{a}	5.93 ± 0.12^{cde}	6.47 ± 0.19^{cd}	7.00 ± 0.06^{bc}

It can be observed from Table 7.4 that crossbred pastes had higher microbial growth than the wagyu pastes, which might be attributed to the higher moisture content and water activity of the former pastes than the latter. The improved antimicrobial activity of nanoemulsions to the neat oils has been attributed to reduced particle size and increased surface area to contact the microbes. In addition, the decreased particle size of essential oil through nanoemulsion formation and better penetration of compounds into microbial cells can also be linked to increased antimicrobial activity has been documented by various studies. Consistent with our results, Dini et al. (2020) reported that chitosan film with cumin essential oil nanoemulsions (in combination with gamma-radiation treatment) showed improved antimicrobial effects against meat microorganisms and inoculated pathogenic microbes (*Listeria monocytogenes Salmonella typhimurium* and *Escherichia coli* O157:H7) on beef loins.

7.3.10.3. Lipid oxidation analysis

The changes in lipid oxidation values during refrigerated storage of meat pastes, either with or without antioxidants, are presented in Figure 7.12 (Appendix D**)**. Both storage time and treatment of preservatives significantly influenced the TBARS values of meat pastes.

On day 0 of storage, TBARS values were significantly higher ($p \leq 0.05$) in control wagyu paste than in essential oil, nanoemulsion, and nanocapsules-treated pastes. During the storage period, TBARS values increased in all control and treated wagyu pastes, whilst the rate of lipid oxidation was higher in control, sodium nitrite, and control nanocapsules-treated wagyu pastes. Comparing the free, nanoemulsions and nanocapsules-form of mānuka and rosemary oil, nanoemulsions showed the best antioxidant effect in wagyu beef and resulted in the lowest TBARS values. This could be due to the reduced particle size and increased surface area in the case of nanoemulsions. The improved antioxidant activity and stability of nanoemulsions than free oils would allow reducing the concentration to be added in food formulations.

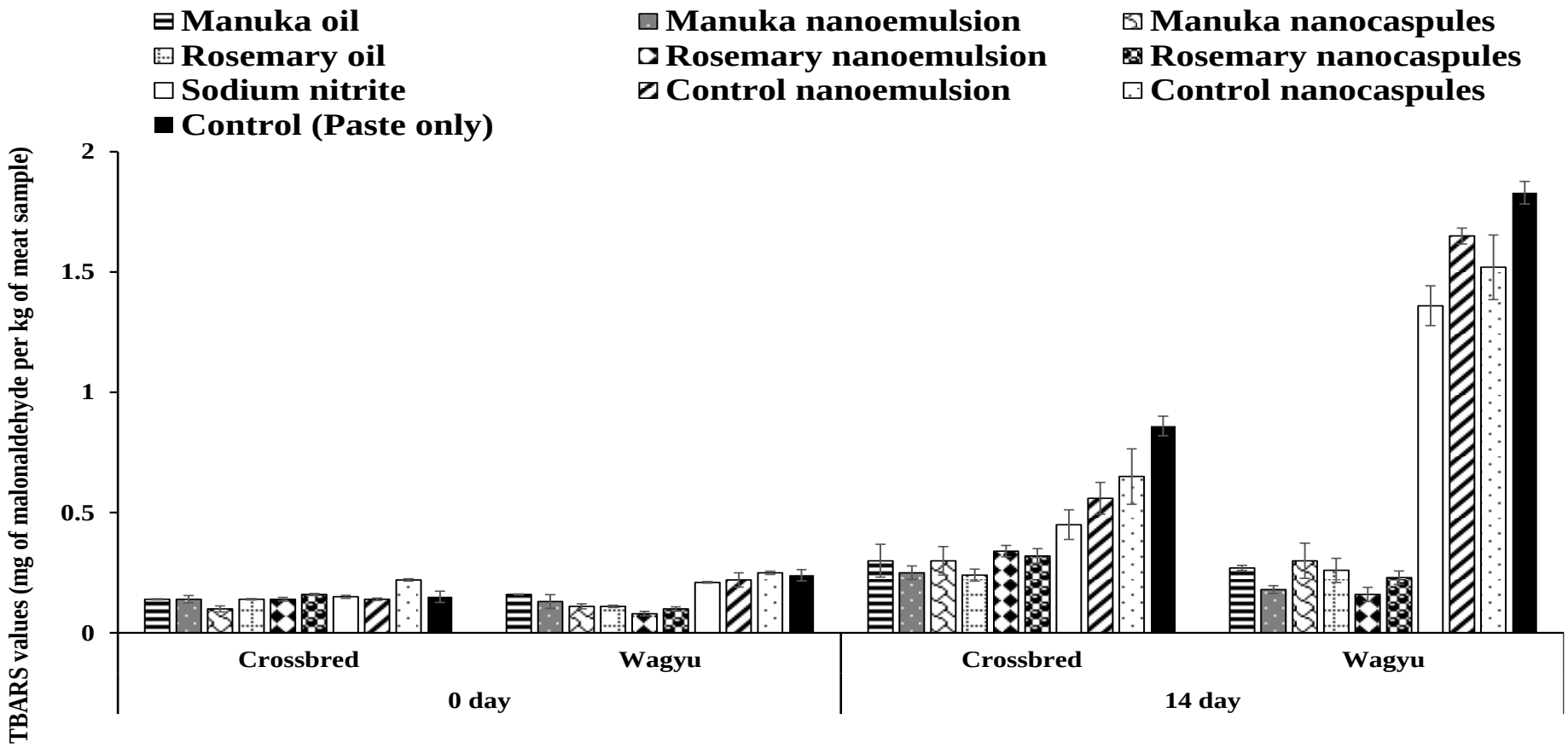


Figure 7.12. The changes in TBARS values for wagyu and crossbred beef paste with or without any added antioxidant agent during storage at 4 °C for 14 days.

TBARS values: 2-Thiobarbituric acid reactive substances (TBARS) values

For TBARS analysis of crossbred beef pastes, no significant difference was observed in treatments with free oil, nanoemulsified, and nanoencapsulated or not, at the beginning of the storage period (on the 0^{th} day). Like wagyu beef pastes, TBARS values of crossbred continuously increased during storage due to the continuous production of lipid oxidation products. In free, nanoemulsion, and nanocapsules-treated crossbred beef pastes, the formation of lipid oxidation products was lower, thereby exhibiting significantly lower ($p \leq 0.05$) TBARS values than the sodium nitrite, meat-only, control nanoemulsions, and nanocapsules-treated samples. Unlike the wagyu beef pastes, no significant difference ($p \leq 0.05$) in essential oil treatment of crossbred pastes, either mānuka or rosemary oil, with free, nanoemulsified and nanoencapsulated forms was noticed. Interestingly, a relation between the octanol-water partitioning coefficients results with the antioxidant potential of mānuka oil in low (crossbred) and high (wagyu) fat meat systems has been observed (discussed in Chapter 4). The higher antioxidant of the mānuka oil in wagyu pastes than the crossbred pastes can be related to the solubility and absorption of compounds in the fat of the meat. Due to their interactions with fats, oils reduced lipid oxidation in high-fat meat pastes.

During the storage, control wagyu pastes appeared more vulnerable to lipid oxidation than crossbred beef pastes, possibly due to their higher monounsaturated fatty acid content. Bermingham et al. (2021) have already reported higher unsaturated fatty acid and neutral lipid content in wagyu beef tenderloins than Angus beef. Generally, lipid oxidation values increase with an increase in storage period due to the increased production of secondary oxidation products. It seems that all essential oil treatments were effective in controlling lipid oxidation in both beef pastes, as observed from the lower TBARS values than the controls. The reason may be the presence of bioactive compounds, which are responsible for the antioxidant activity of essential oils, as documented in the literature (Porter & Wilkins, 1999; Kaur et al., 2021). Mainly, phenolic compounds such as thymol, eugenol, and carvacrol have been related

to the antioxidant activity of essential, while some minor compounds like p-cymene and γ-terpinene also possess significant antioxidant characteristics (Ghaderi-Ghahfarokhi et al., 2016).

Several research studies have reported that essential oils encapsulated in nanoemulsions and nanocapsules have strong antioxidant activity in beef and beef products. Similar to our findings, in a research study by Ghaderi-Ghahfarokhi et al. (2016), a significant reduction of TBARS values was observed for free and encapsulated thyme essential oil-treated beef burgers than the control burgers during 8 days of chilled storage. In another study, thyme essential oil-chitosan nanoemulsions, thymol-chitosan nanoemulsions and chitosan nanoemulsions exhibited reduced TBARS, pH and improved effect against colour degradation of refrigerated pork compared to the control (Wang et al., 2022).

7.3.10.4.Colour

The colour is an essential quality indicator used by consumers to determine the freshness of beef, thereby influencing purchasing behaviour. The changes in colour values of wagyu and crossbred beef paste with and without preservative treatments are presented in Table 7.5. Depending on the addition of the antioxidants, both meat pastes exhibited different colour characteristics during the storage period of 14 days at 4 °C.

At the beginning of storage, the highest L^* values of wagyu beef were observed than those treated with essential oils and crossbred pastes. There was a significant reduction ($p \leq 0.05$) in L^* values of crossbred and wagyu pastes during the storage period, either with or without antioxidants. This could be due to the fact that samples were decomposed through microbial growth, endogenous enzymes, and myoglobin oxidation into brown methemoglobin through long-term oxygen contact. Changes in protein structure and conformation during oxidation lead

to a change in the brightness of meat and, thus, changes in the *L** values of pastes (Vital et al., 2016; Zhang et al., 2022).

As presented in Table 6.5, the *b** values of crossbred beef pastes containing essential oils were close to those obtained from the control nanoemulsions and nanocapsules-treated samples. Interestingly, different values of yellowness and blueness (*b**) for control crossbred and wagyu pastes were noticed in the initial phase of the storage period. It could be related to the concentration of β-carotene deposited in the fat of some beef carcasses relative to other beef or cattle breeds (Jaborek et al., 2019). During the storage, a gradual decrease in *b** values of all treatments of crossbred beef was observed; however, this decrease was lower for encapsulated and nonencapsulated essential oils containing pastes than the untreated pastes (control). Wagyu paste showed a similar pattern to crossbred pastes in a decrease of *b** values. No significant difference in nanoemulsified, nanoencapsulated and free oil treatment was detected in the different forms of essential oils.

A significant and continuous *a** (redness) value reduction was seen in both wagyu and crossbred pastes. During the storage, a sharp drop in *a** of control wagyu and crossbred pastes was noticed by more than 90 % and 50 %, respectively. Even if this decrease was observed in free and encapsulated essential oil-treated samples, it was lower than in the controls, indicating that essential oils tended to stabilise the red colour due to their antioxidant effect against protein oxidation. A sudden decrease in redness values of pastes in the first week and then constant or higher *a** values of mānuka and rosemary-nanocapsules treated samples could be related to the gradual release (data not shown) of essential oils/bioactive compounds from the nanocarriers, thus retarding protein oxidation.

The results of the change in colour values of nanoencapsulated and nanoemulsified oil treatment of meat are consistent with those previously reported by Wang et al. (2020) and Noori

et al. (2018). Wang et al. (2020) documented that eugenol nanocapsules can reduce colour changes in *L**, *a** and *b** levels of chilled pork during refrigerated storage (16 days at 4 °C). Lowest colour changes (ΔE) in chicken breast fillets treated ginger essential oil nanoemulsions after 12 days storage at 4 °C was observed by Noori et al. (2018).

Table 7.5. The changes in colour values for wagyu and crossbred beef pastes with or without any added antioxidant agent during storage at 4 °C for 14 days.

		Treatments										
Days	**Meat**	**MO**	**ME**	**MC**	**RO**	**RE**	**RC**	**SN**	**CC**	**CE**	**C**	**SEM**
L*												
0	Crossbred	51.9^{aA}	52.4^{aB}	52.5^{aB}	51.9^{aA}	52.4^{aA}	51.5^{abA}	46.6^{dB}	51.0^{bB}	49.9^{cB}	51.5^{aB}	0.21
	Wagyu	51.9^{bA}	54^{bA}	51.8^{bA}	51.3^{bA}	52.8^{bA}	51.5^{bcA}	50.5^{cA}	53.7^{bA}	51.9^{bA}	56.0^{aA}	0.56
7	Crossbred	45.9^{dA}	48.5^{aA}	47.7^{aA}	47.4^{aA}	47.3^{aA}	47.6^{abA}	43.3^{eB}	45.8^{dB}	45.4^{dA}	46.9^{dB}	0.28
	Wagyu	45.1^{bA}	46.9^{bB}	46.0^{bB}	45.8^{bB}	46.8^{bB}	46.6^{bB}	45.8^{bA}	46.9^{bA}	46.6^{bA}	49.2^{aA}	0.39
14	Crossbred	44.2^{bB}	47.8^{aB}	47.2^{aA}	46.3^{bB}	47.7^{aB}	47.5^{aB}	42.3^{eB}	44.9^{cB}	45.4^{bB}	43.8^{dB}	0.35
	Wagyu	46.1^{cA}	48.5^{aA}	46.6^{bB}	47.4^{bA}	48.9^{bA}	48.4^{abA}	46.3^{bA}	48.8^{bA}	49.3^{aA}	46.9^{cA}	0.48
Storage effect												
	Crossbred	***	***	***	***	***	***	***	***	***	***	***
	Wagyu	***	***	**	***	***	***	*	***	***	***	***
a*												
0	Crossbred	14.0^{dB}	14.7^{cB}	14.9^{cB}	15.7^{aB}	15.8^{aB}	14.7^{cdB}	12.5^{eB}	14.1^{cB}	14.5^{cB}	15.0^{bB}	0.16
	Wagyu	17.4^{dA}	17.8^{dA}	17.8^{dA}	18.9^{bA}	18.6^{bA}	19.2^{aA}	16.6^{eA}	19.1^{aA}	19.5^{aA}	18.5^{bA}	0.24

7	Crossbred	10.6^{aA}	7.2^{cdB}	7.1^{cdB}	8.0^{cdA}	7.5^{cdA}	7.5^{cdA}	7.0^{dA}	9.8^{bcA}	6.9^{dB}	5.9^{eB}	0.52
	Wagyu	7.9^{bB}	8.3^{bA}	8.1^{bA}	7.7^{bA}	7.9^{bA}	7.7^{bA}	7.6^{bA}	8.5^{bB}	7.4^{bA}	10.4^{aA}	0.33
14	Crossbred	5.9^{aB}	6.0^{aB}	7.7^{aB}	5.6^{aB}	8.2^{aB}	8.5^{aB}	5.7^{aB}	6.8^{aB}	6.8$^{a\ A}$	1.8^{bB}	0.89
	Wagyu	10.9^{aA}	10.1^{aA}	12.7^{aA}	12.3^{aA}	12.5^{aA}	11.3^{aA}	8.6^{abA}	7.9^{abA}	4.8^{bB}	7.8^{abB}	1.18
Storage effect												
	Crossbred	**	***	**	**	***	***	***	***	***	***	
	Wagyu	***	**	***	***	***	***	***	***	***	***	
b*												
0	Crossbred	12.9^{aB}	13.5^{aB}	13.8^{aB}	13.2^{aA}	13.4^{aB}	12.2^{bB}	10.2^{dB}	11.2^{cB}	11.5^{cB}	11.9^{cB}	0.14
	Wagyu	14.2^{bA}	15^{aA}	14.3^{bA}	13.6^{cA}	14.3^{bA}	14.4^{bA}	14.4^{bA}	15.2^{aA}	14.8^{bA}	15.4^{aA}	0.19
7	Crossbred	12.1^{aA}	12.6^{aA}	12.6^{aA}	11.6^{bA}	11.8^{bB}	11.8^{bA}	10.1^{cB}	10.9^{cB}	10.6^{cB}	11.1^{bB}	0.19
	Wagyu	11.9^{aA}	12.6^{aA}	12.6^{aA}	11.3^{Ab}	12.6^{aA}	11.7 ^{bA}	11.4^{bA}	12^{aA}	11.7^{bA}	12.9^{aA}	0.18
14	Crossbred	12.4^{aA}	12.5^{aA}	12.4^{aA}	11.5^{bA}	11.7^{bB}	11.6^{bB}	10.2^{cB}	10.1^{cB}	9.5^{dB}	10.7^{cB}	0.17
	Wagyu	12.2^{aA}	12.3^{aA}	12.0^{aA}	11.9^{Aa}	12.6^{aA}	12.5^{Aa}	11.9^{aA}	11.9^{aA}	11.9^{aA}	11.4^{aA}	0.21
Storage effect												
	Crossbred	*	***	***	***	***	*	ns	*	**	ns	
	Wagyu	***	***	***	***	***	***	***	***	***	**	

Treatments- **MO**-Mānuka oil, **RO**- Rosemary oil, **ME**- Mānuka nanoemulsion, **MC**- Mānuka nanocapsules, **RN**- Rosemary nanoemulsion, **RC**- Rosemary nanocapsules, **C**- Control, **CE**- control nanoemulsion, **CC**- control nanocapsules, **MO*RO**= comparison between mānuka oil and rosemary oil, **MO*ME**= comparison between mānuka nanoemulsion and mānuka oil, **MC*ME**= comparison between mānuka nanoemulsion and mānuka nanocapsules**, MO*MC**= comparison between mānuka oil and mānuka nanocapsules, **Storage effect (0^{th}×7^{th} ×14^{th} day)** = comparison between 0^{th},7^{th} and 14^{th} day. **ns**= $p > 0.05$, * = $P \leq 0.05$, **= $p \leq 0.01$, *** = $p \leq 0.001$, **SEM**- Standard error mean.
a-e Means within a row with the same superscript letters are not significantly different ($p \leq 0.05$) between the treatments on the same storage day.
AB Means within a column with the same superscript letters are not significantly different ($p \leq 0.05$) between the meat systems.

7.4. Conclusion

In conclusion, the results exhibited that rosemary oil was more effective against Gram-negative bacteria than mānuka oil, whereas mānuka oil showed more inhibition effect against Gram-positive microbes. The antimicrobial effect of both oils, i.e., decreased cell viability, disrupted cell wall permeability, and released intracellular materials and proteins, was also evidenced through spectrophotometric assays and electron micrographs. Free mānuka and rosemary oils showed better *in vitro* antimicrobial effects against all tested microbes than their nanoforms compared to the emulsified and encapsulated oils.

In wagyu and crossbred beef pastes, emulsions of both oils showed the lowest microbial growth than free oils, nanocapsules, sodium nitrite and controls. Control wagyu pastes showed lower microbial growth than the crossbred pastes and preservatives, and the antimicrobial effect of both essential oils and their nanoentities were also significantly different in both meat pastes. Rosemary oil and its nanoentities showed the lowest growth of Gram-negative microbes than the other treatments, while mānuka oil and its nanoentities were effective against Gram-positive bacteria. The results suggest the usage of mānuka oil and its nanoentities as an alternative to sodium nitrite, particularly against Gram-positive microbes. However, future studies on the effectiveness of these oils against several microbes and their strains from different species are needed to provide concrete evidence of their antimicrobial efficacies. In addition, the present encapsulation and emulsification method may be used for encapsulating other food-grade essential oils.

Chapter 8 Conclusions and future outlook

8.1 Conclusions

The studies discussed in this thesis explored the antioxidant and antimicrobial characteristics of mānuka oil as a natural preservative for meat applications**.** Firstly, mānuka, kānuka and rosemary oils were characterized for their antibacterial and antiradical efficacies (Chapter 3). The octanol-water coefficient values of major compounds in these oils were also elucidated to understand their lipophilicity and affinity for fat (Chapter 4). The preservative effect of this oil was compared against commonly used natural preservatives-rosemary oil, and chemical preservative-sodium nitrate in low and high-fat meat pastes (Chapter 5). Further, nanoemulsions and nanocapsules containing mānuka and rosemary oils were prepared (Chapter 6) and compared for their antioxidant and antimicrobial characteristics in low (crossbred) and high-fat (wagyu) beef pastes against rosemary oil and sodium nitrites (Chapter 7). Interactions and release properties of mānuka and rosemary were also studied from their nanoemulsified and nanoencapsulated form (using sodium alginate and whey proteins as carrier agents) (Chapter 6). The research done in this project aimed to answer the following questions:

8.1.1. Do mānuka and kānuka oils possess antioxidant and antimicrobial characteristics?

Mānuka oil is a complex mixture of monoterpenes hydrocarbons, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes. The composition of the oils was analysed using gas chromatography-mass spectrometry and Fourier transform-infrared spectroscopy. In contrast, kānuka oil possessed monoterpenes hydrocarbon compounds, especially α-pinene, accounting for around 60 % of its chemical composition. Rosemary oil contained oxygenated sesquiterpenes as a major compound, accounting for around 65 % of its composition. As per the available literature, antimicrobial characteristics of mānuka oil are attributed to the β-triketones (Maddocks-Jennings et al., 2005). This study used mānuka oil containing 5, 25 and

40 % triketone levels to examine their effect on antibacterial and antioxidant effects. Disc diffusion and broth dilution methods were used to examine the antimicrobial potential of essential oils.

Mānuka oil with higher triketone contents showed an increased diameter of the zone of inhibition of selected pathogenic Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus)*. However, increased triketone content did not significantly increase ($p \leq 0.05$) the inhibition zone of *Salmonella* and *Escherichia coli.* Mānuka oil showed a 2.5 % concentration (MIC value) to inhibit tested Gram-negative microbes (*Salmonella* and *Escherichia coli*)*;* however, it prevented *Listeria monocytogenes* and *Staphylococcus aureus* at concentrations lower than 0.04 %. It possibly is due to the complex layer of Gram-negative bacteria preventing the penetration of hydrophobic compounds of essential oil in microbial cells. In broth dilution assay, a 2.5 % minimum inhibitory concentration of rosemary was noticed to successfully inhibit *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli.* As per the broth dilution method results, kānuka oil also showed a higher antimicrobial effect against tested Gram-positive microbes with MIC values of 0.63 % than Gram-negative microbes at 2.5 %. Similar to the antimicrobial activity, mānuka oil exhibited higher antiradical activities against DPPH• and Fe^{2+}• than kānuka oil.

About the antioxidant efficacies, the DPPH• and Fe^{2+}• free radical quenching effect of all oils was determined using DPPH and FRAP assays. Compared with the tested synthetic antioxidant, i.e., butylated hydroxytoluene (BHT), mānuka oil's (at 0.1 % concentration) DPPH• and Fe^{2+}• scavenging activity was around 4 and 15 times higher. This study compared the antioxidant and antimicrobial efficacies of mānuka and kānuka oil with rosemary oil. In terms of antioxidant activity, the ability of rosemary oil to scavenge DPPH• and Fe^{2+}• radicals was significantly lower ($p \leq 0.05$) than the mānuka and BHT.

8.1.2. *How will essential oils get partitioned into different phases?*

The octanol-water partition coefficient of major compounds of mānuka oil, such as α-pinene, γ-terpinene, leptospermone, isoleptospermone, and flavesone, was between 2.5 and 5.5, indicating strong affinity of these compounds towards meat-fat than the water. These results were compared with the predicted values of the octanol-water partition coefficient using the EPI suite. In 3 % and 12 % beef-fat and water systems, amounts of these compounds separated in water were very low. These results suggest that if these compounds are added to high-fat systems, their partitioning in other phases and contact with the microbes present in the aqueous phase may be very low. These results also agree with the low water solubility and octanol-air partition coefficient values evaluated using the same software (EPI suite). As the microbial outer membranes are composed of lipid layers, the log *P* values suggest that the key components in mānuka oil will prefer to be in the microbial membranes rather than stay in the surrounding water phases. It is likely to contribute significantly to the antimicrobial behaviour of essential oils. In food systems containing different phases, fat and lipid components will absorb these compounds much more than water, effectively reducing their concentration in the aqueous regions where microorganisms prefer to grow. Thus, due to their absorption in the lipids, high concentrations of the oils will be required to exert a desirable antimicrobial effect in high-fat systems. The antimicrobial and antioxidant effect of mānuka oil in high-fat and low-fat meat pastes was investigated to correlate it with log *P* values.

8.1.3. *Whether mānuka oil possess the same antioxidant and antimicrobial characteristics as natural preservatives, i.e., rosemary oil and chemical preservatives-sodium nitrate, when added to meat systems?*

8.1.4. *How do the mānuka and rosemary oils influence the physiochemical characteristics of low and high-fat meat pastes in comparison to rosemary oil and a commonly used chemical preservative, sodium nitrate?*

Generally, bioactive compounds show higher antimicrobial effects in laboratory broth media than food matrices, owing to the higher water content and absence of interfering compounds, especially fat in the media. Significant differences were observed when mānuka and rosemary oils were examined for their antimicrobial and antioxidant characteristics in low (3 %) and high-fat (12 %) meat matrices or pastes prepared from commercial beef and wagyu tenderloins, respectively.

Regarding antioxidant efficacy, mānuka oil treatment significantly delayed lipid oxidation in wagyu paste, similar to sodium nitrate, and higher than the control and rosemary oil. On the other hand, all commercial beef pastes had an increase in lipid oxidation during storage, whereas these increases were higher in controls than in the treated samples. Regarding the pH of meat matrices, all meat matrices have undergone changes, especially control samples, while essential oils added samples did not have significant changes ($p \leq 0.05$) in pH values. It might be attributed to the inhibition effect of essential oils against microbial growth (lactic acid bacteria and other microbes) and enzymes, thereby inhibiting protein breakdown and pH changes. Both kinds of beef pastes significantly differed regarding colour characteristics, oxidation, and microbial stability. Wagyu pastes appeared more susceptible to lipid oxidation than the latter paste, which may be due to the higher fat level and unsaturated fatty acid contents. Commercial beef paste showed more microbial growth than wagyu paste, which might be related to the higher moisture/water content of the latter beef than the former. The difference in colour characteristics of both pastes (at the beginning of storage) can be associated with the fat differences and β-carotene concentration (banked in the fat of normal beef carcass).

In inoculated meat pastes, mānuka oil successfully delayed the growth of *Listeria monocytogenes* and *Staphylococcus aureus* in both kinds of meat pastes, while this effect was higher in low-fat meat paste than in high-fat meat paste. Rosemary oil inhibited the tested Gram-negative microbes (*Salmonella* and *Escherichia coli*). All control samples inoculated with *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* and *Escherichia coli* reached 7 log cfu/g on the 10^{th} day at 4 °C. While rosemary oil-treated meat samples inoculated with *Salmonella, Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* remained lower than sample 7 log cfu/g until the 16^{th} day, indicating delayed growth of microbes in oils-containing samples. Similarly, mānuka oil containing meat samples with *Listeria monocytogenes* and *Staphylococcus aureus* remained lower than sample 7 log cfu/g until the 16^{th} day.

8.1.5. How do nanoencapsulation and nanoemulsification affect the thermal stability and kinetics of the release of mānuka and rosemary oils?

To answer this question, mānuka and rosemary containing nanoemulsions and nanocapsules were prepared using sodium alginate and whey protein as carrier material and emulsifier, respectively. The prepared nanocapsules and nanoemulsions showed better characteristics, such as improved thermostability and sustained release, than the free oils. Their particle size was in the nano-range between 300 and 500 nm, and zeta potential was in -ve values (-12 and -10 mV), showing the electrical stability of nanoentities. The core-shell type structure of obtained nanocapsules was confirmed using scanning electron microscopy images, and oil droplets distribution in the aqueous phase of nanoemulsion was elucidated using optical microscopy. In the FTIR results, molecular interaction between essential oils and carrier materials led to changes in the intensity and width of peaks, which confirms the encapsulation of oils. In 10 % ethanol, 50 % ethanol and distilled water solutions mimicking aqueous,

alcohol-containing, and fatty foods, respectively, the oil release profile from nanoemulsions and nanocapsules was slower and more controlled than the unencapsulated oil. It shows the difference in solubility and release mechanisms of unencapsulated and encapsulated oils.

Regarding antioxidant potential, lower DPPH•, Fe^{2+}•, ABTS• radical scavenging activities of nanoemulsions and nanocapsules were observed than the free oils. When these nonentities were compared for their antioxidant effect against sodium nitrite in wagyu (23 %) and crossbred beef (2.3 %) pastes during refrigerated storage for 16 days, all treatments exhibited delayed lipid oxidation than the controls. In comparing the free, nanoemulsified and nanoencapsulated oils, nanoemulsions of both oils showed the best antioxidant effect and lowest lipid values in wagyu beef paste, while no significant differences ($p \leq 0.05$) were observed between all forms in crossbred beef.

Changes in colour properties of both meat pastes were noticed, while these changes were greater in the without preservatives added meats pastes than in the control nanoemulsions, control nanocapsules and meat-only pastes.

8.1.6. How do nanoencapsulation and nanoemulsification affect the antioxidant and antimicrobial activity of mānuka and rosemary oils in low and high-fat beef pastes?

After nanoencapsulation and nanoemulsification, the *in vitro* and *in-situ* antimicrobial potential of mānuka and rosemary oils significantly differed from the free oils. Free oils showed a significantly higher ($p \leq 0.05$) antimicrobial effect in disc diffusion assay and broth dilution method than encapsulated oils might be due to the slowed release of essential oils from nanoentities. Rosemary oil was more effective towards *Salmonella* and *Escherichia coli* (Gram-negative microbes) than mānuka oil, while mānuka oil inhibited the tested Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) most effectively. The influence of mānuka and rosemary oils on decreased cell viability, cell wall permeability, and increased

release of intracellular materials and proteins from *Salmonella*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* cells than the untreated cells was observed. These mechanisms were also confirmed through scanning electron microscopy images.

To understand the answer to this question, nanoentities containing mānuka and rosemary were explored for their antimicrobial potential in low (2.3 %) and high-fat (23 %) containing meat matrices prepared from crossbred and wagyu beef tenderloins during storage (4 °C) and compared with sodium nitrite. Results indicated significantly delayed growth of *Salmonella* and *Escherichia coli* in rosemary treated-meat pastes than in mānuka, sodium nitrite-treated and control meat paste samples. On the other hand, mānuka oil treatment inhibited *Listeria monocytogenes* and *Staphylococcus aureus* more effectively than other meat paste treatments. Nanoemsulions containing essential oils showed a better antimicrobial effect than the nanocapsules, which are free oils-treated and control meat pastes.

8.2. Future Outlook

This research examines the potential of mānuka oil as a natural preservative for food application. There are several areas where future research studies can further explore these preservatives, their effectiveness, use in foods and interaction with food products for better understanding, as discussed below:

8.2.1. Exploration of different characteristics of meat products containing mānuka oil

This study examined the antimicrobial and antioxidant potential of mānuka and rosemary oils against selected pathogens in low and high-fat meat pastes. However, other characteristics, such as sensory and texture characteristics, are of paramount importance when considering any additive to add to any food product. The determination of the effect of these oils on the taste, flavour and odour of food products at different concentrations can ensure consumer

acceptability. Future research studies on the safety and toxicity of mānuka oil and its bioactive compounds can provide concrete proof of its safe usage in foods. Chen et al. (2016) confirmed the antimicrobial efficacies of mānuka and kānuka oils with no adverse effect on immune systems (using human cute monocytic THP-1 cells during 48 hours of exposure). Further exploration of the safety of these oils against various cell lines through different methods needs to be performed.

8.2.2. Selection of different food matrices

Different food products may respond to essential oils treatment differently based on their chemical composition. Further, research studies on the effects of mānuka and rosemary oils treatment in a diverse range of foods such as dairy (especially milk and cheese), juices, fruits, bakery, and meat products could be carried out to explore their wide range of usages. The essential oils can be considered as an additive (obeying the legal regulations (EC, 2008) for any additive used in foods) or applied as packaging in either the edible coating or active packaging (complying with regulations (Commission, 2004) for materials intended to come into contact of food). The application of essential oils in edible coating form has been documented by a literature review by Ju et al. (2019). Mānuka oil in the edible film of sodium alginate or any other carrier materials can be further explored for its preservative effect on fresh vegetables and fruits, cheeses and meat cuts, like other essential oils (Guerreiro et al., 2015; Artiga-Artigas et al., 2017; Rezaei & Shahbazi, 2018).

8.2.3. Characterization of different encapsulation systems

In this project, sodium alginate was used as carrier material to prepare nanoemulsions and nanocapsules due to its food-grade, non-toxic, low-cost nature and suitability to encapsulate essential oils in all forms (emulsions, capsules, edible films, etc.). Several novels and promising approaches have been developed in the past few decades, either materials or methods to

encapsulate and emulsify essential oils. These innovations in encapsulation offer a wide range of benefits, including the protection of bioactive from harsh external conditions (pH, temperature), provide stability, sustained release, targeted delivery and masking of undesirable characteristics (taste and odour) (Vinceković et al., 2017). Forthcoming research studies on the encapsulation of mānuka oil using different carrier materials will provide a wide range of opportunities for its applications in foods. Kinetics and modelling studies will facilitate greater accuracy for essential oils released from encapsulated forms than neat oil. Studies on the optimization of the concentration (essential oils, carrier materials and emulsifiers) and conditions (encapsulation or emulsification methods and equipment) required to encapsulate essential oils would be beneficial for their industrial application.

8.2.4. Characterization of antimicrobial potential of mānuka oil against a wide range of microbes

It is vital to mention that the antimicrobial resistance of microbial cells may vary in bacterial strains from different species. Investigation of the antimicrobial potential of mānuka and rosemary oils against a wide range of microbial strains from different species can serve as concrete proof of their antimicrobial activity. In this project, microbes of pathogenic concern only were selected, and future studies on antimicrobial efficacies of essential oils against spoilage, spore-forming, toxin-producing, biofilms-associated microbes and natural microflora of meats can evidence their effect on the shelf-life extension of foods. As spore-forming microbes are the reason for high economic losses in the food and feed industry, these oils may show antimicrobial activity against these spores and lessen food spoilage. It has been reported that essential oils compounds especially phenolic compounds have the ability to interact with membranes of spore-forming microbes like *Bacillus cereus*, and imbalance the vital process of the cell leads to cell death. Although these effects are dependent on the concentration, exposure

time, and bacterial species. In addition, in-depth mechanisms of action and characterization of essential oils against microbial strains and species, can undercover the potential of innovative antioxidant and antimicrobial agents for food applications.

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Appendix D: The changes in lipid oxidation values for wagyu and crossbred beef paste with or without any added antioxidant agent during storage at 4 °C for 14 days.

		Treatments											*p- values*			
Day	Meat	MO	ME	MC	RO	RE	RC	SN	CE	CC	C	SEM	MO*ME	MO*MC	MC*ME	MO*RO
TBARS																
0	Normal	0.14^{bB}	0.14^{bA}	0.14^{bA}	0.14^{bA}	0.14^{bA}	0.16^{bA}	0.15^{bB}	0.14^{bB}	0.22^{aB}	0.15^{bB}	0.005	ns	ns	ns	ns
	Wagyu	0.16^{cA}	0.13^{cA}	0.11^{cdB}	0.11^{cdB}	0.08^{eB}	0.10^{deB}	0.21^{bA}	0.22^{abA}	0.25^{aA}	0.24^{abA}	0.008	ns	*	ns	*
7	Normal	0.26^{cA}	0.19^{cA}	0.21^{cA}	0.21^{cA}	0.24^{cA}	0.22^{cA}	0.60^{aA}	0.45^{bB}	0.46^{bA}	0.65^{aA}	0.014	ns	ns	ns	ns
	Wagyu	0.22^{dB}	0.14^{eB}	0.16^{eB}	0.23^{dA}	0.13^{eB}	0.23^{dA}	0.58^{abA}	0.54^{bcA}	0.53^{cA}	0.62^{aA}	0.010	***	**	*	ns
14	Normal	0.3^{deA}	0.25^{eA}	0.30^{deA}	0.24^{eA}	0.34^{deA}	0.32^{deA}	0.45^{cdB}	0.56^{bcB}	0.65^{bB}	1.06^{aB}	0.031	ns	ns	ns	ns
	Wagyu	0.27^{dA}	0.18^{eB}	0.30^{dA}	0.26^{dA}	0.16^{eB}	0.23^{dB}	1.36^{bA}	0.85^{cA}	1.52^{bA}	1.83^{aA}	0.033	***	ns	**	ns
Storage effect																
	Normal	**	**	**	**	***	***	***	***	***	***					
	Wagyu	***	ns	**	**	**	***	***	***	***	***					

Treatments- **MO**-Mānuka oil, **RO**- Rosemary oil, **ME**- Mānuka nanoemulsion, **MC**- Mānuka nanocapsules, **RN**- Rosemary nanoemulsion, **RC**- Rosemary nanocapsules, **C**- Control, **CE**- control nanoemulsion, **CC**- control nanocapsules, **MO*RO**= comparison between mānuka oil and rosemary oil, **MO*ME**= comparison between mānuka nanoemulsion and mānuka oil, **MO*MC**= comparison between mānuka oil and mānuka nanocapsules, and, **MC*ME**= comparison between mānuka nanoemulsion and mānuka nanocapsules, **Storage effect (0^{th}*7^{th} *14^{th} day)** = comparison between 0^{th},7^{th} and 14^{th} day. **ns**= $p > 0.05$, * = $P \leq 0.05$, **= $p \leq 0.01$, *** = $p \leq 0.001$, **SEM**- Standard error mean.

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Name and title of main supervisor:	Dr Lovedeep Kaur
In which chapter is the manuscript/published work?	Chapter 2
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Exploration of the literature review, preparation of original draft and revisions of the draft was done by the student. The main supervisor edited and reviewed the manuscript, guided for the idea and the modifications. Co-supervisors have reviewed, and edited the manuscript.

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