AN INVESTIGATION OF ESSENTIAL OILS AS ANTIMICROBIAL AGENTS AGAINST ANTIBIOTIC-RESISTANT BACTERIA ISOLATED AT SOUTH AFRICAN HOSPICES

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DECLARATION OF INDEPENDENT WORK

I, Gaofetoge Gobodiwang Setlhare, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree PHILOSOPHIAE DOCTOR: ENVIRONMENTAL HEALTH is my own work and has not been submitted before to any institution by myself or any other person in fulfillment of the requirements for the attainment of any qualification.

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SIGNATURE OF STUDENT DATE

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

SUMMARY

In developing countries including South Africa information is limited regarding the use of essential oils in treating antibiotic-resistant pathogenic bacteria. Currently, the battle between humans and the multitudes of infection and disease-causing pathogens continues. Emerging at the battlefield as some of the most significant challenges to human health is bacterial resistance to antibiotics and its rapid rise. This has become a major concern in global public health invigorating the need for new antimicrobial compounds. A rational approach to deal with antibiotic resistance problems requires detailed knowledge of the different biological and non-biological factors that affect the rate and extent of resistance development. Besides this approach, there are other approaches to resolving this challenge. For example, modulating the gut microbial community, either through feed additives (e.g. probiotics and prebiotics) or fecal transplantation could be a promising way to prevent certain diseases; non-antibiotic approaches include phage therapy, bacteriocins, and predatory bacteria which are effective against biofilms and can access recalcitrant infections. However, Allen and colleagues (2014) highlighted limitations of these antibiotic alternatives; these limitations include complex regulatory processes of the Food and Drug Administration (FDA) regarding feed additives, the high cost of vaccines as well as limited cross-protection of vaccines against some pathogens. The challenge with bacteriocins is possible sensitivity to proteolysis; phage therapy specificity begets technical limitation of

administration against multiple subspecies while possible interactions of predatory with the host and their commensal microbiota are as yet unknown. Therefore, plants and their derivatives, such as essential oils, are currently blooming and represent a potential area for future investigations. This new generation of phytopharmaceuticals may shed light on the development of new pharmacological regimes in fighting antibiotic resistance. This study consolidates and describes the observed antagonistic outcome of essential oils against antibiotic-resistant *S. aureus* and *B. cereus*, and highlights the possibilities of essential oils as the potential antimicrobial agent.

In the current study, the bacterial cell wall of antibiotic-resistant *S. aureus* and *B. cereus* was exposed as one of the targets for thyme essential oil. *Thymus vulgaris* essential oil showed high antimicrobial activity against the cell wall, cell membrane, and cytoplasm, and in some cases completely changed the morphology of the cells (antibiotic-resistant *S. aureus* and *B. cereus*). This indicates that the cell envelope became thinner than normal. In addition, using Gram staining, the current results clearly showed Gram-positive cells to be affected by a cell wall active agent (thyme oil) and stained pink like Gram-negative bacterial cells. This is possibly due to a decrease in peptidoglycan thickness or a disturbance in the cell wall during cell growth in the presence of thyme oil.

These observations further indicate the stress placed on bacteria due to exposure to essential oils and this might have resulted in changes in morphology and the formation

of Small Colony Variants (SCV) as observed on the agar plate. Small colony variants (SCV) arise within homogeneous microbial populations, largely in response to various environmental stresses, including essential oils. They display unique phenotypic characteristics conferred in part by possible heritable genetic changes. Characteristically slow growing, SCVs comprise a minor proportion of the population from which they arise but persist by virtue of their inherent resilience and host adaptability.

To further confirm the influence of thyme oil on the bacterial cell wall of antibiotic-resistant strains of *S. aureus* and *B. cereus*, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed. Exposure to thyme oil induced alterations within the bacterial membrane of *S. aureus* as well as *B. cereus*, which resulted in a loss of cell wall integrity, as demonstrated by Gram staining, SEM, and TEM. In addition SEM and TEM micrographs showed loss of cellular contents and irregular cytoplasmic membrane. The intracellular leakage and morphological changes of the two treated bacterial cells indicated that *Thymus vulgaris* essential oil affected the structural organisation of the cytoplasm together with the cell wall of *S. aureus* and *B. cereus* cells. It might be proposed that in the primary phase, thyme oil probably binds to the bacterial cell surface and penetrates the cell wall causing cytoplasmic membrane damage and this leads to cell death. This phenomenon indicates that all these observed changes might be stress induced forming SCVs and that the cell wall is the first target of essential oils and this is also an indication that the tested oil indeed affected the

structural organisation of both antibiotic-resistant organisms (*S. aureus* and *B. cereus*) since this was not observed in the untreated cells.

It was evident in this study that the activity of thyme oil is not attributable to a single event but involves a series of events both on the cell surface and within the cytoplasm. The disruption of the cell wall and membrane integrity observed through SEM and TEM electron micrographs was also found to result in reduced saturated and unsaturated fatty acids as observed in the fatty acids profile assessment. Based on the results, it is apparent that thyme essential oil firstly acts on the cell wall and disrupts the outer membrane of antibiotic-resistant *S. aureus* and *B. cereus* which could lead to dispersion of the desaturase enzymes and allows them to act on the membrane fatty acids. In addition to direct effects on the fatty acids of the outer membrane, it is believed that thyme oil affected enzymes that are involved in fatty acid synthesis. The tested oil caused a major decrease in unsaturated fatty acids and this could be due to disrupted fatty acyl-CoA desaturase enzyme affected by the essential oil. However, further investigation needs to be done to certainly prove this hypothesis.

Again, since lipids are the principal form of stored energy in *S. aureus* and *B. cereus* and are major constituents of cellular membranes. It is believed that once fatty acids were depleted due to damaged cell membrane as shown in SEM and TEM electron micrographs, the disrupted cell wall and membrane would inevitably lead to cell death. Therefore, it is speculated from the findings of this study that the membrane disruption

effect of the essential oil, and the less amount of fatty acids is the consequence of the inhibited bacterial growth.

Except for fatty acids, total proteins were also found to be affected by thyme oil. In the proteomic analysis, a total of three proteins from *S. aureus* and two proteins from *B. cereus* bacteria demonstrated reduced expression levels, upon treatment with *Thymus vulgaris* essential oil. The reduced protein expression of total proteins for both antibiotic-resistant *S. aureus* and *B. cereus* could be due to the depletion of fatty acids on the cell membrane. Since lipids serve as anchors for proteins, it is clear from the results that the disrupted fatty acids inevitably results in a damaged bacterial protein with reduced expression levels.

Most importantly this study has revealed the importance of using essential oils as possible alternative antimicrobial agents. During microbial analysis, thyme essential oil damaged the cellular membrane of both antibiotic-resistant *S. aureus* and *B. cereus*, which led to cell death. Additionally, the depletion in the lipid profile and protein profile shown after the treatments of the resting cells is strictly related to the presence of thyme oil compounds. Therefore, this shows that *Thymus vulgaris* essential oil has the capability to target numerous bacterial sites (particularly the cell membrane, cytoplasm, lipids, enzymes, and proteins). In addition, from the findings of this study, some of the advantages of using natural antimicrobials such as thyme essential oils were further identified and those factors include: in the health sector the possibility of reducing total

dependence on antibiotics and using with essential oils as alternatives can be seen; for the food industry potential use in controlling cross-contaminations by food-borne pathogens as well as improvising food preservation technology can be observed. Thyme essential oil is traditionally believed to be rich in phytochemicals showing rich bioactivity. Their compounds identified in this study could be of interest to the local industry as well as to the general population and could be actively explored for various commercial applications. However, more research will have to be conducted to assess the antimicrobial effects of thyme oil in food matrices since the current study exposed the effects of the tested oil on bacteria without interference from matrices such as food products. Thyme essential oil is therefore considered a potential natural antimicrobial agent. Based on these facts, the present study focused mainly on exploring the diverse mechanisms of action of *Thymus vulgaris* essential oil and its components against two specific antibiotic-resistant food-borne pathogens namely *S. aureus* and *B. cereus*. The scientific details provided in this study on these aspects are expected to be useful for the commercial exploitation of essential oils to develop natural preservative and disinfectant preparations with applicability in the food and pharmaceutical industries.

CHAPTER 1: ESSENTIAL OILS, AN ALTERNATIVE APPROACH IN FIGHTING BACTERIAL ANTIBIOTIC RESISTANCE: A LITERATURE REVIEW

ESSENTIAL OILS, AN ALTERNATIVE APPROACH IN FIGHTING BACTERIAL ANTIBIOTIC RESISTANCE: A LITERATURE REVIEW

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1.1 GENERAL BACKGROUND

The introduction of antimicrobials transformed human and animal health systems by revolutionising the weaponry in the battle against infectious diseases, resulting in improved survival for both humans and their domestic animals (Jansen, 2012; Nigam *et al*., 2014). However, this health triumph was immediately diminished by the subsequent realisation that bacterial populations could quickly modify themselves to resist antimicrobials, propagate resistance traits, and even share resistance genes with other contemporary bacteria within their environment. This has resulted in challenges in the health sector where more than half of healthcare associated infections are due to antibiotic-resistant pathogens such as *Salmonella* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa*., Methicillin-resistant *Staphylococcus aureus* (MRSA)., *Bacillus cereus* amongst others (Nigam *et al*., 2014).

Bacillus cereus is one of the important opportunistic pathogens associated with nosocomial infections. From September 1990 to October 1990, 15 patients who were admitted to four different departments of the National Taiwan University Hospital, including nine patients in the emergency department, three in the hematology/oncology ward, two in the surgical intensive care unit, and one in a pediatric ward, were found to have positive blood (14 patients) or pleural effusion (1 patient) cultures for *Bcereus* (Hsueh *et al*., 1999). These isolates were found to be commonly resistant to penicillin and cephalosporins (Hsueh *et al*., 1999). After this study, resistance was observed by other researchers such as Owusu-Kwarteng *et al*. (2017). In South Africa, Nkhebenyane *et al*. (2012) also reported resistant *B. cereus* isolates from the registered South African

HIV/AIDS hospices. The isolates were commonly resistant to cefoxitin, tetracycline, oxacillin and nalidixic acid (Nkhebenyane *et al*., 2012). The emergence of these multidrug-resistant (MDR) strains is a challenging clinical problem as infections caused by *B. cereus* are difficult to treat and often require combination therapy which is often expensive (Hsueh *et al*., 1999; Nkhebenyane *et al*., 2012; Owusu-Kwarteng *et al*., 2017). Moreover, Khan and Khan (2016) stated that increases in the rate of antimicrobial resistant *B. cereus* results in the use of much more expensive drugs, prolonged hospitalisations coupled with higher healthcare costs.

Except for *B. cereus*, *Staphylococcus aureus* is another resistant pathogen commonly found in healthcare settings. *Staphylococcus aureus* is a human pathogen that causes diseases in patients ranging from relatively mild infections of the skin and soft tissue to life-threatening sepsis. The emergence of strains resistant to methicillin and other antimicrobial agents has become a major concern, especially in the hospital environment, because of the higher mortality rates (Dulon *et al*., 2011; Odai, 2016). Significant increases in methicillin resistance in clinical strains of *S. aureus* isolates between 2000 and 2016 in European countries have been shown particularly Western Europe. Methicillin- resistant *Staphylococcus aureus* (MRSA) prevalence varied widely, from less than 1% to greater than 20% (Dulon *et al*., 2011; Odai, 2016).

Moreover, Falagas *et al*. (2013) and Odai (2016) assessed the prevalence of methicillin resistance in *Staphylococcus aureus* isolates in Africa; thirty-two studies were included.

In Tunisia, the prevalence of MRSA increased from 16% to 41% between 2002–2007, while in Libya it was 31% in 2007. In South Africa, the prevalence of MRSA decreased from 36% in 2006 to 24% during 2007–2011, probably due to the implementation of effective infection control policies. In Botswana, the prevalence varied from 23–44% between 2000 and 2007. In Algeria and Egypt, the prevalence was 45% and 52% between 2003 and 2005, respectively. In Nigeria, the prevalence was greater in the northern than the southern part of the country. In Ethiopia and the Ivory Coast, the prevalence was 55% and 39%, respectively. The prevalence of MRSA was lower than 50% in most of the African countries, although it appears to have risen since 2000 in many African countries, except for South Africa (Falagas *et al*., 2013; Odai, 2016).

Other than Europe and African countries, there were more than 80% observed cases of invasive MRSA reported in the United States (Klevens *et al*., 2006). Most MRSA infections were health care–associated: 58.4% were community-onset infections, 26.6% were hospital-onset infections; 13.7% were community-associated infections, and 16% were deaths among patients with MRSA infection (Klevens *et al*., 2006). Klevens and colleagues (2006) also reported that 20% of bloodstream infections in the hospital setting were caused by resistant strains of *S. aureus* and that prevalence of MRSA infections is usually associated with greater lengths of stay, higher mortality and increased costs. However, a substantial decrease (31.2%) in the national burden of invasive MRSA infections has been observed in the United States between 2005 and 2013, with the largest decreases among hospital-onset infections (54.2%) and the smallest among community-associated infections (5.0%) (Dantes *et al*., 2013).

In healthcare settings, chloramphenicol and tetracycline are other antimicrobial agents considered as the most important and mostly used (Taka *et al*., 2012; Khan *et al*., 2015). These antimicrobial agents were regarded as highly effective, broad-spectrum agents against many Gram-positive and Gram-negative bacteria, including most anaerobic organisms up until bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Proteus mirabilis*, Enterobacteriaceae, etc. developed resistance strategies against them (Taka *et al*., 2012; Khan *et al*., 2015). Many bacterial strains are now resistant to the effects of antibiotics that once could abolish them. Every population of bacteria may have some individuals that are resistant and somehow modify the cell wall in such a way that antibiotics are unable to penetrate. Moreover, the careless use of the drugs has given some resistant bacteria the upper hand in the fight against disease (Davies and Davies, 2010; Taka *et al*., 2012; Khan *et al*., 2015).

1.2 BACTERIAL RESISTANCE STRATEGIES AGAINST ANTIBIOTICS

In healthcare settings, most of the microorganisms are able to survive and multiply in the presence of antimicrobial agents that would usually inhibit that particular kind of microorganism (Halon, 2005; WHO, 2014). Bacterial organisms have the ability to enable themselves to out-compete and out-survive their microbial neighbours and overcome host strategies (Halon, 2005; WHO, 2014). To survive in the presence of antibiotics, bacterial organisms usually disrupt few of the essential steps required for the effective action of the antimicrobial agent (Figure 1.1). For example, a variety of Gram-negative bacteria including *P. aeruginosa* and *Enterobacter aerogenes* reduce the uptake of certain antibiotics, such as aminoglycosides and beta-lactams, by modifying

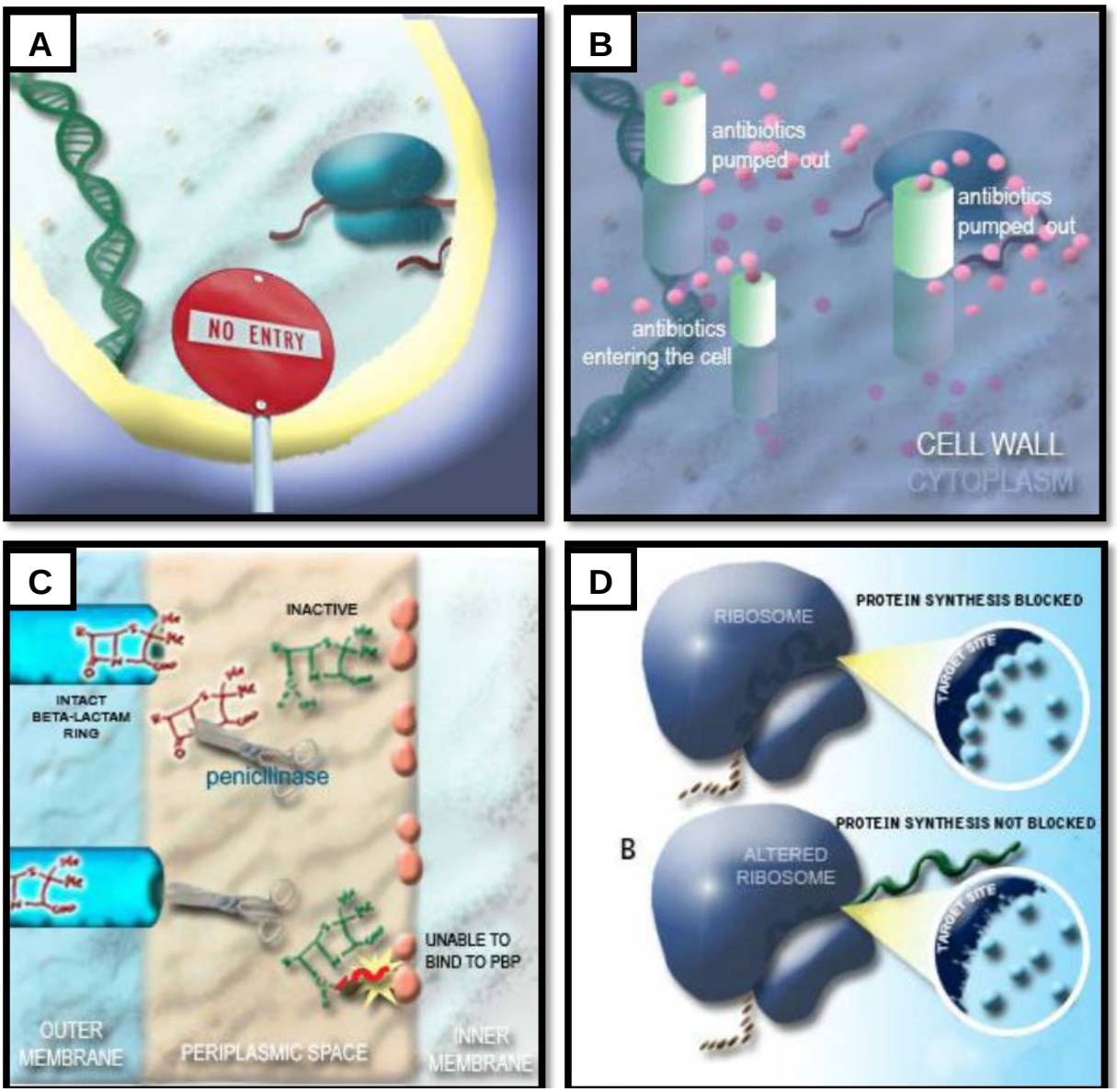

Figure 1.1: Figures A-D indicating four major bacterial acquired resistance strategies against antibiotics. A – By prevention of the antimicrobial from reaching its target by reducing its ability to penetrate into the cell; B – By expulsion of the antimicrobial agents from the cell via general or specific efflux pumps; C – By inactivation of antimicrobial agents via modification or degradation; D – By modification of the antimicrobial target within the bacteria (Adapted from Kumar and Schweizer, 2005; Lambert, 2005).

the cell membrane porin channel frequency, size, and selectivity (Kumar and Schweizer, 2005; Lambert, 2005; Nazzaro *et al*., 2013; Hamilton and Wenlock, 2016). Prohibiting entry in this manner by the bacteria will prevent antibiotics from reaching their intended targets, for aminoglycosides and beta-lactams, which are the ribosomes and the penicillin-binding proteins (PBPs) respectively (Figure 1.1-A) (Kumar and Schweizer, 2005; Lambert, 2005; Hamilton and Wenlock, 2016).

The second mode bacteria use to protect themselves against antibiotics is by the expulsion of the antimicrobial agents from the cell via general or specific efflux pumps (Walsh, 2000; Aeschlmann, 2003; Rouveix, 2007; Yap *et al*., 2014). Bacteria such as *E. coli*, *B. cereus*, *S. aureus* and *S. pneumoniae*, have total proteins that act as an export or efflux pump for certain antimicrobials, extruding the antibiotic out of the cell (Figure 1.1-B). In addition, some efflux pumps emit specific antimicrobial agents such as lincosamides, streptogramins, macrolides and tetracycline (Walsh, 2000; Aeschlmann, 2003; Rouveix, 2007; Hamilton and Wenlock, 2016).

Another technique by which bacteria (Gram-positive and/or Gram-negative) protect themselves is by terminating the active component of the antibiotic (Figure 1.1-C). A common example is the hydrolytic deactivation of the beta-lactam ring in penicillin and cephalosporins by the bacterial enzyme beta-lactamase (Wright, 2005; Hamilton and Wenlock, 2016). The inactivated penicilloic acid will then be ineffective in binding to PBPs (penicillin binding proteins), thereby protecting the process of cell wall synthesis

(see Table 1.1). Moreover, bacteria can protect themselves by reprogramming or camouflaging critical target sites to avoid recognition (Figure 1.1-D). As a result, in spite of the presence of intact and active antimicrobial compounds, subsequent binding or inhibition will not take place (Wright, 2005; Hamilton and Wenlock, 2016).

Since most of the bacteria have developed major resistance strategies against antibiotics (Table 1.1), this has led to an increase in the search for possible alternatives to control bacterial diseases, especially in humans. Such alternative treatment methods include essential oils as potential antimicrobial agents for the fight against infectious diseases and food-borne illnesses that cause severe health effects and even death especially in patients with weakened immune systems (Voon *et al*., 2012; Swamy *et al*., 2016).

Table 1.1: Mechanisms of resistance against different antimicrobial classes (Forbes *et al*., 1998; Berger-Bachi, 2002; Karam *et al*., 2016)

ANTIMICROBIAL CLASS	MECHANISM OF RESISTANCE	SPECIFIC MEANS TO ACHIEVE RESISTANCE	EXAMPLES OF RESISTANCE
Beta-lactams **Examples:penicillin, ampicillin, mezlocillin,, peperacillin, cefazolin, cefotaxime, ceftazidime, aztreonam, imipenem**	Enzymatic destruction	**Destruction of beta-lactam rings by beta-lactamase enzymes**. With the beta-lactam ring destroyed, the antibiotic will no longer have the ability to bind to PBP (Penicillin-binding protein) and interfere with cell wall synthesis.	Resistance of staphylococci to penicillin; Resistance of Enterobacteriaceae to penicillins, cephalosporins, and aztreonam
	Altered target	**Changes in penicillin binding proteins**. Mutational changes in original PBPs or acquisition of different PBPs will lead to inability of the antibiotic to bind to the PBP and inhibit cell wall synthesis	Resistance of staphylococci to methicillin and oxacillin
	Decreased uptake	**Porin channel formation is decreased.** Since this is where beta-lactams cross the outer membrane to reach the PBP of Gram-negative bacteria, a change in the number or character of these channels can reduce beta-lactam uptake.	Resistance of *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* to imipenem
Glycopeptides Example: vancomycin	Altered target	**Alteration in the molecular structure of cell wall precursor components** decreases binding of vancomycin so that cell wall synthesis is able to continue.	Resistance of enterococci to vancomycin
Aminoglycosides Examples: gentamicin, tobramycin, amikacin, netilmicin, streptomycin, kanamycin	Enzymatic modification	**Modifying enzymes** alter various sites on the aminoglycoside molecule so that the ability of this drug to bind the ribosome and halt protein synthesis is greatly diminished or lost entirely.	Resistance of many Gram-positive and Gram-negative bacteria to aminoglycosides
	Decreased uptake	**Change in number or character of porin channels** (through which aminoglycosides cross the outer membrane to reach the ribosomes of Gram-negative bacteria) so that aminoglycoside uptake is diminished.	Resistance of a variety of Gram-negative bacteria to aminoglycosides

	Altered target	**Modification of ribosomal proteins or of 16s rRNA.** This reduces the ability of aminoglycoside to successfully bind and inhibit protein synthesis	Resistance of *Mycobacterium sp* to streptomycin
Quinolones Examples: ciprofloxacin, levofloxacin, norfloxacin, lomefloxacin	Decreased uptake	**Alterations in the outer membrane** diminish uptake of drug and/or activation of an "efflux" pump that removes quinolones before intracellular concentration is sufficient for inhibiting DNA metabolism.	Resistance of Gram-negative and staphylococci (efflux mechanism only) to various quinolones
	Altered target	**Changes in DNA gyrase subunits** decrease the ability of quinolones to bind this enzyme and interfere with DNA processes	Gram-negative and Gram-positive resistance to various

1.3 ESSENTIAL OILS

Essential oils (EOs) also described as volatile or ethereal oils (Guenther, 1948; Nazzaro *et al*., 2013; Chi, 2013; Swamy *et al*., 2016) are aromatic oily liquids obtained from plant material such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots. They can usually be obtained by expression, fermentation, enfleurage or extraction but the method of steam distillation is commonly used for commercial production of EOs (Nazzaro *et al*., 2013; Chi, 2013; Swamy *et al*., 2016). In addition, EOs differ from other oils (fixed oils such as olive oil) as they are volatile and evaporate when left open, and have certain therapeutic properties which can be used to promote health and well-being of patients in healthcare settings (Voon *et al*., 2012; Swamy *et al*., 2016). Essential oils are employed in aromatherapy and for the treatment of several diseases including cardiovascular disease, diabetes, Alzheimer's and cancer (Swamy *et al*., 2016). Antimicrobial properties (antiviral, antimycotic, antitoxigenic and antiparasitic) of EOs are known to be effective against various bacterial species including antimicrobial-resistant microbial pathogens (Burt, 2004; Swamy *et al*., 2016).

1.3.1 Essential oils as possible antimicrobial agents

In developing countries, infectious diseases and food-borne illnesses can cause severe health problems which can even lead to death when not treated by correct antimicrobial agents. It has been estimated that approximately 30% of people in developing countries suffer from food-borne illnesses each year, from 2000 up until 2014 about two million people died from gastrointestinal illnesses (WHO, 2002;

Jahan, 2012; Kadariya *et al*., 2014). The continual emergence of antibiotic-resistant pathogens has prompted researchers' all over the world to search for new antimicrobial agents that are more effective against the resistant bacterial pathogens (Nascimento *et al*., 2000; Thaller *et al*., 2010; Kadariya *et al*., 2014). To improve the effectiveness of antimicrobial drugs researchers started focusing on a new class of antimicrobial compounds called essential oils (EOs) that can successfully work on multi-targeted sites on organisms (Esterhuizen *et al*., 2006; Alka *et al*., 2010; Swamy *et al*., 2016). Essential oils are traditionally believed to be rich in phytochemicals showing rich bioactivity. These active compounds in plants are of interest to the pharmaceutical industry as well as to the general population and are actively being explored for many commercial applications (such as tea, bakery products, and more) (Alka *et al*., 2010; Swamy *et al*., 2016).

Essential oils are also considered to be potential natural antimicrobial agents used for preventing and treating various ailments and food-borne illnesses (Voon *et al*., 2012; Swamy *et al*., 2016). Since 1948 to date, numerous plants with therapeutic value have been used in traditional medicine to cure some of the common disorders and degenerative diseases in humans as well as in animals (Guenther, 1948; Zhao *et al*., 2009; Hosseinzadeh *et al*., 2015). In addition, essential oils are known to have pharmaceutical properties (analgesic, anti-inflammatory, antioxidant, anticancer, antitumor, anti-hyperglycemic, astringent activities, etc.) which play an important role in the fight against the resistant microbial pathogens (Zhao *et al*., 2009; Hosseinzadeh *et al*., 2015). Hamedo and Abdelmigid (2009) reported that antimicrobials derived from plant materials are often regarded as natural and safe when compared to synthetic chemicals. Recently, plant-based medicine has become

more popular due to consumers' awareness with regard to the use of synthetic chemical preparations and use of artificial antimicrobial preservatives, especially in recent food protection practices (Hosseinzadeh *et al*., 2015).

1.3.2 Mechanisms of action of the essential oils against microbes

The antimicrobial activity of EOs, similar to all natural extracts is dependent on their chemical composition and the amount of phenolic compounds (Voon *et al*., 2012; Lopez-Romero *et al*., 2015). In addition, different amounts of specific compounds can affect the antimicrobial activity of EOs. For example, high concentrations of cinnamic aldehyde, eugenol or citral confer antimicrobial properties to EOs (Voon *et al*., 2012; Lopez-Romero *et al*., 2015). The monoterpenes and phenols present in thyme, sage and rosemary EOs possess noticeable antimicrobial, antifungal and antiviral activity (Lopez-Romero *et al*., 2015). Some EOs, such as those found in basil, sage, hyssop, rosemary, oregano, and marjoram, are active against *E. coli*, *S. aureus*, *B. cereus* and *Salmonella* spp. but are less effective against *Pseudomonas* spp. due to the formation of exopolysaccharides that increase resistance to EOs (Nazzaro *et al*., 2013; Semeniuc *et al*., 2016).

As demonstrated by most of the previous reviews (Bouhdid *et al*., 2010; Nazzaro *et al*., 2013), EOs have shown to affect both the external envelope of the cell and the cytoplasm. In fact, the mechanisms of action of the essential oils include degradation of the cell wall, damaging the cytoplasmic membrane and cytoplasm coagulation. Lopez-Romero *et al*. (2015) also described the effects of EOs on the outer

membrane disruption. The hydrophobic nature of EOs allows them to penetrate microbial cells and cause alterations in its structure and functionality. This could explain why EOs are generally most effective, with some exceptions (Yap *et al*., 2014) against both Gram-positive and Gram-negative microorganisms.

It was also reported (Nazarro *et al*., 2013; Yap *et al*., 2014) that even though the cell membrane is the first target of EOs, the mechanism of action of the EO is not isolated but instead different biochemical and structural mechanisms are involved at multiple sites within the cell and on the cell surface. These mechanisms include chemical modifications of the cell membrane, cytoplasm, enzymes, and proteins, as they can completely change the conformation of the microbial cell. However, as noted in the experimental studies of Bouhdid *et al*. (2010); Olajuyigbe and Ashafa (2014) and De Rapper *et al*. (2016) there is still little research done in South Africa on “the possible mechanisms of action of the EOs against antibiotic-resistant bacteria”. In particular, on how essential oils act on the cell surface of antibiotic-resistant *S. aureus* and *B. cereus*, causing the disruption of the bacterial membrane which eventually leads to cell death. Furthermore, more needs to be done to harness the various ideas for identifying differentially expressed proteins induced by essential oils. Therefore, this study seeks to expand on existing literature by reporting on the current efficient and effective methods that can be used when identifying a cascade of reactions involving the entire bacterial cell.

1.4 RATIONALE

1.4.1 Problem statement

The emergence of antimicrobial resistance in both medical and food disciplines has become a serious problem worldwide (Cattaneo *et al*., 2008; Nkhebenyane, 2010). The most alarming development today (Ventola, 2015) is the rate at which acquired antibiotic resistance often develops and how quickly it spreads across the globe and among different species of bacteria. In healthcare settings, most bacterial pathogens with multiple-drug (MDR) resistance have become resistant to antibiotics which were previously quite efficacious (Ehrlich, 1907; Fleming, 1945; Howden *et al*., 2010; Ventola, 2015). Some of the most problematic MDR organisms that are encountered currently especially in hospitals include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumoniae* bearing extended-spectrum β-lactamases (ESBL), vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant MRSA, extensively drug-resistant (XDR) *Mycobacterium tuberculosis*, multi-drug resistant *Salmonella* enteric serovar Typhimurium and *Bacillus cereus* (Alekshun and Levy, 2007; Khan and Khan, 2016). *Bacillus cereus*, have emerged in recent years as a major cause of nosocomial infections primarily in patients with weakened immune systems especially those in intensive care units with severe burns, on mechanical ventilation or those who are catheterized (Karlowsky *et al*., 2003; Hanlon, 2005; Khan and Khan, 2016).

A significant feature of these nosocomial infections is that most *B. cereus* are highly resistant to the majority of the currently used antibiotics, making them extremely

difficult to treat (Khan and Khan, 2016). Resistance from antibiotics occurs from both an intrinsic insusceptibility resulting from the bacterial cell structure, together with a gradual acquisition of genetic determinants of resistance over time and probably developed as a result of the extensive use of broad-spectrum antibiotics (Hanlon, 2005; Ventola, 2015). Such abilities have seriously compromised the usefulness of antibiotics in the war against microorganisms and warn of a future when antibiotics may have very limited usefulness to control bacterial infection (Cattaneo *et al*., 2008; Jansen, 2012; Ventola, 2015). Therefore, to improve the effectiveness of antimicrobial drugs essential oils (EOs) are reported to successfully work on multi-targeted sites or organisms (Esterhuizen *et al*., 2006; Alka *et al*., 2010; Semeniuc *et al*., 2016).

The reason the study was conducted using bacteria isolated from hospices is due to the emergence of multidrug-resistant bacterial species (*Acinetobacter* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus*, *Mycobacterium tuberculosis*, *Salmonella* spp.) which were found to be major concerns in healthcare settings. These bacterial pathogens have resistance traits that make them survive in the presence of an antibiotic (Beger-Bachi, 2002; Davies and Davies, 2010; Blair *et al*., 2015). They usually disrupt one or more crucial steps required for the effective action of the antibiotics. They do this by preventing antibiotic access into the bacterial cell or perhaps removal or even degradation of the active component of the antimicrobial agent. The emergence of these multidrug-resistant bacterial species in healthcare settings is a challenging clinical problem that should not be overlooked but should be greatly emphasised (Beger-Bachi, 2002; Davies and Davies, 2010;

Blair *et al*., 2015). Therefore, in order to do so, new antimicrobial agents such as essential oils need to be put forth or considered to improve the effectiveness of antimicrobial drugs against the resistant microbial pathogens. In addition, some of the advantages of using these natural antimicrobials (essential oils) include: reducing total dependence on antibiotics, reducing the development of antibiotic resistance by pathogenic microorganisms and strengthening the immune system in humans.

1.4.2 Study aim

The overall aim was to investigate the antimicrobial activity of *Thymus vulgaris* essential oils against antibiotic-resistant bacteria isolated at South African Hospices.

1.4.3 The objectives of the study

To achieve the overall aim, the objectives of this study were:

- to assess the activity of antimicrobial compounds using biochemical tests.
- to investigate the morphological changes induced by *Thymus vulgaris* essential oil on the bacterial cell wall.
- to assess the effect of *Thymus vulgaris* essential oil on fatty acids profile of the cell membrane.
- to assess changes in total protein expression following exposure to *Thymus vulgaris* essential oil.

In order to understand the overall aim of this study, the following aspects were considered: antibiotic resistance, essential oils and their components, potential antibacterial targets such as cell membrane, cytoplasm, lipids, and proteins.

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CHAPTER 2: MORPHOLOGICAL AND CHEMICAL CHANGES INDUCED BY *THYMUS VULGARIS* ESSENTIAL OIL ON *STAPHYLOCOCCUS AUREUS* CELL WALL

MORPHOLOGICAL AND CHEMICAL CHANGES INDUCED BY *THYMUS VULGARIS* ESSENTIAL OIL ON *STAPHYLOCOCCUS AUREUS* CELL WALL

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2.1 ABSTRACT

Aims: To investigate the action of *Thymus vulgaris* essential oil on *Staphylococcus aureus* cell wall with emphasis on changes in fatty acid and protein composition.

Methods and Results: The effect of thyme essential oil on *S. aureus* was evaluated by bio-assay preparation and minimal inhibitory concentrations (MICs). Changes in the cell wall were assessed using Gram staining, scanning electron microscopy (SEM), transmission electron microscopy (TEM), while gas chromatography (GC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to assess the effect of the oil on fatty acid and protein composition, respectively. Exposure to thyme oil induced alterations in the bacterial membrane of *S. aureus*, which led to the loss of cell wall integrity, as demonstrated by Gram staining, SEM, and TEM. Chemical changes were investigated by assessing changes in the fatty acid profile of *S. aureus* cells exposed to essential oils. Treatment with thyme oil depleted both saturated and unsaturated fatty acids of *S. aureus*. Next, by using proteomic approach, three bacterial proteins with reduced expression levels, upon treatment with thyme oil were observed.

Conclusions: Thyme oil damages the cellular membrane of an antibiotic-resistant strain of *S. aureus*, which eventually leads to cell death. Thyme essential oil also shows effective antimicrobial activity. Evidence provided in this study indicates that thyme essential oil might enhance the chances of developing new conventional and natural antimicrobial agents (drugs as well as food preservatives) and be good alternatives to synthetic chemicals.

Keywords: Antimicrobial activity, *Thymus vulgaris* essential oil, *S. aureus* cell wall.

2.2 INTRODUCTION

Resistance to antibiotics, especially among staphylococcal strains, is currently a major threat to public health (Dulon *et al*., 2011; Edmondson *et al*., 2011; WHO, 2014). Since resistance by certain strains of *Staphylococcus* to multiple antibiotics like methicillin emerged in the late 1970s (Dulon *et al*., 2011; WHO, 2014), many strategies to control antibiotic resistance have been proposed (Kumar and Schweizer, 2005; Lambert, 2005; Edmondson *et al*., 2011). Considering current therapeutic regimens, vancomycin usage has proven to be the most reliable to treat resistant staphylococcal infections (Sakoulas *et al*., 2006; Howden *et al*., 2010; Rodvold and McConeghy, 2014). However, some staphylococcal strains have become resistant to vancomycin - indicating a dire need for new alternative therapeutic approaches (Denis *et al*., 2002; Sakoulas *et al*., 2006; Howden *et al*., 2010; WHO, 2014). Alternative treatment methods include essential oils as potential antimicrobial agents for the fight against infectious diseases and food-borne illnesses (Voon *et al*., 2012; Swamy *et al*., 2016).

Essential oils are complex volatile compounds, synthesised naturally in different plant parts during the process of secondary metabolism (Faleiro and Miguel, 2013; Swamy *et al*., 2016). Essential oils have great potential in the field of biomedicine as they effectively eliminate several bacterial, fungal, and viral pathogens. The presence of different types of aldehydes, phenolics, terpenes, and other antimicrobial compounds means that the essential oils are effective against a diverse range of pathogens (Rubiolo *et al*., 2010; Swamy *et al*., 2016). The reactivity of

essential oil such as *Thymus vulgaris* depends upon the nature, composition, and orientation of its functional groups (Voon *et al*., 2012; Swamy *et al*., 2016).

Thymus vulgaris is a herb widely used for medicinal purposes and has been used since ancient times to achieve healing, for food preservation and other useful effects (Stahl-Biskup and Sáez, 2002; Thosar *et al*., 2013; Swamy *et al*., 2016). Currently, *T. vulgaris* EO is classified with EOs with the most pronounced antimicrobial activity (Iten *et al*., 2009; Yap *et al*., 2014). It was shown to be very active against many food spoilage and pathogenic bacteria. The high antimicrobial activity of thyme oil and its components, such as thymol, carvacrol, and others was demonstrated against *S. aureus* (Sokovic *et al*., 2010; Jovanka *et al*., 2011; Yap *et al*., 2014) including methicillin-resistant isolates (Tohidpour *et al.* 2010; Yap *et al*., 2014). It is therefore considered an effective alternative antimicrobial agent against *S. aureus*. However, to use thyme oil as a food additive and or medicinal herb, the mechanism through which it exerts its antibacterial activity should be elucidated. Until now, limited work has been done in South Africa on the effect of thyme essential oil on antibiotic-resistant *S. aureus*. Consequently, the purpose of this study became to assess the effect of *T. vulgaris* essential oil on antibiotic-resistant bacterial cells of *S. aureus*.

2.3 MATERIALS AND METHODS

2.3.1 Antibacterial product and chemicals

2.3.1.1 Essential oil characterisation

The essential oil extracted from *Thymus vulgaris* was purchased from local suppliers. Thyme oil was characterised according to the method of Kirbaslar *et al*. (2009). Briefly, the oil was dissolved in hexane (10% in hexane) and injected in a Finnigan Focus Gas Chromatograph (GC) at a split ratio of 50:1; the injector temperature was set at 230˚C. The GC was equipped with an AB-1MS (30M X0.25mm id 0.25µm) capillary column. Helium was used as carrier gas at a constant flow of 1mL min^{-1}. The temperature programme was set at 40˚C for four minutes and then raised at 5˚C min^{-1} to 200˚C and then held at 200˚C for 1 minute and then raised at 5˚C to 220˚C where it was held for 10 min. Mass analysis of the oils was done using a Finnigan Focus DSQ mass spectrometer. The ion source was at 250˚C with an ionisation voltage of 70eV and mass scan range of 50-650 amu. Individual GC peaks and mass spectra were identified by searching commercial libraries; this was followed by expert matching of MS data.

2.3.2 Microorganisms

An environmental strain of *Staphylococcus aureus* was obtained from a South African hospice and subcultured weekly on plate count agar (PCA) (Merck, SA) at 37°C throughout the study. The selected *S. aureus* strain was tested for antibiotic resistance in a previous study and found resistant towards cefoxitin, tetracycline and nalidixic acid (Nkhebenyane *et al*., 2012). To prepare inocula, cultures were grown

overnight at 37°C; bacterial density was then adjusted to approximately 10^8 colony forming units (CFU) per ml for bio-assay preparation and 10^5 CFU.ml^{-1} for microdilution method with sterile saline solution. Bacterial counts were confirmed by plating out on plate count agar, plates were incubated at 37°C for 24 h.

2.3.3 Bio-assay preparation

The bacterium was suspended in sterilised distilled water (dH_20) and 0.2 ml spread out on PCA (0.5% m.v^{-1} agar). This produced a homogenous lawn across the surface of the agar (Kock *et al*., 2009). Next, a well (0.5 cm in diameter and depth) was constructed at the centre of the Petri dish and 46 µl of two concentrations of thyme oil were added, that is, diluted thyme oil (2ml of essential oil into 100ml of ethanol) and undiluted thyme oil. Ethanol (96%) was also added alone to the wells as a control. All plates were incubated at 37°C until different textured growth zones were observed (usually after 24 h). To avoid evaporation of the essential oil from plates, the tested oil was allowed to diffuse in the agar before incubation.

2.3.4 Inhibitory effect of thyme oil on *S. aureus* isolates

Minimal inhibitory concentrations (MICs) were determined using the broth microdilution assay, as previously described (Wong *et al*., 2014), with a slight modification. Briefly, a final bacterial inoculum of 5 × 10^5 CFU.ml^{-1} was prepared using Mueller-Hinton Broth (MHB) and aliquoted into a 96-well sterile microtitre plate. *Thymus vulgaris* essential oil was added to the first row of wells and serial dilutions were performed to achieve final concentrations of 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2

and 0.1 mg.ml^{-1}. The sealed plate was then incubated at 37ºC overnight. The experiment was run twice in duplicate for each concentration. To indicate growth after 24 hours of incubation, 40 µl of p-iodonitrotetrazolium violet (INT) [Sigma] solution (showing a pink to violet colour) was added to each well. The plate was then incubated at 37°C for 10 – 30 minutes. Growth was indicated by colour change ranging from pink to violet.

2.3.5 Gram stain preparation

Gram stain was performed on *S. aureus* cells according to the method of El-Garnal *et al*. (2009). Briefly, the slide with the specimen was flooded with Gram stain solutions (crystal violet, iodine, decolorizer, and safranin) (Merck, SA) and rinsed off with distilled water accordingly. Afterwards, the slide was microscopically examined using Nikon E200 Eclipse Light microscope for bacterial organisms under 100X objective and the Gram reaction of the organism observed was described.

2.3.6 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was carried out according to Van Wyk and Wingfield (1991). During bio-assay preparation, cells exposed to thyme oil and control samples were fixed using 3% v/v of a sodium phosphate buffered glutardialdehyde (Sigma-Aldrich, St. Louis, Mo., U.S.A.) solution at pH 7.0 and a similarly buffered solution (1% m.v^{-1}) of osmium tetroxide (Sigma-Aldrich, St. Louis, Mo., U.S.A.) for 1 h. Subsequently, the material was dehydrated in a graded series of the ethanol solution. Next, the ethanol-dehydrated material was critical-point dried,

mounted and coated with gold to make it electrically conductive. This preparation was then examined using a Shimadzu SX500 SEM (Shimadzu, Tokyo, Japan).

2.3.7 Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM; van Wyk and Wingfield 1991), bio-assay preparation was performed to treat *S. aureus* cells. Afterwards, bacterial cells exposed to thyme oil and control samples were fixed using the same protocol described for SEM. After fixation, the material was dehydrated in a graded acetone (Merck, Darmstadt, Germany) series (50%, 70%, 95%, 2X 100%). Dehydration lasted 30 min for each step. Next, the material was embedded in an epoxy resin (Spurr 1969) and allowed to polymerise in an oven at 70°C for 8 h. Thin sections were made using an LKB III Ultramicrotome (Stockholm, Sweden) and stained with uranyl acetate for 5 min and lead citrate for 1 min. Finally, these sections were viewed with a Phillips EM 100 transmission electron microscope (Eindhoven, the Netherlands). These procedures were performed twice in at least duplicate.

2.3.8 Identification of fatty acids by GC-FID

2.3.8.1 Treatment of cells

Firstly, bio-assay preparation was performed and cells exposed to thyme oil and control samples were incubated at 37°C until different textured growth zones were observed (usually after 24 h). Afterwards, fatty acids were extracted using a fatty acid extraction kit (Sigma-Aldrich, SA). Briefly, lipids (0.15 g) were weighed and 3 ml of extraction solvent (Sigma-Aldrich, SA) was added to each sample (both treated

and untreated samples). After cell suspension of treated and untreated (control) samples, 0.5 ml of aqueous buffer was added to the homogeneous mixture of each sample. Next, the solution was poured into the syringe, plunger attached and pushed to elute lipids into the collecting tube for each sample (the experiment was performed twice in duplicate). Then, the total lipid extract for each sample was now transesterified and analysed by GC-FID.

2.3.8.2 Analysis of the Fatty Acid Composition

The extracted fatty acids were methylated to fatty acid methyl esters (FAMEs) with boron trifluoride/methanol complex (5 ml of 20% BF_3/MeOH reagent) (Merck, SA) followed by heating in 2.5 ml toluene (Merck, SA) at 100°C for 45 min under gentle mixing. Afterwards, distilled water (12.5 ml) was added at room temperature, and the FAMEs were extracted with 5 ml of hexane (Merck, SA). The hexane fraction was dried in nitrogen gas, resuspended in 500 µl hexane and the solution was filtered prior analysis. The fatty acids were analysed using Finnigan Focus GC (Thermo Fisher Scientific, Waltham, Massachusetts, US) with flame ionisation detector and a 25 m x 0.32 mm ID SGE capillary column BPX70, 0.25 µm film (SGE, Melbourne, Victoria, Australia). The temperature conditions were 100°C for 5 min, 100-240°C at a rate of 3°C/min and at 240°C for 20 min. The samples were injected at 225°C and detected at 285°C with helium (linear flow of 20 $cm.s^{-1}$) as the carrier gas and split ratio of 1:50. A 37-Component FAME mixture (Sigma-Aldrich, SA) was used to identify the fatty acids.

2.3.9 Preparation of proteins

2.3.9.1 Isolation of bacterial protein for SDS-PAGE analysis

During bio-assay preparation, cells exposed to thyme oil and control samples were incubated at 37°C until different textured growth zones were observed (usually after 24 h). Afterwards, bacterial proteins were determined using the protein isolation method, as previously described (Wong *et al*., 2014), with a slight modification. Briefly, bacterial cells (0.15 g) were weighed and 4 ml of B-PER bacterial protein extraction reagent (Thermo Scientific) was added to the cell pellet (0.15 g). After cell suspension of both treated and untreated (control) samples, homogeneous mixture for each sample was incubated for 10-15 minutes at room temperature. Then, lysate for each sample (treated and untreated cells) were centrifuged at 15, 000 x g for 5 minutes to separate soluble proteins from insoluble proteins. After obtaining the soluble proteins, SDS-PAGE gel electrophoresis was then performed. This procedure was performed twice in at least duplicate.

2.3.9.2 SDS-PAGE gel electrophoresis

SDS-PAGE gel electrophoresis was performed using 12% resolving gel [1.5 M Tris-HCl, pH 8.8 (Bio Basic Canada, Inc), 10% SDS (Fisher Scientific), 40% bis-acrylamide] and 4% stacking gel [0.5 M Tris-HCl (pH 6.9), 10% SDS, 40% bis-acrylamide]. Before loading bacterial proteins (1 to 40 µl) into the wells, bacterial proteins were treated with dithiothreitol (DTT) and boiled. For ease of molecular weight estimation and comparison, protein ladder (Spectra Multicolor Broad-Range Protein Ladder, Thermo Scientific) was loaded (1 to 40 µl) onto each gel. The SDS-PAGE gels were ran using a constant electric current (135 mV) until the

bromophenol blue dye front reached the bottom of the gel plate. Protein gels were then stained with Coomassie Brilliant Blue R-250 (Fisher Scientific) staining solution for an hour, followed by overnight destaining in distilled water. Next, a gel documentation system was used for the imaging and documentation of proteins suspended within polyacrylamide gel.

2.4 RESULTS

Thymus vulgaris essential oil was characterised by the following compounds: borneol (0.5%), camphene (1.0%), caryophyllene (1.2%), caryophyllene oxide (1.4%), pinocarvone (1.5%), γ-Terpinene (3.0%), Thymol (32.1%), α-Pinene (5.1%) and ρ-Cymene (20.4%). Similar results where high amounts of thymol and α-Pinene were observed were reported by Manou *et al*. (1998), Angelov *et al*. (2013) and Nazzaro *et al*. (2013). These compounds were shown to have high antimicrobial activity against *B. cereus*, *S. aureus* and *P. aeruginosa* (Angelov *et al*., 2013; Nazzaro *et al*., 2013). Their antimicrobial activity results in structural and functional alterations in the cytoplasmic membrane that can damage the outer and inner membranes; they can also interact with proteins and intracellular targets. The interaction of these compounds with the membrane was reported to affect membrane permeability and results in the release of K+ ions and ATP (Manou *et al*., 1998; Angelov *et al*., 2013; Nazarro *et al*., 2013).

Except for their composition, a bio-assay showing the antimicrobial activity of thyme oil against antibiotic-resistant *S. aureus* was conducted. The undiluted *Thymus vulgaris* essential oil was observed with an inhibition zone of 52 mm for treated *S. aureus* cells (Figure 2.1) and the diluted essential oil with an inhibition zone of 47 mm for treated *S. aureus* cells on an agar plate (Figure not shown). For inhibitory effect of thyme oil on *S. aureus* isolates, the undiluted *Thymus vulgaris* essential oil completely inhibited bacterial growth due to its high antimicrobial activity (Table 2.1) and the diluted essential oil showed an MIC of 0.8 mcg/ml (Table 2.1) for treated *S. aureus* cells (Table 2.1). Additionally, different growth patterns were observed on the agar plate treated with thyme oil (Figure 2.1), the zone close to the well was characterised by single pale looking colonies devoid of pigmentation while the zone close to the periphery of the plate was characterised by golden staphylococci cells observed in treated sample (Figure 2.1). The golden colour is an eponymous feature of the human pathogen *Staphylococcus aureus* that shields the organism from oxidation-based clearance (Liu *et al*., 2005; Lan *et al*., 2010). Therefore, the results observed in Figure 2.1 clearly demonstrate that thyme oil has completely disturbed the cell wall of *S. aureus* leading to a lack of pigmentation. This loss of pigmentation might be linked to the environmental stress and the phenotypic changes that became associated with the formation of small colony variants (SCVs). It might be proposed that these SCVs bacteria observed (Figure 2.1) emerged within apparently homogeneous microbial populations, largely in response to environmental stress induced by exposure to thyme oil.

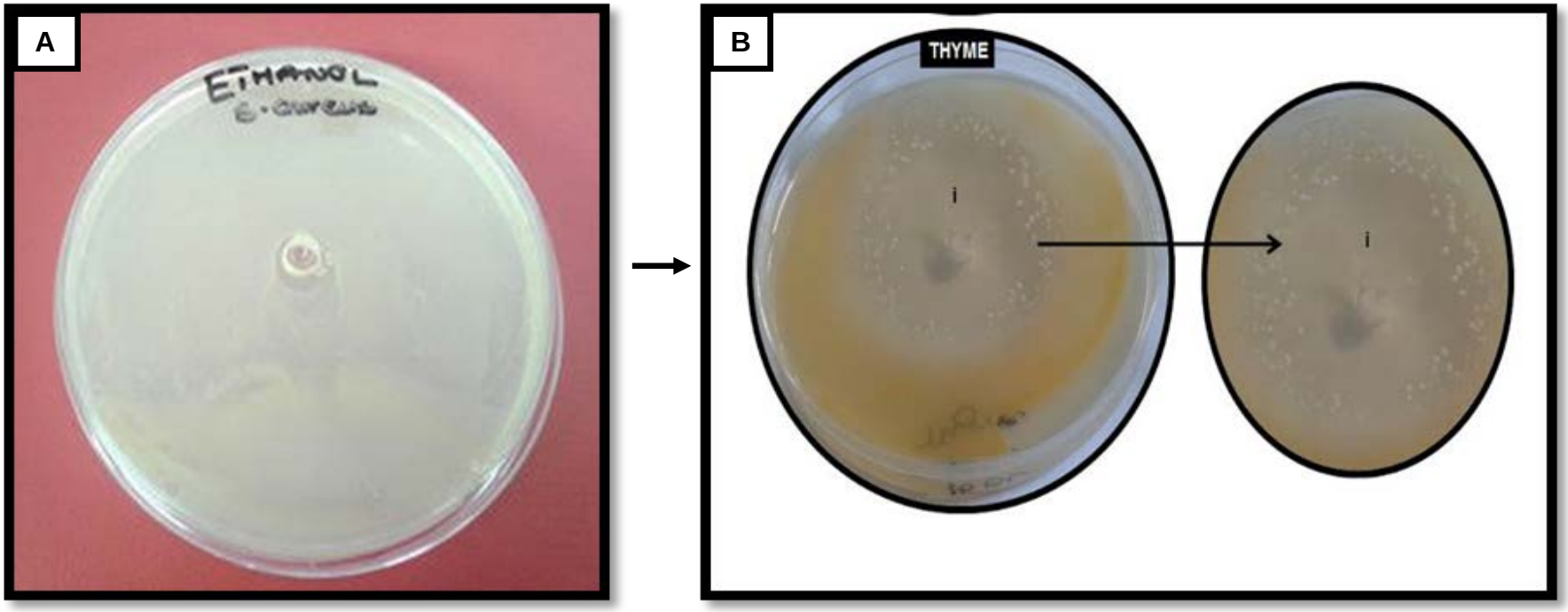

Figure 2.1: A bio-assay showing (A) untreated (control) *S. aureus* cells with no antimicrobial activity and (B) treated *S. aureus* cells with the antimicrobial activity of thyme oil against *S. aureus* on an agar plate. I – designates the zone of inhibition. The periphery of the agar plate shows golden Staphylococcal cells while close to the inhibition zones, pale cells lacking pigmentation due to environmental stress can be observed

Table 2.1: The minimum inhibitory concentration (MIC) of thyme oil against *S. aureus* isolate

S. aureus Isolate	**Dilution of thyme essential oil (µg/ml)**							
	≥ 12.5	**≥ 6.3**	**≥ 3.1**	**≥ 1.6**	**≥ 0.8**	**≥ 0.4**	**≥ 0.2**	**≥ 0.1**
Control	+	+	+	+	+	+	+	+
Diluted	–	–	–	–	–	+	+	+
Undiluted	–	–	–	–	–	–	–	–

Data are reported as '+'indicates growth of bacteria (not sensitive to thyme oil), '–'indicates inhibition of growth of bacteria (sensitive to thyme oil).

To assess the cellular damage produced by the oil on the bacteria, a single colony close to the zone of inhibition was picked and Gram staining was performed. The results (Figure 2.2) showed Gram-positive cells to be affected by a cell wall active agent (thyme oil) and stained pink like Gram-negative bacterial cells. *S. aureus* is a facultative anaerobic Gram-positive coccal bacterium, purple in colour appearing as grape-like clusters when viewed through a microscope following Gram staining (Hanselman *et al*., 2009). However, results found in this study (Figure 2.2) showed *S. aureus* cells staining pink instead of purple as anticipated, this is possibly due to a decrease in peptidoglycan thickness or a disturbance in the cell wall during cell growth in the presence of thyme oil as observed elsewhere (Silhavy *et al*., 2010).

The results observed from Gram staining (Figure 2.2) clearly demonstrate that *Thymus vulgaris* essential oil affected both the cell wall and cell membrane making them permeable and allowing the crystal violet to wash out of cells causing them to de-stain and subsequently stain red during the counterstaining step in the Gram staining procedure and appear as Gram negative. To confirm that thyme oil has certainly affected the bacterial cell wall of antibiotic-resistant strain of *S. aureus*, scanning electron microscopy (SEM) was performed.

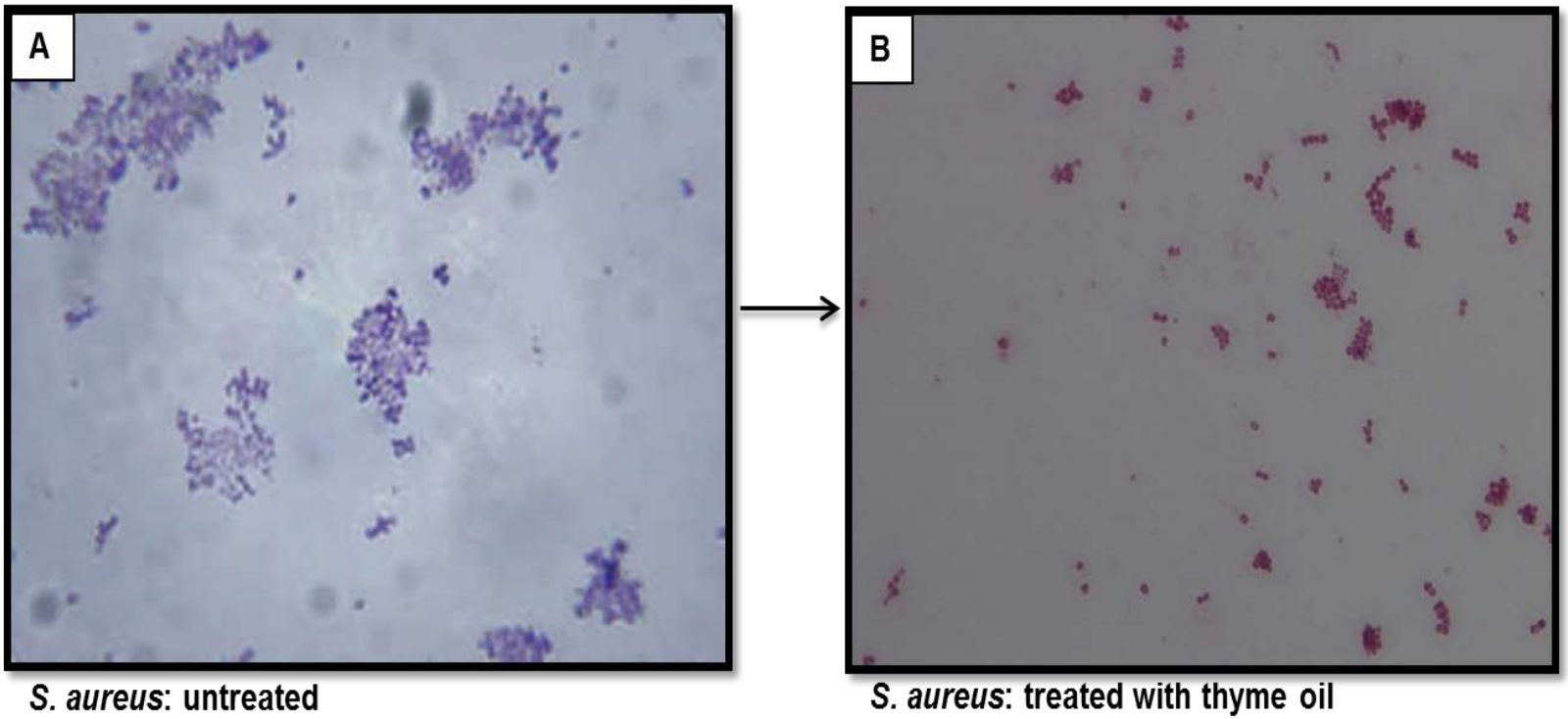

Figure 2.2: Gram stain light micrographs showing (A) untreated (control) *S. aureus* cells staining purple and (B) treated *S. aureus* cells staining pink indicating the negative effect of thyme essential oil on the bacteria.

The effect of the oil treatment on bacterial ultrastructure was assessed using SEM and TEM. The oil caused major structural changes in *S. aureus* cells (Figure 2.3 and 2.4). In fact, when *S. aureus* bacterium was treated with thyme oil, the results observed (Figure 2.3 B) exposed a damaged cell wall (DCW) with the formation of holes on the cell surface and incomplete cell division (ICD). In addition, a significant amount of cytoplasmic materials or loss of cellular contents (LCC) was observed outside the cells (Figure 2.3 B). Scanning electron micrographs also revealed that the size of the untreated cells (Figure 2.3 A) were different from that of treated cells (Figure 2.3 C).

Transmission electron micrographs also demonstrated damaged bacterial cells (Figure 2.4) similar to what was observed with SEM. The treated cells showed membrane disruption that caused cell wall deformation (Figure 2.4 B and C), slight roughness of the cells (Figure 2.4 B), coagulation of intracellular contents (Figure 2.3 B) and the presence of incomplete cell division (Figure 2.4 C). Based on the results found in this study, the incomplete cell division observed (Figure 2.3 B and Figure 2.4 C) might be caused by thyme oil acting on proteins present in bacteria since this was not observed in the control (Figure 2.3 A and Figure 2.4 A). Moreover, an irregular cytoplasmic membrane with dark and densely stained cytoplasmic contents (Figure 2.4 B and C) was observed inside the cell and loss of the intracellular material could be linked to the presence of fatty acids.

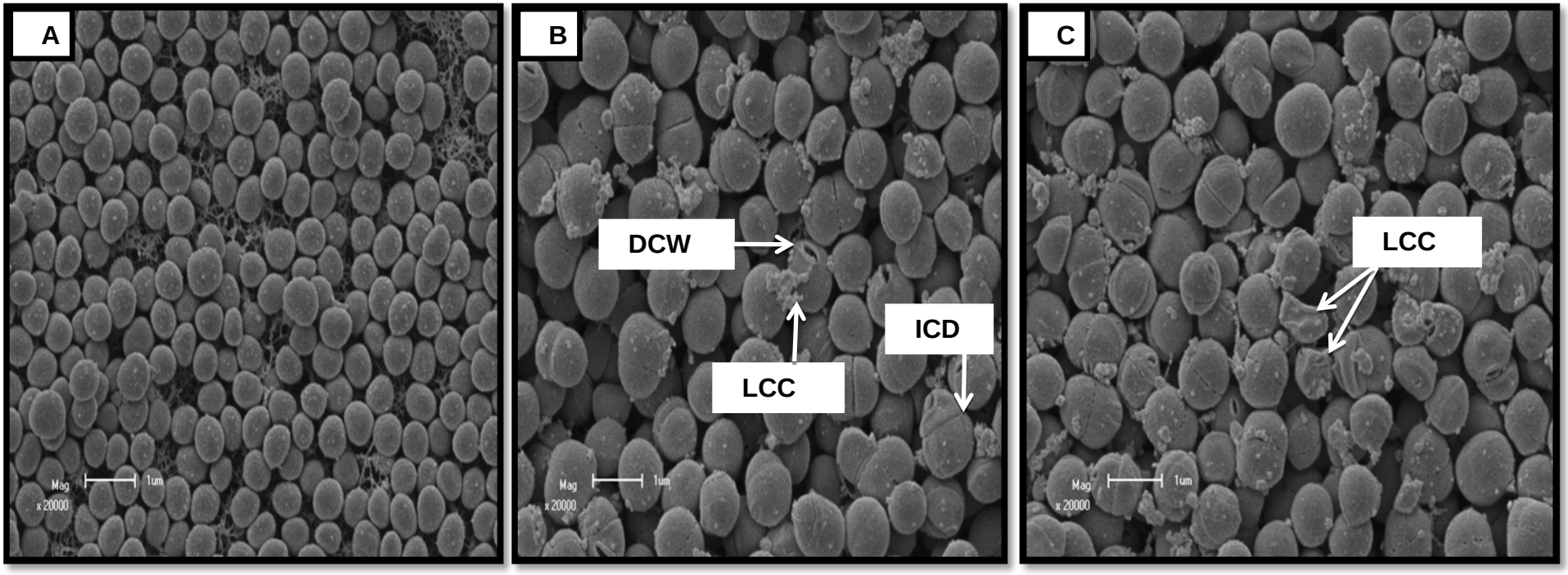

Figure 2.3: (A) control cells and (B-C) different types of injuries induced by thyme oil on the bacterial cell wall and membrane structure. DCW - Damaged cell wall with the formation of holes on the cell surface; LCC - Loss of cellular contents; ICD – Incomplete cell division.

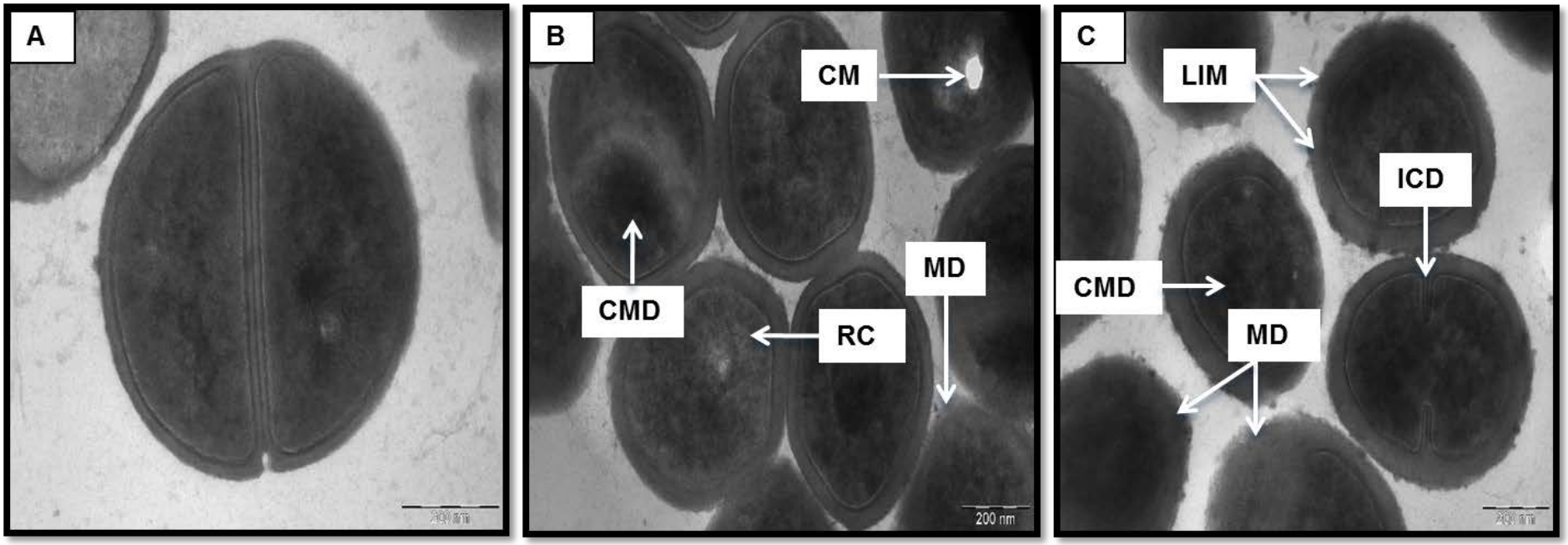

Figure 2.4: (A) control cells and (B-C) morphological changes of *S. aureus* cells after exposure to thyme oil. MD - Membrane disruption or cell wall deformation; LIM - Loss of intracellular material; RC -The slight roughness of the cell; CMD - The presence of cytoplasmic membrane damage; ICD – Incomplete cell division inside the cell; CM- Coagulated material.

To confirm that loss of intracellular material observed might be linked to fatty acids, total lipid extraction was performed (Figure 2.5) to assess changes in fatty acid profile. Fatty acids are abundant in the host and can be used as building blocks by Gram-positive *S. aureus* pathogen to maintain the lipid biosynthesis pathway (Morvan *et al*., 2016). However, once fatty acids are affected somehow after treatment with thyme oil as observed in Figure 2.5, the bacterial structure is inevitably disturbed and this makes the bacteria less pathogenic. The unsaturated C18: 2n6 (cis), 18: 3n3 (cis) and 18: 3n6 (cis) fatty acids composition showed a decrease in treated *S. aureus* cells when compared to untreated cells (Figure 2.5). In addition, unsaturated C16: 1 and C18: 2n6 (trans) fatty acids were completely depleted when using the undiluted thyme oil in comparison to diluted oil and control (Figure 2.5) showing that the more concentrated the oil is, the more degradation of fatty acids occurs. Other than unsaturated fatty acids, saturated C16:0 and C18:0 fatty acids also showed a decrease in fatty acids after exposure to thyme oil. Generally, there was a decrease of all fatty acids when compared to the control.

In addition, the reorganisation of the lipid profile of *S. aureus* cells (Figure 2.5) after treatment with thyme oil portray the bacterial fatty acid synthesis pathway as an optimal target for antibacterial agents (Nazzaro *et al*., 2013; Aleksic and Knezevic, 2014). Fatty acid synthesis involves the production of fatty acids from acetyl-CoA and NADPH with malonyl-CoA, through the action of enzymes called fatty acid synthases (Mashima *et al*., 2009; Ali *et al*., 2016). Therefore, once the metabolic pathways (such as the synthesis of palmitate acetyl- CoA, the elongation of palmitate

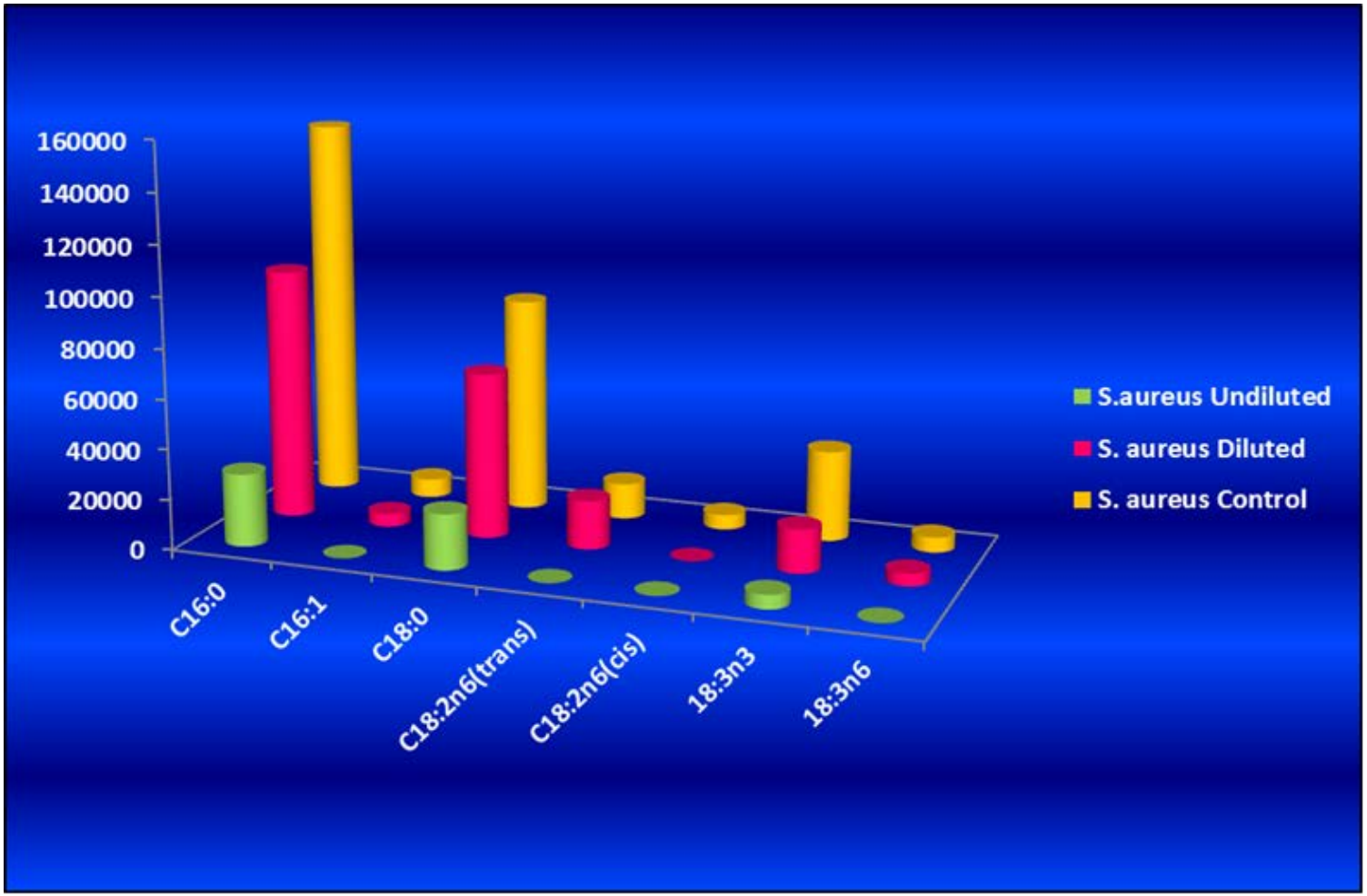

Figure 2.5: Fatty acid profile of *S. aureus* cells affected by *Thymus vulgaris* essential oil. Standard deviations for treated (n=135113) and untreated samples (n=306496) are as indicated. 'y-axis' indicates Peak Intensity.

and in desaturation of fatty acids) is disrupted, saturated C16:0 and C18:0 as well as unsaturated C18:2 and C18:3 fatty acids decrease (Figure 2.5). These results about the loss of fatty acids as a consequence of treatment with thyme oil (Figure 2.5) were also in accordance with the studies of Nazzaro *et al*., (2013). In addition to direct effects on the fatty acids of the outer membrane, thyme oil may also affect the enzymes that are involved in fatty acid synthesis. The oil caused a major decrease in unsaturated C18: 2n6 (cis), 18: 3n3 (cis) and 18: 3n6 (cis) fatty acids (Figure 2.5) and this could be due to disruption of the fatty acyl-CoA desaturase enzyme affected by the essential oil. Nazzaro *et al*. (2013) also stated that thyme oil is capable of affecting this multicomponent membrane desaturase enzyme that is generally employed by cells to produce unsaturated fatty acids. To confirm that thyme oil has affected the expression levels of proteins, a protein analysis was performed using a proteomic approach.

By using proteomic approach, the aim was to identify differentially expressed bacterial cellular proteins and the focus was using multidrug resistant *Staphylococcus aureus* as the model. Following treatment with the *Thymus vulgaris* essential oil, bacterial cellular proteins were extracted and prepared using Bacterial Protein Extraction Reagents (Thermo Scientific) and ammonium sulphate precipitation. In the negative control, *Thymus vulgaris* essential oil was substituted with solvent (ethanol) alone as a treatment. Purified bacterial proteins were then separated on denaturing polyacrylamide electrophoresis gels. Figure 2.6 showed the total protein expression profile of *S. aureus*, following exposure to *Thymus vulgaris* essential oil.

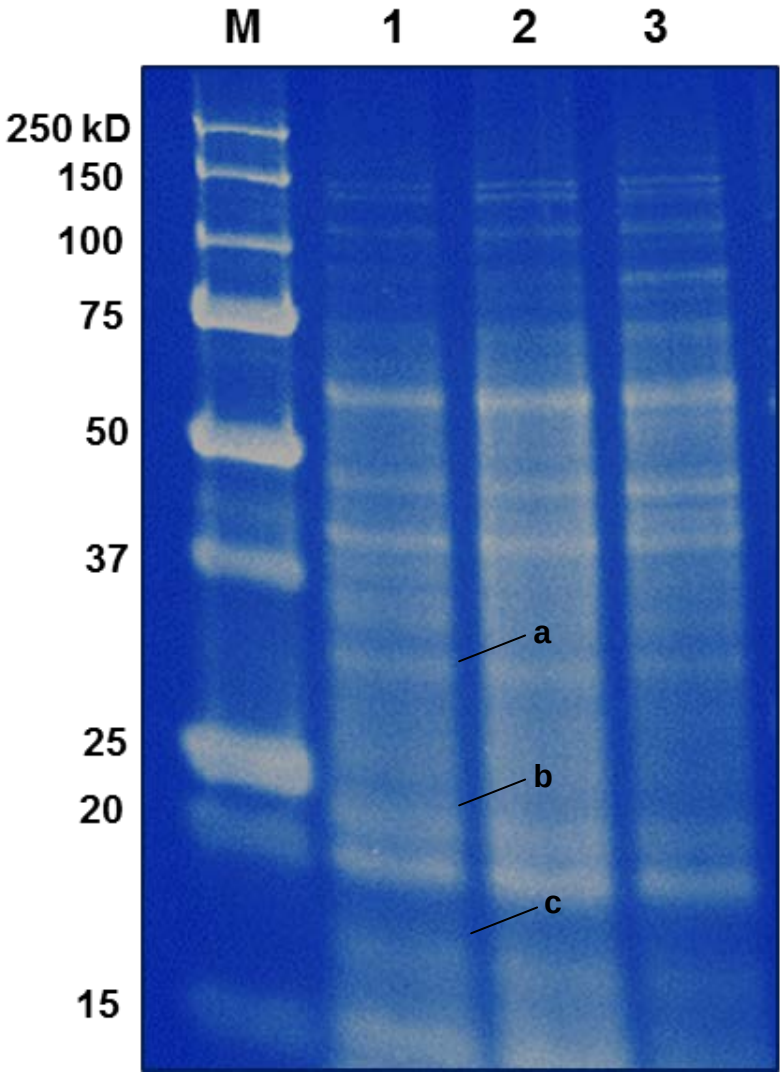

Figure 2.6: Protein expression profiles of *S. aureus*, following exposure to *Thymus vulgaris* essential oil. M.-.Marker; 1 - the absence of *Thymus vulgaris* essential oil in bacterial culture medium; 2 - the presence of diluted *Thymus vulgaris* essential oil in bacterial culture medium; 3 - the presence of undiluted *Thymus vulgaris* essential oil in the bacterial culture medium. Labels (a to c) indicate differentially expressed total protein bands upon treatment with *Thymus vulgaris* essential oil.

Here, a total of three differentially expressed total proteins (as indicated by letters a to c) were observed. In addition, the total proteins were completely depleted with faint bands when using the undiluted thyme oil in comparison to diluted oil and the control (Figure 2.6) showing that the more concentrated the oil is the more degradation of proteins occurs.

2.5 DISCUSSION

Thymus vulgaris essential oil showed strong antibacterial activity against an antibiotic-resistant strain of *S. aureus.* This activity may be associated with the presence of thymol and ρ-cymene identified in this study, which was the major components of the oil, however these compounds will have to be tested in future in isolation. Thymol and ρ-cymene are active against a range of Gram-positive bacteria including *S. aureus* (Faleiro and Miguel, 2013). To establish whether this inhibitory activity affected cell morphology and integrity Light microscopy (Gram staining), SEM and TEM were performed. These approaches were used to study the effect of antimicrobial activity on cell wall and membrane integrity (Hyldgaard *et al*., 2012).

Staphylococcus aureus membrane damage and breakdown of the permeability barrier observed through electron micrographs demonstrates possible dissipation of the potassium (K^+) gradient across the cell membrane (Bouhdid *et al*., 2010). This could have been caused by membrane disruption observed. Potassium is the most abundant intracellular cation in all living microorganisms, including bacteria, and is important for many essential cellular functions (Bouhdid *et al*., 2010). Moreover, the results in Figure 2.3 B reveal that thyme oil could have possibly induced leakage of

intracellular K^+ from cells of *S. aureus*. These results are similar to previous studies that reported the capability of essential oils and their components to alter the permeability of bacterial cells to cations like K^+ (Hada *et al*. 2003; Walsh *et al*. 2003; Inoue *et al*. 2004; Bouhdid *et al.* 2009).

In addition, slight roughness of the cell (Figure 2.4 B) was observed and the roughness was indeed associated with the perforation (presence of holes) on the cell wall with the release of intracellular material and subsequent cell wall deformation. All these changes led to cell death. This study would be the first study done in South Africa where the effect of thyme essential oil on an antibiotic-resistant strain of *S. aureus* was analysed at this morphological level. Moreover, the intracellular leakage and morphological changes of the treated bacterial cells (Figure 2.3 and Figure 2.4) indicated that thyme oil affected the structural organisation of the cytoplasm together with the cell wall of treated *S. aureus*. It might be proposed that in the primary phase, the tested oil probably binds the bacterial cell surface and penetrates the cell wall causing cytoplasmic membrane damage (Figure 2.4 B and C) and this led to cell death. This is an indication that the tested oil indeed affected the structural organisation of antibiotic-resistant *S. aureus* since this was not observed in the control (Figure 2.3 A and Figure 2.4 A).

The major sites of action of thyme oil on treated cells appeared to be the cell cytoplasm, cell wall, and cell division. According to the results found in this study, the incomplete cell division observed (Figure 2.3 B and Figure 2.4 C) in *S. aureus* cells might be caused by thyme oil acting on proteins present in bacteria. Bacterial cell

division is regulated by FtsZ, a prokaryotic homolog of tubulin. FtsZ assembles into a Z-ring at the site of cell division; thymol can decrease the *in vitro* assembly reaction and bundling of FtsZ (Nazzaro *et al*., 2013). It also can perturb the Z-ring morphology *in vivo* and reduce the frequency of the Z-ring per unit of cell length of *S. aureus* which eventually causes cell division to stop (Nazzaro *et al*., 2013). Surprisingly, the incomplete cell division was not observed in *B. cereus* cells (see Chapter 3) and the differences registered between the two bacteria could be mainly due to differences in the genes and proteins that direct cell division in both bacteria as these could differ from specie to specie. Moreover, these results about incomplete cell division as a possible consequence of mutated Ftsz protein (Figure 2.3 B and Figure 2.4 C) were also observed in studies by Hyldgaard *et al*. (2012). The presence of coagulated material was also observed (Figure 2.4 B). The coagulated material is thought to be a precipitate of abnormal proteins or denatured membrane probably induced by *Thymus vulgaris* essential oil (Gustafson *et al*.,1998; Becerril *et al*., 2007).

Thyme oil also has the ability to bind to the penicillin-binding protein and interfere with cell wall synthesis that many antibiotics fail to target (Friedman, 2015). Since 1970, bacteria have developed resistance strategies against antibiotics. For example, bacteria uses the beta-lactamase enzyme to destroy the beta-lactam ring and once the beta-lactam ring is destroyed the antibiotic would not be able to bind to the penicillin-binding-protein (PBP) and interfere with cell wall synthesis (Dulon *et al*., 2011; WHO, 2014). Therefore, the results found in this study show the potential of thyme oil in dealing with antibiotic-resistant infections. To confirm whether there was indeed an effect on proteins, these results together with other studies such as

proteomics was followed up in this study. In the proteomic analysis, a total of three differentially expressed proteins were identified (Figure 2.6). To the best of our knowledge, this represents the first time such proteins with reduced expression levels are being reported in the literature, upon exposure to *Thymus vulgaris* essential oil. Moreover, based on the bacterial secretomics profile (Figure 2.6) it might be speculated that *Thymus vulgaris* essential oil may exert its inhibitory action via the bacterial protein translation pathway since the protein translation process is critically essential to the bacteria survival, and it is highly conserved across different species of bacteria including *S. aureus* (Wong *et al*., 2014).

Results in the current study indicate that the lipid biosynthesis pathway appears to be one of the important targets for the development of novel antimicrobials. Due to the hydrophobic character of thyme oil constituents, the cytoplasmic membrane appears to be a suitable site of their action, influencing the percentage of UFAs and altering its structure. In this study, after exposure of *S. aureus* cells to the compounds, the percentage of UFAs was found to be lower than SFAs (Figure 2.5). This result would support a mechanism of action of these compounds against the outer cell envelope, most probably interacting with the membrane lipid profile and causing membrane structural alterations noticeable by SEM examination (Figure 2.3). In addition, after the cells were treated with *Thymus vulgaris* essential oil, similar modifications of the lipid profile: a decrease of saturated C16 (and shorter length) fatty acids, a decrease of the saturated C18, and a corresponding decrease of the unsaturated C18 fatty acids were observed (Figure 2.5). The decrease in the amount of SFAs in the membrane lipid bilayer (Figure 2.5) when compared to

untreated cells results in a gain of membrane fluidity and a consequent decrease in membrane rigidity as appreciable by SEM examination (Figure 2.3).

Again, it is important to understand that the activity of thyme oil and their components is not attributable to a single event; most of the components of the EO (Hyldgaard *et al*., 2012) firstly act on the cell wall, penetrates through the outer membrane and increase its permeability which leads to dispersion of the desaturase enzymes and allows them to act on the membrane fatty acids (Hyldgaard *et al*., 2012). In this study, it can be proposed that *S. aureus* cells defective in the production of SFAs and UFAs can continue to grow and synthesise phospholipids, but eventually begin to lose its membrane integrity.and subsequently divide abnormally. Therefore, from these observations (Figure 2.5 and Figure 2.6) it is clear that thyme oil acts on the membrane, altering its lipid profile together with the protein profile, increasing the surface areas of the membrane, and altering its structure. However, it is also able to penetrate the deeper part of the cell resulting in cell death (Hyldgaard *et al*., 2012; Nazzaro *et al*., 2013).

The knowledge gained from this study (Figure 2.1 - Figure 2.6) about the mode of action of thyme oil against *S. aureus* means that it is now possible to have a complete picture of the bacterial cellular targets of *Thymus vulgaris* essential oil. Most importantly, this knowledge is found to be very useful since up until today there is a lack of information documented in South Africa regarding strategies to use for controlling antibiotic-resistant *S. aureus* strains.

2.6 CONCLUSIONS

Findings in the current study reveal the mode of action of *Thymus vulgaris* essential oil on *Staphylococcus aureus* morphology. The current findings demonstrate that thyme essential oil damage the cellular membrane of antibiotic-resistant *S. aureus*, which leads to cell death. Additionally, the reorganisation of the lipid profile and protein profile shown after the treatments of the resting cells is strictly related to the presence of thyme oil compounds. As a result, this shows that *Thymus vulgaris* essential oil has the capability to target the bacterial sites of *S. aureus* that antibiotics such as cefoxitin, tetracycline, and nalidixic acid failed to target. Thyme essential oil is therefore considered a potential antimicrobial agent. Moreover, from adequate scientific evidence provided in this study, it can be concluded that thyme essential oil might enhance the chances of developing new conventional and natural antimicrobial agents (drugs as well as food preservatives) and be good alternatives to replace synthetic chemicals.

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CHAPTER 3: ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL EXTRACTED FROM *THYMUS VULGARIS* ON ANTIBIOTIC-RESISTANT *BACILLUS CEREUS*

ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL EXTRACTED FROM *THYMUS VULGARIS* ON ANTIBIOTIC-RESISTANT *BACILLUS CEREUS*

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3.1 ABSTRACT

Aims: To assess cellular damage induced by *Thymus vulgaris* essential oil in antibiotic-resistant food-borne pathogen *Bacillus cereus*.

Methods and Results: GC-MS analysis of thyme oil was performed to determine oil composition. Bio-assays were applied to evaluate the effect of thyme oil on *B. cereus* and determine the minimal inhibitory concentrations (MICs). Changes in cell morphology were assessed using Gram staining, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Consequently, changes in lipid and protein profile were also investigated. Exposing the bacteria to thyme oil caused alterations in the bacterial membrane, which led to the loss of cell wall integrity, as demonstrated by Gram staining, SEM, and TEM. In addition, loss of cellular contents, irregular cytoplasmic membrane, and the presence coagulated material, as indicated by SEM and TEM were observed. Moreover, the oil demonstrated a decrease in bacterial saturated C16:0 and C18:0 and unsaturated C16:1, C18:1n9, C18: 2n6 (cis), C18: 2n6 (trans) and 18: 3n6 fatty acids in comparison to the control sample. Other than fatty acids, two differentially expressed bacterial proteins were identified via proteomic approach.

Conclusions: The results of this study revealed that thyme oil and its components affect bacterial growth by targeting various cellular structures and eventually metabolic processes; therefore, they can be used as a potential source of active ingredients for food preservatives.

Keywords: Antibacterial properties, Essential oils, Fatty acids, Food-borne pathogens, Protein expression.

3.2 INTRODUCTION

Food-borne bacterial agents are the leading cause of severe and fatal food-borne illnesses. More than 90% of food-poisoning illnesses are caused by species of *Bacillus* (Nyenje and Ndip, 2013) and this has financial implications for the foodstuff industry. *Bacillus cereus* contamination affects the food industry in a dual manner, firstly it deteriorates food products by causing spoilage and secondly through production of toxins which endangers consumer's health upon consumption of contaminated foods (Singh and Chaturvedi, 2015). Under certain conditions, strains of this species produce haemolysins, phospholipases C, and also emetic toxins and enterotoxins that cause food poisoning (Ceuppens *et al*., 2013). The danger posed by some strains of this microorganism is increased by its ability to adapt to chemicals, heat and cold environments (Carlin *et al*., 2010; Hassan *et al*., 2010).

Bacillus cereus had so far been considered to be exclusively endospore-forming (Carlin *et al*., 2010). In response to harsh conditions, the bacteria form protective endospores enabling them to remain dormant for extended periods, then they reactivate to become fully functioning bacteria when conditions are favourable again (Carlin *et al*., 2010; Ceuppens *et al*., 2013). Researchers (Tuchscherr *et al*., 2010; Frenzel *et al*., 2015; Johns *et al*., 2015) have also found that *B. cereus* has an alternative life cycle in the form of so-called small colony variants (SCVs). These SCVs grow slower than the original form of *B. cereus* and form in response to various environmental stresses, including essential oils. They display unique phenotypic characteristics conferred in part by heritable genetic changes as also noted by Johns *et al*. 2015.

Interestingly, one species of bacteria that has been known for years (Alharbi, 2013) to be a multi-resistant hospital pathogen and which poses a life-threatening risk for immunocompromised individuals, in particular, is *Staphylococcus aureus*. Those bacteria also form SCVs (Frenzel *et al*., 2015) in response to environmental stresses, but unlike *B. cereus* they are capable of reverting to its original state after antimicrobial treatment. For *B. cereus*, the adaptation to a small colony variant appears to be final as noted by Frenzel *et al*. 2015. It is believed that the SCV formation in *B. cereus* functions differently than in *S. aureus* when exposed to harsh conditions. The ability to form SCVs appears to be of environmental significance for *B. cereus* (Frenzel *et al*., 2015). In addition Poole (2012) believes that this alternative life cycle allows the bacteria to remain pathogenic with the progressive formation of SCVs in response to threatening stress factors and possibly essential oil exposure. However, more research needs to be done about the possibility of these bacteria forming SCVs due to exposure to essential oils and the possibility of recovery of these bacterial cells after treatment with essential oils.

Another common cause of antibiotic resistance in *B. cereus* is an increased abundance of β-lactamases (Fenselau *et al*., 2008; Bottone, 2010). Beta-lactamases are enzymes that destruct beta-lactam ring. With the beta-lactam ring destroyed, the antibiotic will no longer have the ability to bind to PBP (Penicillin-binding protein) and interfere with cell wall synthesis. In addition, Schlegelova and colleagues (2013) reported that the production of β-lactamases is one of potential virulence factors that make the producing strains resistant even to the 3rd generation of cephalosporins (Schlegelova *et al*., 2013).

Moreover, according to studies done by Nkhebenyane (2010), *B. cereus* was found to be resistant to cefoxitin, tetracycline, oxacillin and nalidixic acid. Resistance of *B. cereus* to cefoxitin was evident (7%), followed by nalidixic acid (30%), while oxacillin and tetracycline were both in the range of 62%. Microbial resistance to antibiotics in health care centres emerged soon after the first use of these agents in the treatment of infectious diseases, and continues to pose a challenge for the health care sector (Nkhebenyane, 2010). Resistance which was once primarily associated with health care institutions, has firmly emerged as a problem in the wider community. This is attested by the spread of infection by antibiotic-resistant *B. cereus*, often resulting in death (Boarder *et al*., 2016). Antimicrobial resistance of the investigated pathogen is a great concern keeping in mind the immune status of the patients at the hospices. The primary cause of antibiotic resistance could be the overuse of antibiotics both in clinical and veterinary practice as well as when the bacteria change or mutate, in ways that reduce or erase the antibiotic's effect on them (Nkhebenyane, 2010; Boarder *et al*., 2016). Therefore, these results led to the aim of this study which is to investigate the effects of *Thymus vulgaris* essential oil on antibiotic-resistant *Bacillus cereus* cells.

Essential oils are plant extracts that exhibit antimicrobial activity, and also possess antioxidative properties (Nazzaro *et al*., 2013). If used in food products, these aromatic oily liquids obtained from a variety of plant materials can reduce the incidence of food-borne diseases and retard lipid oxidation as well (Rasooli, 2007; Nazzaro *et al*., 2013). According to Preedy and Watson (2014) thyme oil (among the essential oils) exhibited the most effective antibacterial activity against *B. cereus* and such an activity could be strictly related to their chemical composition. In fact, thymol,

also found in thyme oil, exhibited a good antibacterial effect, mainly against *B. cereus* (Preedy and Watson, 2014). Therefore thyme oil is considered as an effective alternative antibacterial agent against antibiotic-resistant *B. cereus*. However, to use thyme oil as a food additive and or medicinal herb, the mechanism through which it exerts its antibacterial activity should be clearly elucidated and this forms the basis of this study.

3.3 MATERIALS AND METHODS

3.3.1 Antibacterial product and chemicals

3.3.1.1 Essential oil characterisation

The essential oil extracted from *Thymus vulgaris* was provided by local suppliers. Thyme essential oil was characterised according to the method of Kirbaslar *et al*. (2009). Briefly, the oil was dissolved in hexane (10% in hexane) and injected in a Finnigan Focus Gas Chromatograph (GC) at a split ratio of 50:1; the injector temperature was set at 230˚C. The GC was equipped with an AB-1MS (30M X0.25mm id 0.25µm) capillary column. Helium was used as carrier gas at a constant flow of 1mL min^{-1}. The temperature programme was set at 40˚C for four minutes and then raised at 5˚C min^{-1} to 200˚C and then held at 200˚C for 1 minute and then raised at 5˚C to 220˚C where it was held for 10 min. Mass analysis of the oils was done using a Finnigan Focus DSQ mass spectrometer. The ion source was at 250˚C with an ionisation voltage of 70eV and mass scan range of 50-650 amu. Individual GC peaks and mass spectra were identified by searching commercial libraries; this was followed by expert matching of MS data.

3.3.2 Microorganisms

The *Bacillus cereus* strain was obtained from a South African hospice and subcultured weekly on plate count agar (PCA) (Merck, SA) at 37°C throughout the study. The selected antibiotic-resistant *B. cereus* strain was previously tested for antibiotic resistance and found resistant towards cefoxitin, tetracycline, oxacillin and nalidixic acid (Nkhebenyane *et al*., 2012). To prepare inocula, cultures were grown overnight at 37°C; bacterial density was then adjusted to approximately 10^8 colony forming units (CFU) per ml for bio-assay preparation and 10^5 CFU.ml^{-1} for microdilution method with sterile saline solution. Bacterial counts were confirmed by plating out on plate count agar, plates were incubated at 37°C for 24 h.

3.3.3 Bio-assay preparation

The *Bacillus cereus* was suspended in sterilised distilled water (dH_20) and 0.2 ml spread out on PCA (0.5% m.v^{-1} agar). This produced a homogenous lawn across the surface of the agar (Kock *et al*., 2009). Subsequently, a well (0.5 cm in diameter and depth) was constructed at the centre of the Petri dish and 46 µl of two concentrations of thyme oil were added, that is, diluted thyme oil (2ml of essential oil into 100ml of ethanol) and undiluted thyme oil. Ethanol (96%) was also added alone to the wells as a control. Afterwards, all plates were incubated at 37°C for 24 h to observe different textured growth zones. To avoid evaporation of the essential oil from plates, the tested oil was allowed to diffuse in the agar before incubation.

3.3.4 Antibacterial assay of isolated *Bacillus cereus*

The investigation of the antibacterial effects of thyme essential oil was performed on antibiotic-resistant *Bacillus cereus*. Susceptibility of a bacterial strain to essential oil was investigated by broth microdilution method (Wong *et al*., 2014). Broth microdilution method was performed in sterile U-bottom microtiter plates (Spektar, Serbia). The inoculum density was set to 0.5 McFarland, diluted 10 times in sterile saline and 5 µl of this suspension was inoculated into 0.1 ml of Mueller-Hinton Broth (Becton, Dickinson and Company, Sparks, USA). The tested oil was added to the first row of wells, and serial dilutions were performed to achieve final concentrations of 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2 and 0.1 $mg.ml^{-1}$. After inoculation, plates were incubated at 37°C for 24 hours. The experiment was run twice in duplicate for each concentration. Minimal inhibitory concentration (MIC) was determined as the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism in broth dilution susceptibility test

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3.3.5 Gram stain preparation

Gram stain was performed on *B. cereus* cells according to the method of El-Garnal *et al*. (2009). Briefly, the slide with the specimen was flooded with Gram stain solutions (crystal violet, iodine, decolorizer, and safranin) (Merck, SA) and rinsed off with distilled water accordingly. Next, the slide was microscopically examined using Nikon E200 Eclipse Light microscope for bacterial organisms under 100X objective and the Gram reaction of the organism observed was defined.

3.3.6 Scanning electron microscopy (SEM)

Through bio-assay preparation, bacterial cells were collected from agar plates and immediately fixed with 3% sodium buffered (0.1 M, pH 7.0) glutardialdehyde (Merck, Darmstadt, Germany) for 3 h. The suspension was rinsed once by centrifugation with the same buffer to remove excess aldehyde fixative and then post-fixed with 1% aqueous osmium tetroxide (Merck, Darmstadt, Germany) in similar buffer solution. The suspension was rinsed twice by centrifugation to remove excess osmium solution and dehydrated by using a graded ethanol sequence (50%, 70%, 95%, 100% × 2 for 30 min per step). The cell and ethanol suspension was centrifuged between each dehydration step. The cell pellet was finally transferred to 5µm critical point dryer baskets (Biorad, London, UK) for the critical point drying process. The dried pellet of *B. cereus* cells was dispersed over a thin layer of epoxy glue (Prately, Gauteng, South Africa) on SEM stubs for mounting. The material was coated with 200 nm gold in a sputter coater (Biorad, London, UK) and viewed using a Shimadzu SX500 scanning electron microscope (SEM, Shimadzu, Tokyo, Japan) (Van Wyk and Wingfield, 1991).

3.3.7 Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM; Leeuw 2010), treated and untreated bacterial cells using bio-assay preparation, were fixed using the same protocol described for SEM. After washing, the material was dehydrated in a graded acetone (Merck, Darmstadt, Germany) series (50%, 70%, 95%, 2X 100%). Dehydration lasted 30 min for each step. Then the material was embedded in an expoxy resin (Spurr 1969) and allowed to polymerise in an oven at 70°C for 8 h. Thin sections

were made using a LKB III Ultramicrotome (Stockholm, Sweden) and stained with uranyl acetate for 5 min and lead citrate for 1 min. Finally, these sections were viewed with a Phillips EM 100 transmission electron microscope (Eindhoven, the Netherlands). These procedures were performed twice in at least duplicate.

3.3.8 Identification of fatty acids by GC-FID

3.3.8.1 Total lipid extraction.

For bio-assay preparation, cells exposed to thyme oil and control samples were incubated at 37°C until different textured growth zones were observed (usually after 24 h). Next, fatty acids were extracted using a fatty acid extraction kit (Sigma-Aldrich, SA). Briefly, lipids (0.15 g) were weighed and 3 ml of extraction solvent (Sigma-Aldrich, SA) was added to each sample (both treated and untreated samples). After cell suspension of treated and untreated (control) samples, 0.5 ml of aqueous buffer was added to the homogeneous mixture of each sample. Following that, the solution was poured into the syringe, plunger attached and pushed to elute lipids into the collecting tube for each sample (the eluted solvent now contained the total lipid extract). Then, the total lipid extract for each sample was now transesterified and analysed by GC-FID. This experiment was performed twice in duplicate.

3.3.8.2 Analysis of the Fatty Acid Composition

After fatty acids were extracted using a fatty acid extraction kit (Sigma-Aldrich, SA), the extracted lipids were methylated to fatty acid methyl esters (FAMEs) with boron trifluoride/methanol complex (5 ml of 20% BF_3/MeOH reagent) (Merck, SA) followed

by heating in 2.5 ml toluene (Merck, SA) at 100°C for 45 min under gentle mix. At room temperature, distilled water (12.5 ml) was added and the FAMEs were extracted with 5 ml of hexane (Merck, SA). The hexane fraction was dried in nitrogen gas, resuspended in 500 µl hexane and the solution was filtered prior analysis. The fatty acids were analysed using Finnigan Focus GC (Thermo Fisher Scientific, Waltham, Massachusetts, US) with flame ionisation detector and a 25 m x 0.32 mm ID SGE capillary column BPX70, 0.25 µm film (SGE, Melbourne, Victoria, Australia). The temperature conditions were 100°C for 5 min, 100-240°C at a rate of 3°C/min and at 240°C for 20 min. The samples were injected at 225°C and detected at 285°C with helium (linear flow of 20 $cm.s^{-1}$) as the carrier gas and split ratio of 1:50. A 37-Component FAME mixture (Sigma-Aldrich, SA) was used to identify the fatty acids.

3.3.9 Preparation of proteins

3.3.9.1 Isolation of bacterial protein for SDS-PAGE analysis

Throughout bio-assay preparation, cells exposed to thyme oil and control samples were incubated at 37°C until different textured growth zones were observed (usually after 24 h). Afterwards, bacterial proteins were determined using the protein isolation method, as previously described (Wong *et al*., 2014), with a slight modification. Briefly, bacterial cells (0.15 g) were weighed and 4 ml of B-PER bacterial protein extraction reagent (Thermo Scientific) was added to the cell pellet (0.15 g). After cell suspension of both treated and untreated (control) samples, homogeneous mixture for each sample was incubated for 10-15 minutes at room temperature. Then, lysate for each sample (treated and untreated cells) were centrifuged at 15, 000 x g for 5 minutes to separate soluble proteins from insoluble proteins. After obtaining the

soluble proteins, SDS-PAGE gel electrophoresis was then performed. This procedure was performed twice in at least duplicate.

3.3.9.2 SDS-PAGE gel electrophoresis

SDS-PAGE gel electrophoresis was performed using 12% resolving gel [1.5 M Tris-HCl, pH 8.8 (Bio Basic Canada, Inc), 10% SDS (Fisher Scientific), 40% bis-acrylamide] and 4% stacking gel [0.5 M Tris-HCl (pH 6.9), 10% SDS, 40% bis-acrylamide]. Before being loaded into the wells, bacterial proteins were treated with dithiothreitol (DTT) and boiled. For ease of molecular weight estimation and comparison, protein ladder (Spectra Multicolor Broad-Range Protein Ladder, Thermo Scientific) was loaded onto each gel. The SDS-PAGE gels were run using constant electric current (135 mV) until the bromophenol blue dye front reached the bottom of the gel plate. Protein gels were then stained with Coomassie Brilliant Blue R-250 (Fisher Scientific) staining solution for an hour, followed by overnight destaining in distilled water. Next, a gel documentation system was used for the imaging and documentation of proteins suspended within polyacrylamide gel.

3.4 RESULTS

Thymus vulgaris essential oil was characterised by the following compounds: borneol (0.5%), camphene (1.0%), caryophyllene (1.2%), caryophyllene oxide (1.4%), pinocarvone (1.5%), γ-Terpinene (3.0%), Thymol (32.1%), α-Pinene (5.1%) and ρ-Cymene (20.4%). Similar results were obtained by Nikolić *et al.* (2014). In their study EO extracted from *Thymus vulgaris*, thymol was also the major constituent

(49.1%), along with p-cymene (20.1%). In addition, the chemical profile of thyme EO sample in the present study is also in agreement with the report of Boskovic *et al.* (2015).

Thymol compounds have shown to have high antimicrobial activity against antibiotic-resistant *B. cereus* (Nazzaro *et al*., 2013; Boskovic *et al.*, 2015). For example, the antimicrobial mechanism of thymol is mainly based on its ability to disintegrate the cell membrane of *B. cereus* and increase the permeability of the cytoplasmic membrane to ATP (Lambert *et al*., 2001; Nazzaro *et al*., 2013). Moreover, α-Pinene and ρ-Cymene have been reported to deplete the intracellular ATP pool, change the membrane potential, and increase the permeability of the cytoplasmic membrane to potassium ions in *B. cereus* (Lambert *et al*., 2001; Nazzaro *et al*., 2013).

During bio-assay analysis, undiluted *Thymus vulgaris* essential oil showed antimicrobial activity on the agar plate (Figure 3.1) with an inhibition zone of 37 mm and the diluted essential oil with an inhibition zone of 32 mm for treated *B. cereus* cells on an agar plate (Figure not shown). For inhibitory effect of thyme oil on *B. cereus* isolates, undiluted *Thymus vulgaris* essential oil completely inhibited bacterial growth (and showed no minimum inhibitory concentration (MIC) due to its high antimicrobial activity (Table 3.1) and the diluted essential oil showed an MIC of 0.8 mcg/ml for treated *B. cereus* cells (Table 3.1). In addition, to determine the cellular damage produced by the oil on the *B. cereus* cells, a single colony close to the zone of inhibition from the agar plate (Figure 3.2) was picked and Gram staining was

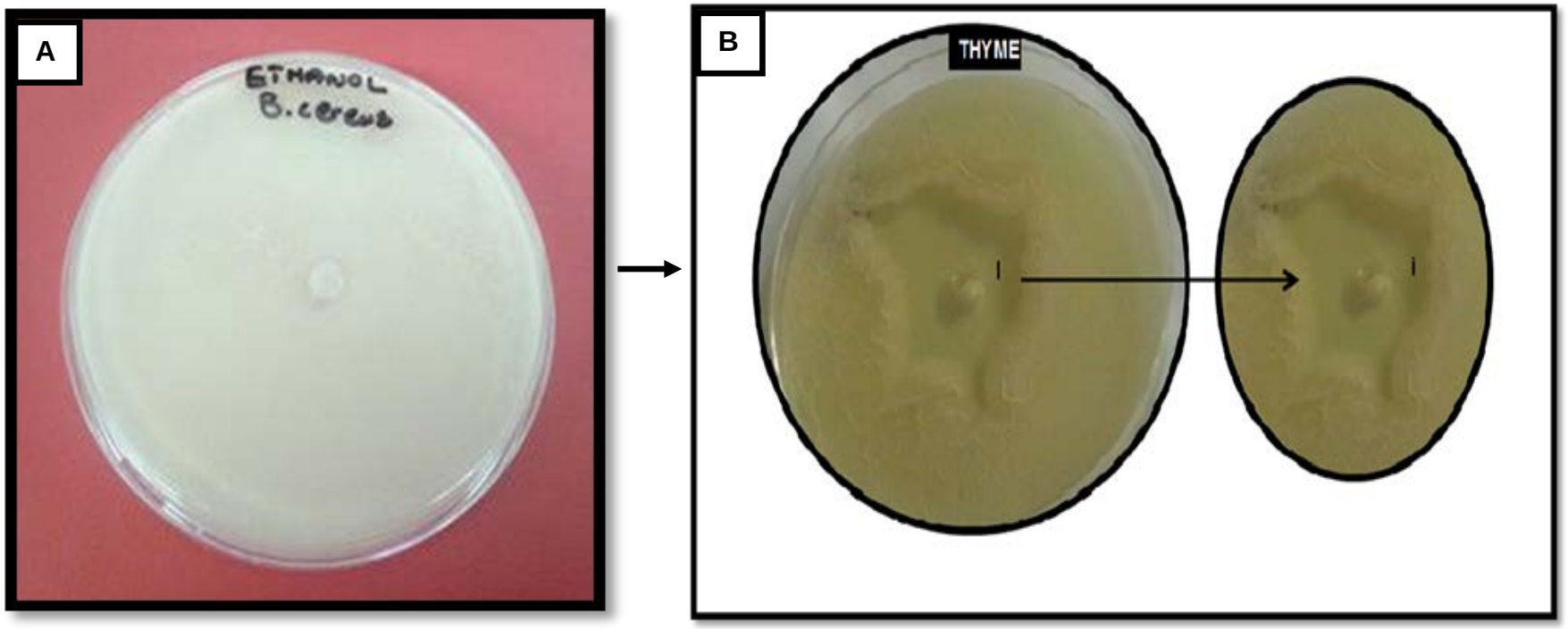
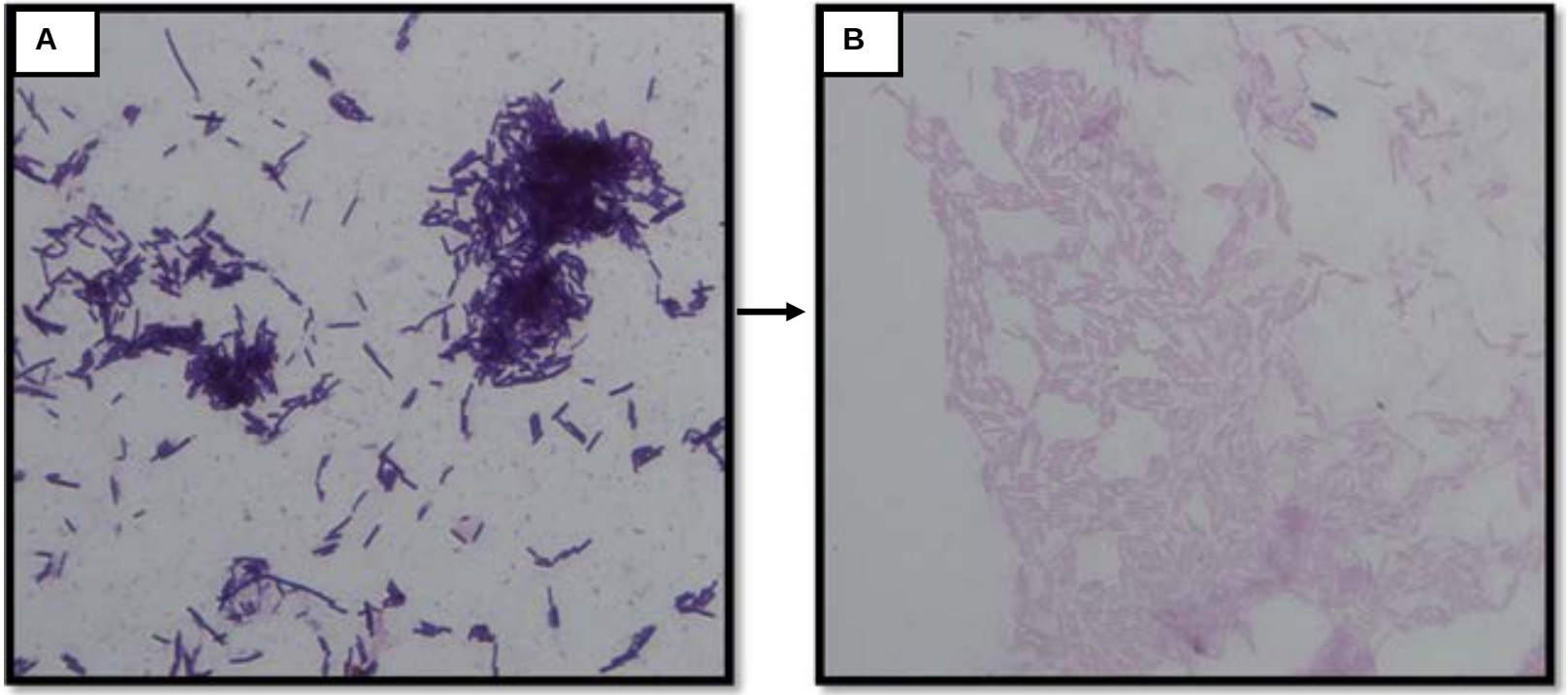


Figure 3.1: A bio-assay showing (A) untreated (control) *B. cereus* cells with no antimicrobial activity and (B) treated *B. cereus* cells with the antimicrobial activity of thyme oil against antibiotic-resistant *B. cereus* on an agar plate. I – designates zone of inhibition.

Table 3.1: The minimum inhibitory concentration (MIC) of thyme oil against *B. cereus* isolate

B. cereus	**Dilution of thyme essential oil (µg/ml)**							
Isolate								
	≥ 12.5	**≥ 6.3**	**≥ 3.1**	**≥ 1.6**	**≥ 0.8**	**≥ 0.4**	**≥ 0.2**	**≥ 0.1**
Control	+	+	+	+	+	+	+	+
Diluted	–	–	–	–	–	+	+	+
Undiluted	–	–	–	–	–	–	–	–

Data are reported as '+'indicates growth of bacteria (not sensitive to thyme oil), '–'indicates inhibition of growth of bacteria (sensitive to thyme oil).



***B. cereus*: untreated** ***B. cereus*: treated with thyme oil**

Figure 3.2: Gram stain light micrographs showing (A) untreated (control) *B. cereus* cells staining purple and (B) treated *B. cereus* cells staining pink

performed. The results (Figure 3.2) showed Gram-positive cells to be affected during Gram stain and stained pink like Gram-negative bacterial cells. These observations might be an indication of SCVs after exposure to essential oil. Characteristically slow growing, SCVs comprise a minor proportion of the population from which they arise but persist by virtue of their inherent resilience and host adaptability (Johns *et al*., 2015). *B. cereus* is an aerobic Gram-positive rod-shaped bacterium, purple in colour when viewed through a microscope after Gram staining (Bottone, 2010). Unlike Gram-negative bacteria**,** Gram-positive bacteria retain the violet dye due to their thick peptidoglycan layer and stain dark violet or purple (Fayaz*et al*., 2010). However, results observed in this study (Figure 3.2) showed *B. cereus* cells staining pink instead of purple as expected, this is possibly due to disturbance in the cell wall, absence of outer membrane and a decrease in peptidoglycan thickness during cell growth in the presence of thyme oil as observed elsewhere (Silhavy *et al*., 2010).

Since, *B. cereus* cells did not retain the violet dye and colored red or pink (Figure 3.2) this could be due to the effect of *Thymus vulgaris* essential oil on the cell wall, making them porous and allowing the crystal violet to wash out of cells causing them to de-stain and subsequently stain pink during the counterstaining step in the Gram staining procedure and appear as Gram negative. To confirm whether thyme oil has certainly affected the bacterial cell wall of antibiotic-resistant *B. cereus*, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed (Figure 3.3 and 3.4).

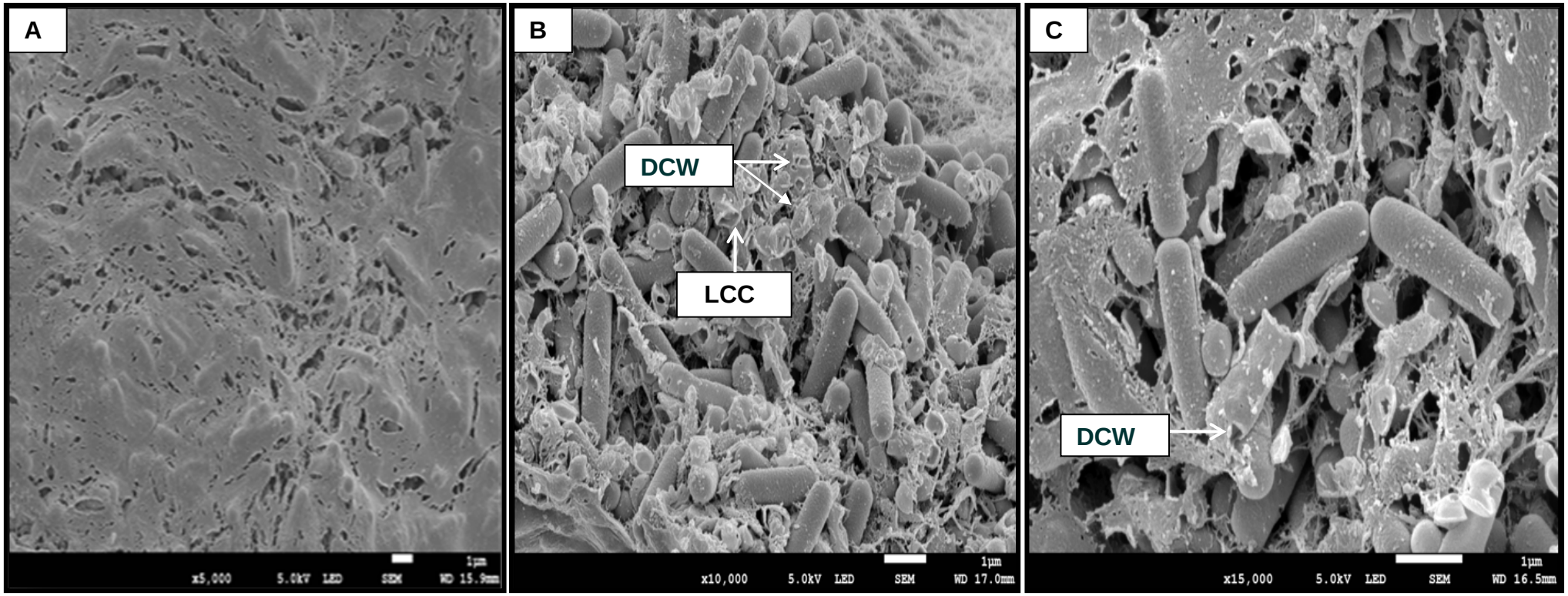


Figure 3.3: (A) control cells and (B-C) different types of injuries induced by thyme oil on the bacterial cell wall and membrane structure. DCW - Damaged cell wall with formation of holes on the cell surface; LCC - Loss of cellular contents.

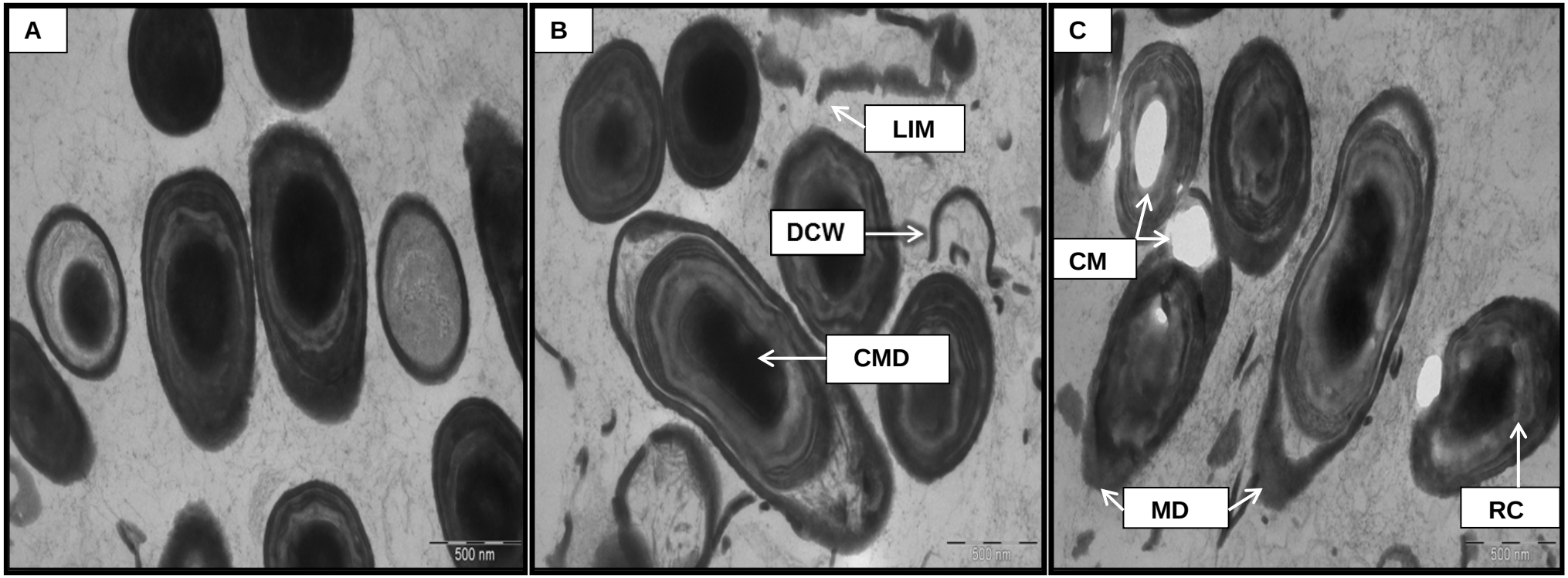


Figure 3.4: (A) control cells and (B-C) morphological changes of *B. cereus* cells after exposure to thyme oil. MD - Membrane disruption or cell wall deformation; LIM - Loss of intracellular material; RC -The slight roughness of the cell; CMD - The presence of cytoplasmic membrane damage; CM- Coagulated material; DCW- Damaged cell wall.

Exposure to thyme oil induced alterations in the bacterial membrane of antibiotic-resistant *B. cereus*, which led to the damaged cell wall (DCW) with the formation of holes on the cell surface, as demonstrated by scanning electron micrographs (Figure 3.3 C). In addition, loss of cellular contents (LCC) was observed (Figure 3.3 B). This could be an indication that the cells responded to thyme essential oil by forming SCVs. Small colony variants of *B. cereus* remain to be some of the best characterised, and when examined by electron microscopy are revealed to be a heterogeneous population of differing size, including “empty” cells (Johns *et al*., 2015). Moreover, the morphological change of thyme EO sample in the present study is also in agreement with the report of Johns *et al.* (2015).

Other than morphological changes observed using SEM, transmission electron micrographs also demonstrated damaged bacterial cells (Figure 3.4). The treated cells showed membrane disruption that caused cell wall deformation (Figure 3.4 C), slight roughness of the cells (Figure 3.4 C), coagulation of intracellular contents (Figure 3.4 C), and irregular cytoplasmic membrane with dark and densely stained cytoplasmic contents (Figure 3.4 B). In addition, loss of intracellular material (Figure 3.3 B and 3.4 B) which could possibly be lipid contents was observed outside the cell. Lipids are fatty substances that are used by bacteria as stores of energy and play a structural role in the cell membrane (Hall, 2015). Therefore, to confirm that lipid contents were certainly affected after treatment with thyme oil, changes in the lipid profile were investigated using a lipid extraction kit and gas chromatography (Figure 3.5).

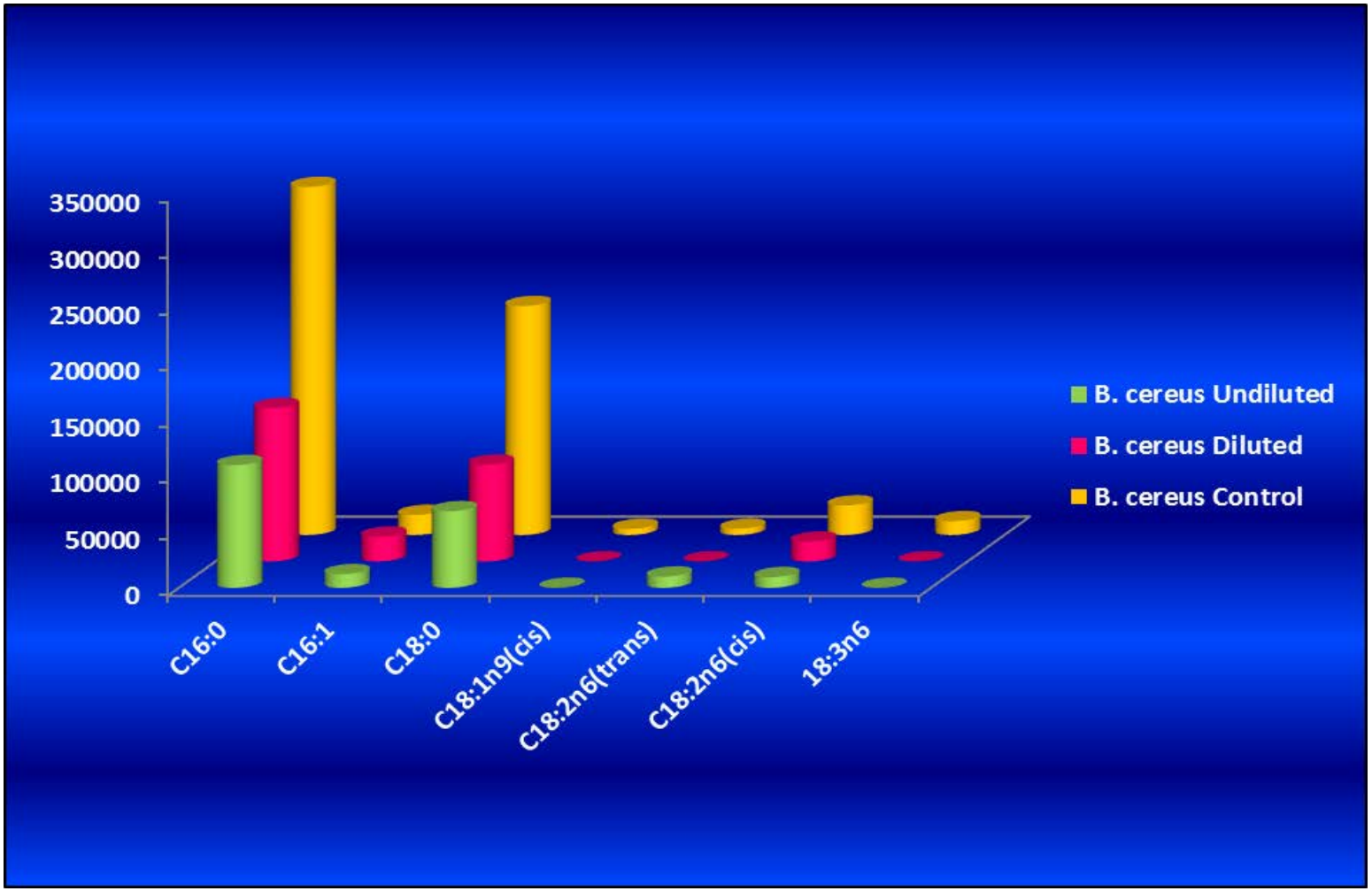


Figure 3.5: Fatty acid profile of antibiotic-resistant *B. cereus* affected by *Thymus vulgaris* essential oil. Standard deviations for treated (n=239087) and untreated samples (n=579893) are as indicated. 'y-axis' indicates Peak Intensity.

Bacillus cereus is known to synthesise unsaturated fatty acids (UFAs) as well as saturated (SFAs) fatty acids which are major constituents of cellular membrane (Cifré., *et al*., 2013; Diomandé *et al*., 2015). However, after treatment with thyme oil, both the saturated and unsaturated fatty acids were reduced when compared to the control (Figure 3.6). In addition, unsaturated C18:1n 9(cis) and 18:3n6 fatty acids were completely depleted after exposure to thyme oil. These observations (Figure 3.5) clearly demonstrate the negative effects of thyme oil and their components against antibiotic-resistant *B. cereus*. The antimicrobial effect of thyme essential oil might be due to a perturbation of the lipid fractions of the bacterial plasma membrane, which might be affected by the membrane disruption and leakage of intracellular materials observed by SEM and TEM micrograms (Figure 3.3 and 3.4). Moreover, a decrease in the amount of SFAs fatty acids when compared to untreated cells (Figure 3.5) results in a gain of membrane fluidity and as a consequent decrease in membrane rigidity as noticeable by SEM examination (Figure 3.3).

The other action of thyme oil on the cell membrane might be the inhibition of toxin secretion. Faleiro (2011) also reported that the exposure of *B. cereus* to thymol resulted in inhibition of diarrheal toxin production. From the results obtained in this study (Figure 3.4 and 3.5), structural modification on the cell membrane induced by thyme oil might explain the inhibition of toxin production (Faleiro, 2011). However, it is hoped that further works in this direction will be performed to prove this conjecture. The secretion of toxins is usually prevented by modifications in the bacterial membrane due to the attachment of the essential oil that disturb the phospholipid

bilayer with consequences to the trans-membrane transport process limiting in this way the release of toxins to the contiguous environment (Faleiro, 2011).

Two bacterial proteins with reduced expression levels upon treatment with *Thymus vulgaris* were identified (Figure 3.6). In addition, the bacterial proteins were completely depleted with faint bands when using the undiluted thyme oil in comparison to diluted oil and control (Figure 3.6) showing that the more concentrated the oil is, the more reduction of proteins occurs. Moreover, from the results obtained in this study, it is clear that the antimicrobial activity of thyme oil is not attributed to a single mechanism. Instead, different biochemical and structural mechanisms are involved at multiple sites within the cell and on the cell surface. These mechanisms include chemical modifications of the cell membrane, cytoplasm, fatty acids and proteins as observed in Figure 3.3 – Figure 3.6.

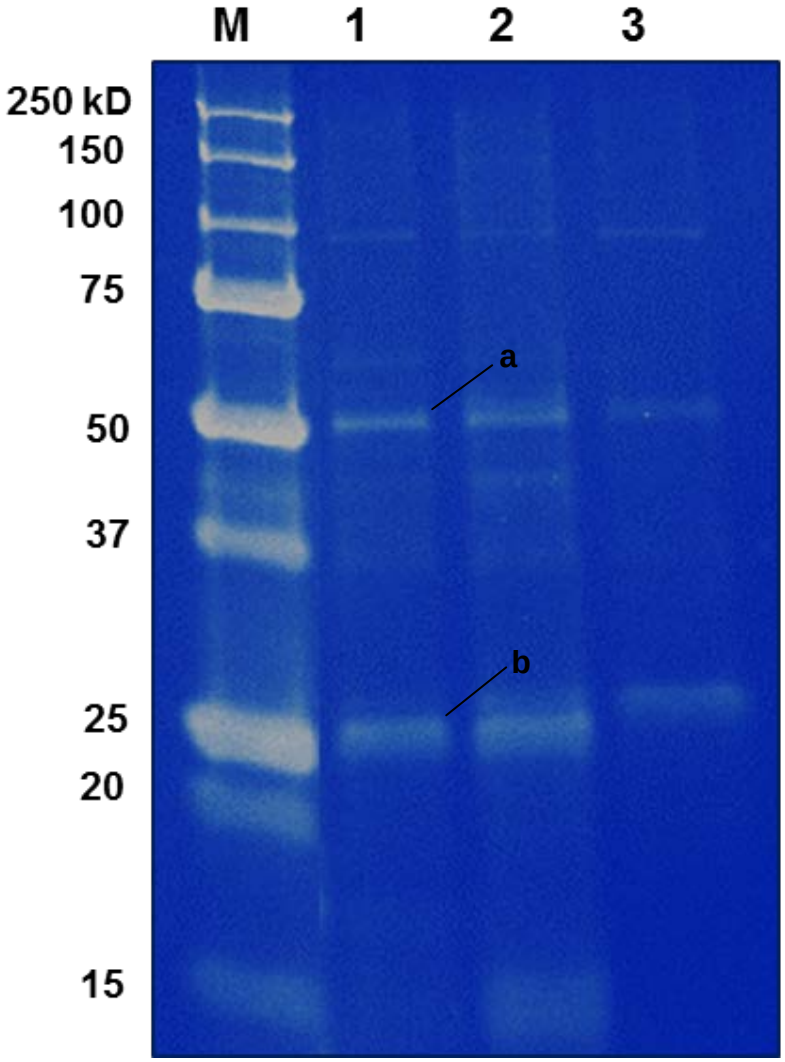


Figure 3.6: Protein expression profiles of antibiotic-resistant *B. cereus*, following exposure to *Thymus vulgaris* essential oil. M.-.Marker; 1 - the absence of *Thymus vulgaris* essential oil in bacterial culture medium; 2 - the presence of diluted *Thymus vulgaris* essential oil in bacterial culture medium; 3 - the presence of undiluted *Thymus vulgaris* essential oil in the bacterial culture medium. Labels (a to b) indicate differentially expressed protein bands upon treatment with *Thymus vulgaris* essential oil.

3.5 DISCUSSION

Essential oils have the strongest antibacterial properties when they contain a high percentage of phenolic compounds (Nazzaro *et al*., 2013). Essential oils (EOs) used in this study contained a high concentration of phenols, which exhibited a greater antimicrobial effect than tested antibiotics (cefoxitin, tetracycline, oxacillin and nalidixic acid) against antibiotic-resistant *B. cereus*. Antimicrobial mechanism of thymol, which is the major constituent of the EOs used, is commonly based on their ability to disrupt the cell wall and disintegrate the cell membrane of antibiotic-resistant *B. cereus* as demonstrated by Gram staining, SEM, and TEM (Figure 3.2, Figure 3.3 and Figure 3.4).

In addition, the disruption in membrane integrity (Figure 3.4 C); the intracellular leakage (Figure 3.3 B and Figure 3.4 B), and morphological changes (Figure 3.3 C and Figure 3.4 B) of the treated bacterial cells indicated that thyme oil affected the structural organisation of the cytoplasm together with the cell wall of treated antibiotic-resistant *B. cereus.* Moreover, slight roughness of the cell (Figure 3.4 C) was observed and the roughness was indeed associated with the perforation on the cell wall with the release of intracellular material and subsequent cell wall deformation. The results observed using SEM and TEM (Figure 3.3 and Figure 3.4) clearly showed the morphological changes on the bacterial cells after essential oil treatment (Figure 3.3 B, C and Figure 3.4 B, C).

It might be proposed that in the primary phase, the tested oil firstly act on the cell wall which is the first target of essential oils and disrupts its membrane ability (Figure 3.3 and Figure 3.4) which leads to dispersion of the desaturase enzymes and allows them to act on the membrane fatty acids. Fatty acids are the principal form of stored energy in most organisms including *B. cereus* and major constituents of cellular membranes (Turner *et al*., 2014). Once fatty acids are depleted as observed in Figure 3.5, the metabolic energy is inevitably disturbed and this led to cell death. Therefore, it is clear from the results (Figure 3.4 and Figure 3.5) obtained in this study that the lipid biosynthesis pathway is greatly affected by *Thymus vulgaris* essential oil.

Total proteins were also found to be affected by *Thymus vulgaris* essential oil. In the proteomic analysis, two bacterial proteins presented with reduced expression levels, upon treatment with *Thymus vulgaris* essential oil (Figure 3.6). The reduced protein expression of bacterial proteins for antibiotic-resistant *B. cereus* could be due to the depletion of fatty acids on the cell membrane. Lipids serve as anchors for proteins (covalently attached fatty acids, prenyl groups, and phosphatidylinositol) (Paoletti and Kritchevsky, 2015). For that reason, it is clear from the results that the disrupted fatty acids could inevitably results in damaged bacterial proteins (Paoletti and Kritchevsky, 2015).

Moreover, thyme oil acted on proteins present in antibiotic-resistant *B. cereus* and affected bacterial protein synthesis. Based on the results, it might be speculated that thyme oil was able to affect one of the proteins responsible for cell wall synthesis

such as a penicillin-binding protein. *Bacillus cereus* produces β-lactamases (Fenselau *et al*., 2008; Bottone, 2010) enzymes that destruct beta-lactam ring, with the beta-lactam ring destroyed, the antibiotic such as penicillin will no longer have the ability to bind to PBP (Penicillin-binding protein) and interfere with cell wall synthesis (Dulon *et al*., 2011; WHO, 2014). However, unlike penicillin, an essential oil used in this study has shown (Figure 3.6) its ability to bind the bacterial cell surface and penetrates the cell wall causing a decrease in proteins, which eventually leads to cell death. The presence of coagulated material was also observed (Figure 3.4 C). The coagulated material is thought to be a precipitate of abnormal proteins or denatured membrane induced by thyme essential oil (Becerril *et al*., 2007).

In general, thyme oil acts to inhibit the growth of *B. cereus* cells and possibly inhibit the production of toxic bacterial metabolites. Most EOs have a more powerful effect on Gram-positive bacteria such as *B. cereus* than Gram-negative species (Nazzaro *et al*., 2013), and this effect is most likely due to differences in the cell membrane compositions (Figure 3.3, Figure 3.4 and Figure 3.5). Compared with Gram-negative bacteria, *Bacillus* species are more susceptible against antimicrobials because of their penetrable cell wall and lack of outer membrane (Chorianopoulos *et al*., 2008; Nazzaro *et al*., 2013). This chapter consolidates and describes the observed antagonistic outcome of *Thymus vulgaris* essential oil against antibiotic-resistant *B. cereus*, and highlights the possibilities of thyme oil as the potential antimicrobial agent.

3.6 CONCLUSIONS

In the present study, *Thymus vulgaris* essential oil showed high antimicrobial activity towards antibiotic-resistant *B. cereus*. This human pathogen tested showed an appreciable sensitivity towards the essential oil used in the experiment (Figure 3.1). Thyme essential oil was found to damage the integrity of the cell membrane, the key element for the fundamental biological activities taking place within the *B. cereus* cell. After treatment with the oil, the membrane failed to represent an effective barrier between the cytoplasm and the external environment as observed by SEM and TEM micrographs, which eventually leads to cell death. From the results observed, thyme oil also depleted both saturated and unsaturated fatty acids of antibiotic-resistant *B. cereus*. In addition, the decrease in the amount of saturated fatty acids when compared to untreated cells results in a gain of membrane fluidity and as a consequent decrease in membrane rigidity as noticeable by SEM examination (Figure 3.3). Moreover, it was evident in this study that thyme oil has the capability to target the bacterial sites (particularly the cell wall, cell membrane, cytoplasm, lipids, enzymes, and proteins) of *B. cereus* that antibiotics such as cefoxitin, oxacillin, tetracycline, and nalidixic acid failed to target. In the proteomic analysis, two proteins with reduced expression levels appearing as faint bands (Figure 3.6) were identified, upon treatment with *Thymus vulgaris* essential oil. Moreover, from the scientific data provided in this study, it can be concluded that inhibition of these bacterial targets may likely prove fatal to the invading *B. cereus* strains, by rendering the bacteria vulnerable in an acidic environment (human stomach), unable to replicate (by blocking the translation pathway) and reduced virulence (by repressing the expression of membrane damaging exotoxin).

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CHAPTER 4: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 INTRODUCTION

For many years, the battle between humans and the multitudes of spoilage microbes and food-borne pathogens as well as disease-causing pathogens continues (Boire, Riedel and Parrish, 2013; WHO, 2014). Emerging at the battlefield as the most significant challenge to human health is bacterial resistance to antibiotics and its rapid rise (Boire, Riedel and Parrish, 2013; WHO, 2014). This has become a major concern in global public health invigorating the need for new antimicrobial compounds. A rational approach to deal with antibiotic resistance problems requires detailed knowledge of the different biological and non-biological factors that affect the rate and extent of resistance development. Plants and their derivatives, such as essential oils, are currently blooming and represent a potential area for future investigations (Swamy *et al*., 2016). This new generation of phytopharmaceuticals may shed light on the development of new pharmacological regimes in combating antibiotic resistance (Swamy *et al*., 2016). To describe the observed antagonistic outcome of essential oils and their components, and highlight the possibilities of essential oils as potential antimicrobial agents, important factors need to be considered when designing preventative measures. The objectives of this study were as follows:

- To assess the activity of antimicrobial compounds using biochemical tests.
- To investigate the morphological changes induced by *Thymus vulgaris* essential oil on the bacterial cell wall.
- To assess the effect of thyme essential oil on fatty acids profile of the cell membrane.

- To identify potential antibacterial protein targets following exposure to *Thymus vulgaris* essential oil using proteomics.

In order to attend to these objectives, the following aspects were considered: Antibiotic-resistant bacteria, essential oils and their components, potential antibacterial targets such as cell membrane, cytoplasm, lipids, and proteins.

The study has been arranged in three sequential parts: where one chapter covered literature review and two focused on respective research chapter as shown.

- Chapter 1: Gathering information from a review of the literature relating to essential oils as possible antimicrobial agents against antibiotic-resistant bacteria.
- Chapter 2: Investigating the action of *Thymus vulgaris* essential oil on *Staphylococcus aureus* cell wall, including cell membrane, cytoplasm, lipids, and proteins, by means of Gram staining, scanning electron microscopy (SEM), transmission electron microscopy (TEM), gas chromatography and proteomics.
- Chapter 3: Studying the cellular damage induced by *Thymus vulgaris* essential oil in antibiotic-resistant *Bacillus cereus*.

4.2 GENERAL DISCUSSION

During observations as discussed in Chapter 2 and Chapter 3, the bacterial cell wall of antibiotic-resistant *S. aureus* and *B. cereus* was found to be one of the most promising niches for thyme essential oil targets. Moreover, the permeable nature of

the Gram-positive envelope contribute to the simplicity of this task since essential oils (unlike antibiotics) are able to penetrate through the cell wall and affect a cascade of reactions involving the entire bacterial cell. Thyme essential oil showed antimicrobial activity against both bacteria (*S. aureus* and *B. cereus*) by targeting (see Chapter 2 and 3) the cell wall, cell membrane and cytoplasm, and in some cases completely changed the morphology of the cells. To assess the cellular damage produced by the oil on both bacteria, a single colony close to the zone of inhibition that might represent SCV was picked and Gram staining was performed. The results (see Chapter 2 and 3) showed Gram-positive cells of both *S. aureus* and *B. cereus* to be affected by a cell wall active agent (thyme oil) and stained pink like Gram-negative bacterial cells.

From literature, it is clear that both bacteria are Gram-positive coccal and/or rod bacteria, purple in colour when viewed through a microscope following Gram staining (Hanselman *et al*., 2009). However, results found in this study (see Chapter 2 and 3) showed both bacterial cells staining pink instead of purple as anticipated, this is possibly due to a decrease in peptidoglycan thickness induced by environmental stress or a disturbance in the cell wall during cell growth in the presence of thyme oil (Silhavy *et al*., 2010). Moreover, the results found during Gram staining clearly demonstrate that *Thymus vulgaris* essential oil affected the cell wall and cell membrane of both bacteria making them porous and allowing the crystal violet to wash out of cells causing them to de-stain and subsequently stain red during the counterstaining step in the Gram staining procedure and appear as Gram negative.

To confirm that thyme oil has certainly affected the bacterial cell wall of antibiotic-resistant *S. aureus* and *B. cereus*, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed (see Chapter 2 and 3). Exposure to thyme oil induced alterations in the bacterial membrane of *S. aureus* as well as *B. cereus*, which led to the loss of cell wall integrity, as demonstrated by Gram staining, SEM, and TEM. In addition, loss of cellular contents and irregular cytoplasmic membrane of the cell that could represent SCVs, as indicated by SEM and TEM were observed.

Moreover, the intracellular leakage and morphological changes of the two treated bacterial cells (see Chapter 2 and 3) indicated that thyme oil affected the structural organisation of the cytoplasm together with the cell wall of treated *S. aureus* and *B. cereus* cells. It is proposed that in the primary phase, the tested oil probably binds the bacterial cell surface and penetrates the cell wall causing cytoplasmic membrane damage and this leads to cell death. This phenomenon indicates that all these observed changes might be stress induced forming SCVs and that the cell wall is the first target of essential oils and this is also an indication that the tested oil indeed affected the structural organisation of both antibiotic-resistant organisms (*S. aureus* and *B. cereus*) since this was not observed in the control sample (see Chapter 2 and 3).

Again, it was evident in this study (see Chapter 2 and 3) that the activity of thyme oil and their components is not attributable to a single event but instead involves a series of events both on the cell surface and within the cytoplasm. The disruption of

the cell wall and membrane integrity observed through SEM and TEM electron micrographs was also found to result in reduced saturated and unsaturated fatty acids as observed in the fatty acids profile (see Chapter 2 and 3). Based on the results, it is apparent that thyme essential oil firstly acts on the cell wall and disrupts the outer membrane of antibiotic-resistant *S. aureus* and *B. cereus* which could lead to dispersion of the desaturase enzymes and allows them to act on the membrane fatty acids. In addition to direct effects on the fatty acids of the outer membrane, it is believed that thyme oil affected enzymes that are involved in fatty acid synthesis. However, further investigation needs to be done to certainly prove this hypothesis. The oil caused a major decrease in unsaturated C18: 2n6 (cis), and 18: 3n6 (cis) fatty acids (as shown in Chapter 2 and 3) and this could be due to disrupted fatty acyl-CoA desaturase enzyme affected by the essential oil. Nazzaro and colleagues (2013) also stated that thyme oil is capable of affecting this multicomponent membrane desaturase enzyme that is generally employed by cells to produce unsaturated fatty acids.

Furthermore, since lipids are the principal form of stored energy in most organisms including *S. aureus* and *B. cereus* and are major constituents of cellular membranes (Turner *et al*., 2014). It is believed that once fatty acids are depleted due to damaged cell membrane as shown in Chapter 2 and 3, the disrupted cell wall and membrane would inevitably lead to cell death. Therefore, it is clear from the findings of this study that the membrane disruption effect of the essential oil, and the less amount of fatty acids is the consequence of the inhibited bacterial growth.

Total proteins were also found to be affected by *Thymus vulgaris* essential oil. In the proteomic analysis, a total of three proteins from *S. aureus* and two proteins from *B. cereus* organisms with reduced expression levels, upon treatment with *Thymus vulgaris* essential oil were observed (see Chapter 2 and 3). The reduced protein expression of proteins for both antibiotic-resistant *S. aureus* and *B. cereus* could also be due to the depletion of fatty acids on the cell membrane. Lipids serve as anchors for proteins (covalently attached fatty acids, prenyl groups, and phosphatidylinositol) (Paoletti and Kritchevsky, 2015). For that reason, it is clear from the results that the disrupted fatty acids inevitably results in a damaged bacterial protein with reduced expression levels.

Moreover, it might be speculated that thyme oil was able to affect one of these proteins namely FtsZ proteins, fatty acyl-CoA desaturase enzyme, and β-lactamases or Penicillin-binding proteins. Thyme oil might have acted on FtsZ proteins present in *S. aureus* cells and affected cell division as observed in Chapter 2. Bacterial cell division is regulated by FtsZ protein and FtsZ assembles into a Z-ring at the site of cell division. However, the results found in this study showed thyme oil perturbing the Z-ring morphology and reducing the frequency of the Z-ring per unit of cell length of *S. aureus* which eventually caused cell division to stop (Nazzaro *et al*., 2013). Surprisingly, the incomplete cell division was not observed in *B. cereus* cells (see Chapter 3) and the differences registered between the two bacteria could be mainly due to differences in the genes and protein composition.

Secondly, there was a decrease of all fatty acids for both antibiotic-resistant *S. aureus* and *B. cereus* cells when compared to the control (see Chapter 2 and 3). It is believed that fatty acyl-CoA desaturase enzyme could be one of the proteins that were observed with reduced expression levels, upon treatment with *Thymus vulgaris* essential oil. This was shown by a decrease in unsaturated fatty acids (see Chapter 2 and 3) due to a disruption of the fatty acyl-CoA desaturase enzyme as a result of exposure to thyme essential oil. But, further proteomic analysis needs to be done to validate this hypothesis regarding fatty acyl-CoA desaturase enzyme. Moreover, the observed resistance towards penicillin of the selected strains (Nkhebenyane, 2012) it might be proposed that β-lactamases or Penicillin-binding proteins (PBPs) could be other proteins that were affected by *Thymus vulgaris* essential oil. In the primary phase, the thyme oil binds the bacterial cell surface and penetrates the cell wall causing a decrease in PBPs followed by interrupted cell wall synthesis (see Chapter 2 and 3). However to confirm our speculations future work need to be done using MALDI-TOF-TOF MS protein sequencing to identify these differentially expressed proteins.

The current findings clearly demonstrate that *Thymus vulgaris* essential oil has the capability to target the bacterial sites (particularly the cell wall, cell membrane, cytoplasm, lipids, enzymes, and proteins) of both *S. aureus* and *B. cereus* that antibiotics such as cefoxitin, oxacillin tetracycline, and nalidixic acid failed to target. Consequently, the overall results found in this study show the advantage of using thyme oil when compared to antibiotics. It was also evident in this study that the antimicrobial actions of thyme essential oil are linked to one of the most important EO characteristics, its hydrophobicity resulting in increased cell disruption that leads

to depletion of fatty acids and consequent decrease in proteins. It is important to comprehend that a disturbed cell structure may affect other cellular structures in a cascade type of action.

4.3 CONCLUDING REMARKS ON THE PRECEDING CHAPTERS

As discussed in Chapter 2, the mode of action of *Thymus vulgaris* essential oil on *Staphylococcus aureus* morphology was revealed. Thyme essential oil damaged the cellular membrane of antibiotic-resistant *S. aureus*, which leads to cell death. Additionally, the reorganisation of the lipid profile shown after the treatments of the resting cells was strictly related to the presence of thyme oil compounds. It was also evident that depletion of fatty acids could be due to damaged cell membranes inevitably. Therefore, from the results presented in chapter 2, it can be concluded that the membrane disruption effect of the essential oil and the less amount of fatty acids is the consequence of the inhibited bacterial growth. This also shows that *Thymus vulgaris* essential oil has the capability to target the bacterial sites of *S. aureus* that antibiotics, for instance, cefoxitin, tetracycline, and nalidixic acid failed to target. Thyme essential oil is therefore considered a potential antimicrobial agent. Moreover, from adequate scientific evidence provided in this study, it can be concluded that thyme essential oil might enhance the chances of developing new conventional and natural antimicrobial agents (drugs as well as food preservatives) and be good alternatives to replace synthetic chemicals.

In chapter 3, *Thymus vulgaris* essential oil also showed high antimicrobial activity towards antibiotic-resistant *B. cereus*. This human pathogen tested showed an

appreciable sensitivity towards the essential oil used in the experiment. In addition, thyme essential oil was found to damage the integrity of the cell membrane which is the key element for the fundamental biological activities taking place within the cell. After treatment with the oil, the membrane failed to represent an effective barrier between the cytoplasm and the external environment as observed by SEM and TEM micrographs, which eventually leads to cell death. When compared to *S. aureus* observations in Chapter 2, the tested oil showed similar results in the lipid biosynthesis pathway of *B. cereus* cells. Thyme oil depleted both saturated and unsaturated fatty acids of *B. cereus* which results in loss of metabolic energy. Moreover, it was evident in this study that thyme oil has the capability to target the bacterial sites of *B. cereus* that antibiotics such as cefoxitin, oxacillin, nalidixic acid, and tetracycline failed to target as observed in proteomic analysis. Unlike the tested antibiotics, thyme oil showed to degrade the proteins that might act as efflux pumps. Consequently, the results found in this study show the advantage of using thyme oil when compared to antibiotics.

In conclusion, a total of three differentially expressed proteins from *S. aureus* and two differentially expressed proteins from *B. cereus* organisms were identified. Inhibition of these bacterial targets may likely prove fatal to the invading *B. cereus* strains, by rendering the bacteria vulnerable in an acidic environment (human stomach), unable to replicate (by blocking the translation pathway) and reduced virulence (by repressing the expression of membrane damaging exotoxin). Most importantly, from the results presented in Chapter 2 and Chapter 3, it can be concluded that thyme essential oil could play a key role in the fight against infectious

diseases and food-borne illnesses since it showed high antimicrobial activity against both antibiotic-resistant food-borne pathogens (*S. aureus* and *B. cereus*) studied.

4.4 CONCLUSIONS AND RECOMMENDATIONS

Infectious diseases and food-borne illnesses triggered by *S. aureus* and *B. cereus* can cause severe health effects and can even lead to death among the residing population, especially in the developing regions of the world. The continual emergence of antibiotic-resistant microorganisms has prompted researchers in this study to search for new antimicrobial agents that are more effective against the resistant microbial pathogens. Structural modification of the antimicrobials (against which microbial resistance has been developed) is reported to improve the effectiveness of antimicrobial agents against bacteria, fungi, and viruses. Such alternative treatment methods included essential oils as potential antimicrobial agents for the fight against infectious diseases and food-borne illnesses that cause severe health effects and even death especially in patients with weakened immune systems. Therefore it is advised that plants with these potential therapeutic or medicinal values can be successfully utilised for preventing and treating various ailments and food-borne illnesses.

From the results obtained in this study, it can be concluded that essential oils and their components play a significant role in combating bacterial antibiotic resistance and can be used in traditional medicine to cure some of the common disorders and degenerative diseases in humans as well as in animals. The effectiveness of these procedures has been attributed mainly to the presence of active phytochemicals or

bioactive compounds in essential oils observed in this study. Given the scope of searching new antimicrobial agents, antimicrobials derived from *Thymus vulgaris* plant materials are regarded as natural and safe compared to synthetic chemicals. Moreover, plant-based medicine is highly recommended due to the increasing concern of consumers reported in the literature (Djilani and Dicko, 2012) with regard to the use of synthetic chemical preparations and use of artificial antimicrobial preservatives, especially in modern food protection practices.

Based on these facts, the present study focused mainly on providing baseline information on exploring some of the diverse mechanisms of action of the *Thymus vulgaris* essential oil and their components against two specific antibiotic-resistant pathogens (*S. aureus* and *B. cereus*). The scientific details provided in this study on these aspects are hopefully expected to be useful for the commercial exploitation of essential oils to develop natural preservative preparations with applicability in the food and pharmaceutical industries. The results presented in this study further indicate some of the advantages of using natural antimicrobials such as thyme essential oil and those factors include: the possibility of reducing total dependence on antibiotics by replacement with essential oils, controlling cross-contaminations by food-borne pathogens, and improvising food preservation technology. Thyme essential oil is traditionally believed to be rich in phytochemicals exhibiting rich bioactivity. These compounds identified in this study could be of interest to the local industry as well as to the general population and could be actively explored for various commercial applications (such as cleaning antimicrobial agents, food preservatives, and more). However, more research will have to be conducted to assess the antimicrobial effects of thyme oil in food matrices since the current study

exposed the effects of the tested oil on bacteria without interference from matrices such as food products. This essential oil also indicates its efficacy and to possess a broad spectrum of antimicrobial activity against various spoilage and pathogenic microorganisms which are attributed to their bioactive constituents (Nazzaro *et al*., 2013).

4.5 FUTURE RESEARCH

The current study has revealed the following areas for possible future research:

- Determination of which bacterial enzymes or pathways are actually affected by this antibacterial medicinal plant (*Thymus vulgaris* essential oil) using MALDI-TOF-TOF MS protein sequencing. It is hoped that further work in this direction could potentially lead to the discovery of effective therapeutic agents.
- Exploration on the final state of the bacteria forming small colony variants (SCVs) due to exposure to thyme oil. To assess if the initially disturbed/affected bacterial colonies treated with thyme oil does remain disrupted or do they somehow over the course of time regain their structural stability and begin to synthesise.
- Performing *in vivo* studies to assess the antibacterial activity of thyme essential oil against whole living organisms. Example: use of model organisms such as mice and rabbit, where drugs will be directly injected into the body (drug testing). To compare the antimicrobial effect of thyme oil between experimentation outside whole living organism and using a whole living organism.
- Investigating the antimicrobial activity of thyme essential oil in food matrices.
- Developing essential oils based on antimicrobial products that could serve as surface disinfectants in food industries.

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