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MALAYSIAN LEMONGRASS (*Cymbopogon citratus*) (DC.) Stapf AS NATURAL ANTIMICROBIALS FOR ENHANCING FOOD SAFETY: IDENTIFICATION, CHARACTERISATION AND APPLICATION

A thesis presented in partial fulfilment of the requirements for the degree of

Doctoral of Philosophy in Food Microbiology

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Highlights

1. Supercritical fluid extraction (SFE) of the Peha Ayam lemongrass variety at 85 bar produced the highest citral content and exhibited the strongest antibacterial activity, compared with other pressures, confirming the importance of extraction parameters in enhancing bioactive potency.

2. Emulsified forms of lemongrass extract showed significantly improved antibacterial effects compared with non-emulsified forms, against *Bacillus cereus*, especially in soymilk systems, highlighting the role of nanoemulsion technology in overcoming solubility and dispersion limitations of essential oils.

3. Lemongrass nanoemulsion outperformed both citral nanoemulsion and nisin in biofilm disruption and prevention assays, demonstrating its potential as a natural, plant-based alternative for managing *B. cereus* contamination in food systems.

4. Characterisation of lemongrass nanoemulsions revealed good initial physicochemical stability, although performance declined over time due to droplet coalescence. Nevertheless, the whole extract retained bioactivity for longer than citral, supporting the synergistic role of minor constituents.

5. Mechanistic studies confirmed that lemongrass nanoemulsion exerts its antibacterial effect through membrane disruption, Adenosine triphosphate (ATP) depletion, and cytoplasmic leakage, as observed through flow cytometry, microscopy, and transmission electron microscopy (TEM) imaging, reinforcing its efficacy and concentration-dependent mode of action.

Abstract

This study investigates the antimicrobial potential of lemongrass (*Cymbopogon citratus*) nanoemulsion against *Bacillus cereus*, a resilient foodborne pathogen known for its spore-forming ability, toxin production, and biofilm-forming capacity. The research adopts a multi-phase approach encompassing extraction, formulation, comparative efficacy, and mechanistic understanding to explore the viability of lemongrass-based nanoemulsions as natural antimicrobial agents for food safety.

In Chapter 3, supercritical fluid extraction (SFE) was employed to extract essential oils from two Malaysian lemongrass varieties, Gajah and Peha Ayam under varying pressures (85 to 300 bar). Peha Ayam extracted at 85 bar yielded the highest citral content (90.06%) and showed the strongest antibacterial activity. Emulsified extracts demonstrated enhanced antimicrobial efficacy, especially in soy milk, while higher carbohydrate content in rice milk was associated with reduced performance. These findings highlight the importance of extract quality, formulation, and food matrix compatibility in determining antimicrobial effectiveness.

Chapter 4 presents a comparative assessment of lemongrass and citral nanoemulsions against the conventional antimicrobial nisin across three *B. cereus* isolates (ATCC 14579, P4, and M2). While nisin exhibited strong planktonic inhibition, lemongrass nanoemulsion consistently outperformed both nisin and citral in biofilm disruption and prevention assays, suggesting its superior performance in managing biofilm-related contamination in food systems. Chapter 5 further characterizes the nanoemulsions' physicochemical properties, noting strong initial stability and bactericidal activity. However, droplet coalescence over time reduced long-term efficacy. Notably, whole lemongrass nanoemulsions retained bioactivity longer than citral nanoemulsions, indicating the possible synergistic role of minor constituents.

Chapter 6 focuses on the antibacterial mechanism. A series of mechanistic assays revealed that lemongrass nanoemulsion disrupts bacterial membranes, leading to

ATP depletion, membrane depolarization, and cell lysis. These effects were confirmed via flow cytometry, fluorescence microscopy, and transmission electron microscopy (TEM), highlighting its concentration-dependent action and isolate-specific responses. Collectively, this thesis establishes lemongrass nanoemulsion as a promising clean-label antimicrobial with potential applications in food preservation, offering new insights into its formulation, mechanism, and commercial viability.

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- **Mohd Daud, I. S.,** Mahmud Ab Rashid, N. K., Palmer, J., & Flint, S. (2025). From Grass to Guard: Lemongrass Nanoemulsions Ensuring Stable Food Preservation. New Zealand Institute of Food Science and Technology Conference; Poster presentation.

- **Mohd Daud, I. S.,** Mahmud Ab Rashid, N. K., Palmer, J., & Flint, S. (2024). Lemongrass Nanoemulsion as a Potential Biopreservatives to Control *B. cereus* Biofilm in Food. New Zealand Institute of Food Science and Technology Conference; Oral presentation.

- **Mohd Daud, I. S.,** Mahmud Ab Rashid, N. K., Palmer, J., & Flint, S. (2024). Application of Lemongrass Nanoemulsionon *Bacillus cereus* in Different Food Systems New Zealand Institute of Food Science and Technology Conference; Poster presentation.

- **Mohd Daud, I. S.,** Mahmud Ab Rashid, N. K., Palmer, J., & Flint, S. (2024). Lemongrass Nanoemulsion: Potent and stable natural preservative. Mini Microbiology Massey Conference; Oral presentation.

- **Mohd Daud, I. S.,** Mahmud Ab Rashid, N. K., Palmer, J., & Flint, S. (2023). Potential Use of Lemongrass Nanoemulsion and its Major Compound, Citral on Inhibition of *Bacillus cereus*. Mini Microbiology Massey Conference; Oral presentation.

Abbreviations

%EE	Percentage of encapsulation efficiency
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
B. cereus	*Bacillus cereus*
B. thuringiensis	*Bacillus thuringiensis*
BHI	Brain heart infusion
C	Citral
C. citratus	*Cymbopogon citratus*
C. nardus	*Cymbopogon nardus*
C. tropicalis	*Candida tropicalis*
CO_2	Carbon dioxide
DLS	Dynamic-light-scattering
E. coli	*Escherichia coli*
ECHA	European Chemicals Agency
eDNA	Extracellular DNA
EO	Essential oil
EPS	Extracellular polymeric substances
EU	European union
FDA	Food and drug adminstration
FEMA	Flavor and Extract Manufacturers Association
FSC	Forward scatter
G	Gajah
GC-MS	Gas chromatography-Mass spectrometry
GRAS	Generally Recognized as Safe
h	Hour
HCl	Hydrochloric acid
HPLC	High-Performance Liquid Chromatography
IBS	Institute of Bioscience
kg	Kilogram
L. monocytogenes	*Listeria monocytogenes*
LD_{50}	Median lethal dose
LEO	Lemongrass essential oil

LG	Lemongrass
MAHD	Microwave-assisted hydrodistillation
MBC	Minimum bactericidal concentration
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
min	Minute
MPC	Milk protein concentrate
OD	Optical density
PA	Peha ayam
PALS	Phase-analysis light scattering
PDI	Polydispersity index
PI	Propidium iodide
PLA	Polylactic acid
RLU	Relative light unit
rpm	Revolutions per minute
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermis*
S. Typhimurium	*Salmonella* Typhimurium
SFE	Supercritical fluid extraction
SSC	Side scatter
TBC	Total bacteria count
TPC	Total plate count
TSA	Tryptic soy agar
U.S.	United States
UAE	Ultrasonic-assisted extraction
UPM	Universiti Putra Malaysia
USD	United States dollar
WHO	World Health Organization
ζ-potential	Zeta potential

Chapter 1. General introduction

1.0 Introduction

1.1 Relevance and Importance

Food safety continues to be a critical global concern, with foodborne pathogens posing significant threats to public health and economic stability. Among these, *Bacillus cereus* stands out due to its dual role as both a food spoilage organism and a causative agent of gastrointestinal illness (Ghosh et al., 2024). Frequently isolated from soil, raw produce, and food processing environments, *B. cereus* is especially problematic in ready-to-eat and starchy foods such as rice and non-starchy foods such as dairy products (Rahnama et al., 2023). Its ability to form dormant endospores represents a major challenge to food safety, as these spores are highly resistant to heat, desiccation, and conventional disinfectants (Cho & Chung, 2020). These spores allow them to survive common cooking and cleaning processes. Under favourable conditions, the spores germinate into vegetative cells that rapidly multiply and produce harmful toxins (Abee et al., 2011). Notably, *B. cereus* can produce two classes of toxins: an emetic toxin (cereulide) that induces vomiting, and a set of enterotoxins responsible for diarrheal symptoms (Yang et al., 2023). In addition to toxin production, its ability to form biofilms on food-contact surfaces contributes to its environmental persistence and resistance to standard sanitation procedures, increasing the risk of recurring contamination in food processing (Rouzeau-Szynalski et al., 2020).

In parallel with the rise in antimicrobial resistance and consumer rejection of synthetic preservatives, there is growing interest in natural antimicrobial agents as safer and more sustainable alternatives (Mesias et al., 2021). Plant-derived essential oils (EOs), particularly those rich in bioactive compounds, have demonstrated promising antimicrobial and antibiofilm properties (Alvarez-Martinez et al., 2021). However, their application in food systems is often hindered by challenges such as volatility, low water solubility, and strong sensory impacts that may affect product quality (Barradas & de Holanda e Silva, 2020). These limitations call for innovative formulation strategies to improve essential oil's efficacy, stability, and usability for food safety applications.

1.2 Why Lemongrass?

Lemongrass (*Cymbopogon citratus*) has emerged as a particularly promising natural antimicrobial due to its high content of citral, a compound widely recognized for its strong antimicrobial and antibiofilm properties against foodborne pathogens, including *B. cereus* (Mukarram et al., 2021). Traditionally, lemongrass has been used across Southeast Asia, including Malaysia, as a culinary herb and a remedy in traditional medicine and aromatherapy (Abdul Aziz et al., 2023). It also functions as a natural insect repellent (George et al., 2016). Despite its widespread use in daily life, its potential for industrial food preservation remains largely underutilised and underexplored in scientific research. As a tropical plant abundantly cultivated in Malaysia, lemongrass offers multiple practical advantages including affordability, local availability, and sustainability. Moreover, it is classified as Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA) (FDA, 2020), Rosol et al. (2023) further supporting its safety for food-related applications.

Unlike other essential oils with pungent or medicinal odors, lemongrass has a mild and pleasant citrus aroma, increasing its acceptance in consumer foods. Given the increasing demand for clean-label, plant-based antimicrobial solutions, lemongrass represents a valuable raw material for the development of natural preservatives. It also holds commercial promise to contribute to the growing halal-certified, health-focused product sector in Malaysia. The valorisation of lemongrass into high-value antimicrobial formulations could offer economic benefits for the agricultural and food technology sectors, aligning with national development goals for health, innovation, and sustainability.

1.3 Nanoemulsion as a Technological Solution

To overcome the functional limitations of raw essential oils, nanoemulsion technology has emerged as a cutting-edge strategy for enhancing the delivery and performance of natural antimicrobials (Hanan et al., 2024). Nanoemulsions are thermodynamically or kinetically stable colloidal dispersions consisting of nanoscale oil droplets (typically <200 nm) in an aqueous phase, stabilised by emulsifiers or surfactants (Kaur et al., 2024). This nano-scale formulation

enhances the solubility and dispersion of hydrophobic compounds, improves their bioavailability, offers controlled-release, and extends product shelf life (Tanuku et al., 2024). These advantages make nanoemulsions particularly attractive for food preservation, where rapid antimicrobial action and uniform distribution are essential.

Nanoemulsified essential oils demonstrate significantly improved antimicrobial and antibiofilm activity compared to their non-emulsified counterparts (Brito et al., 2024). However, the literature remains limited in terms of direct comparisons with standard natural preservatives such as nisin, particularly under biofilm-relevant conditions. Most existing studies focus on planktonic growth inhibition or shelf-life extension rather than the control of surface-associated biofilms on food-contact materials. For example, Gao et al. (2014) investigated the combined use of nisin and rosemary extract to improve microbial and physicochemical quality during chilled fish storage, yet did not assess biofilm formation or surface-associated microbial persistence. Similarly, current reviews and experimental studies acknowledge the antimicrobial potential of natural preservatives, but systematic, head-to-head comparisons of their antibiofilm efficacy remain limited (Sun et al., 2021).

In addition, limited attention has been given to how key physicochemical properties of nanoemulsions, including particle size, zeta potential, and polydispersity index, influence antimicrobial performance. Collectively, these gaps highlight the need for investigations that compare conventional natural preservatives with nanoemulsion-based systems and evaluate their practical applicability for biofilm control in food safety contexts.

1.4 Aim and Significance

This thesis aims to evaluate the antimicrobial and antibiofilm efficacy of lemongrass-based nanoemulsions against *B. cereus*, with a focus on both physicochemical characterisation and functional performance in planktonic and biofilm states. It seeks to explore the relationship between nanoemulsion formulation properties and biological activity, contributing theoretically to the

field of nano–bio interface research. Technologically, this study employs supercritical fluid extraction to optimise the recovery of bioactive compounds from lemongrass, combined with nanoemulsion techniques to enhance the stability, solubility, and antimicrobial action of the extract.

From an applied perspective, this research supports the development of clean-label, lemongrass-based antimicrobial solutions for food safety, addressing health and sustainability goals. By identifying, characterising, and applying lemongrass nanoemulsions, the study advances plant-based preservation strategies and offers insights for innovation in natural product development, food packaging, and antimicrobial policy particularly in Malaysia and Southeast Asia.

1.5 Research Questions, Objectives and Hypothesis

Research questions

1. How effective is supercritical fluid-extracted lemongrass oil in inhibiting *B. cereus* in different food matrices, and does emulsification improve its antimicrobial performance?
2. How effective are lemongrass and citral nanoemulsions in inhibiting planktonic and biofilm-associated *B. cereus* compared to nisin across different isolates?
3. What are the key physicochemical properties of lemongrass nanoemulsion, and how do they contribute to its stability and potential antimicrobial effectiveness?
4. What are the observed patterns of antimicrobial activity of lemongrass nanoemulsions against planktonic *B. cereus* cells, and what might these suggest about potential modes of action?

Research Objectives

1. To evaluate the antimicrobial efficacy of supercritical fluid-extracted lemongrass oil, in both raw and emulsified forms, against *Bacillus cereus* in selected food systems.
2. To characterise the key physicochemical properties of lemongrass nanoemulsion and examine their contribution to formulation stability and antimicrobial potential.

3. To investigate the antimicrobial and antibiofilm activities of lemongrass, citral nanoemulsions, and nisin against planktonic and biofilm-associated *B. cereus* across multiple isolates.
4. To explore the observed patterns of planktonic *B. cereus* inhibition by lemongrass nanoemulsion and identify potential antimicrobial modes of action based on concentration-dependent activity.

Research Hypotheses

1. Supercritical fluid-extracted lemongrass oil exhibits antimicrobial activity against *B. cereus*, with emulsified formulations showing enhanced efficacy compared to the raw extract in food matrices.
2. The physicochemical properties of lemongrass nanoemulsion such as particle size, polydispersity index (PDI), and zeta potential are positively associated with its stability and antimicrobial effectiveness.
3. Lemongrass and citral nanoemulsions demonstrate superior inhibitory effects on both planktonic and biofilm-associated *B. cereus* compared to nisin, across multiple clinical and reference isolates.
4. The antimicrobial activity of lemongrass nanoemulsion against planktonic *B. cereus* follows a concentration-dependent pattern, suggesting a multi-targeted mechanism involving membrane disruption and enzyme inhibition.

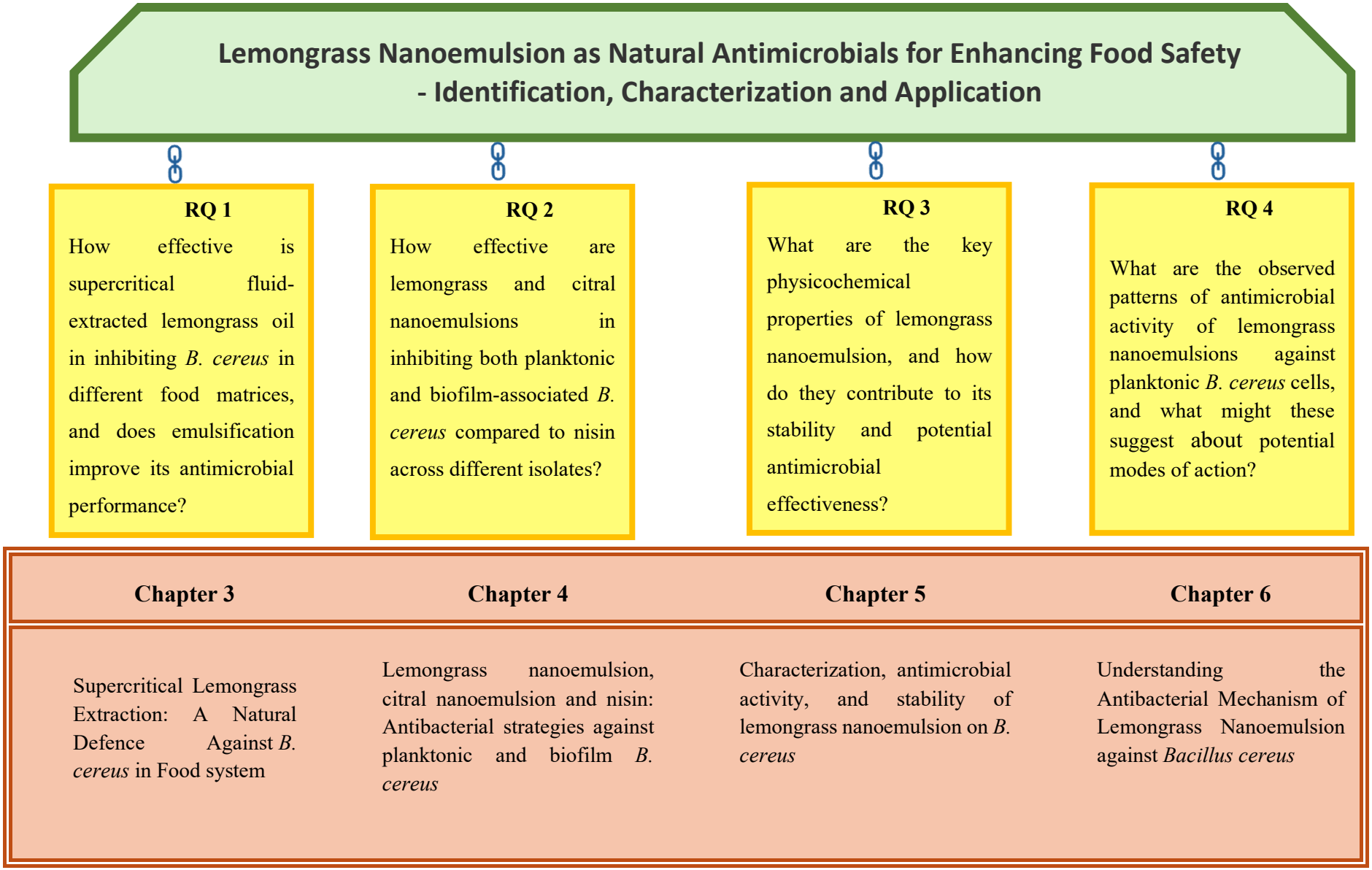

Figure 1.1. A schematic presentation of how the chapters is related to the research question in this study.

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Chapter 2. Literature review

2.1 Overview of Food Safety and the Need for Natural Antimicrobials

2.1.2 Common foodborne pathogens with a focus on *Bacillus cereus*

Food safety remains a critical global issue, with the World Health Organization (WHO) estimating that contaminated food causes approximately 600 million cases of foodborne illnesses and 420,000 deaths annually, with children under five accounting for nearly 40% of the disease burden (WHO, 2024). The challenges associated with food safety arise from multiple factors, including microbial contamination, chemical residues, and physical hazards, all of which compromise food quality and consumer health. Biofilms formed by foodborne pathogens such as *Listeria monocytogenes* on food contact surfaces act as persistent reservoirs of contamination, making them difficult to eradicate with conventional sanitisation methods (Mazaheri et al., 2021). These biofilms also contribute to antimicrobial resistance (AMR), further increasing the incidence and severity of foodborne outbreaks (Pai et al., 2023). With rapid population growth, urbanisation, and globalised food supply chains, ensuring access to safe and nutritious food has become increasingly challenging. Shifts in food production systems and dietary preferences toward minimally processed and ready-to-eat foods increase contamination risks (Bhatia et al., 2024).

Furthermore, emerging and re-emerging pathogens such as *Salmonella*, *L. monocytogenes*, *Escherichia coli,* and *Campylobacter* are evolving to exploit new ecological niches in food matrices (Newell et al., 2010). Climate change adds another layer of complexity by altering agricultural systems, increasing the presence of disease carriers, and enhancing the survival and transmission of foodborne pathogens across global supply chains (Bongben, 2024). Microbial contamination remains the most common cause of foodborne illnesses globally, contributing to significant public health and economic burden. Outbreaks frequently originate from contaminated fresh produce, dairy, and meat products, with pathogens such as *Norovirus*, *Salmonella*, and *B. cereus* among the leading culprits (Warmate & Onarinde, 2023). In the United States alone, the annual economic burden of foodborne illnesses is estimated at over USD 15 billion, accounting for medical costs, productivity losses, and legal liabilities (CDC, 2024).

In regions such as Southeast Asia, sub-Saharan Africa, and countries like Nepal, similar patterns are observed, particularly in low and middle-income settings, where inadequate sanitation, informal food markets, and weak regulatory oversight heighten food safety risks (Subedi et al., 2024; Wallace et al., 2022).

A key mechanism of microbial contamination is cross-contamination. The transfer of pathogens from raw ingredients, utensils, or contaminated surfaces to ready-to-eat foods during processing or handling (Pakdel et al., 2023). This is especially problematic in facilities with poor hygiene practices or inadequate equipment maintenance. For example, *L. monocytogenes* biofilms on stainless steel surfaces in processing plants have been shown to resist standard cleaning agents, allowing persistent contamination across production cycles (Lake et al., 2024). Likewise, fresh produce is frequently contaminated using irrigation water polluted with faecal matter, a common route for *E. coli* and *Salmonella* contamination (Gurtler & Gibson, 2022).

A key pathogen of interest is *B. cereus*, which is capable of withstanding heat treatments and surviving in adverse environmental conditions due to its spore-forming ability. Spores can persist in dry food products such as rice and pasta, and upon rehydration and inadequate storage, vegetative cells can proliferate and produce enterotoxins leading to vomiting or diarrhoea. A notable case occurred in 2021 in China, where rice noodles contaminated with *B. cereus* led to 198 reported cases of foodborne illness (Li et al., 2023). These cases highlight the need to enhance food safety systems through advanced detection methods, strict hygiene measures, and coordinated efforts across sectors.

Importantly, there is growing interest in natural antimicrobial solutions, including plant-derived compounds like essential oils, as sustainable alternatives to chemical preservatives and conventional sanitisation agents. These innovations may offer safer, more consumer-acceptable strategies for controlling microbial contamination and addressing antimicrobial resistance (Karnwal, 2024). Table 2.1 below summarises notable foodborne illness outbreaks from 2008 to 2023, illustrating the diversity of pathogens, contamination sources, and public health impacts across multiple countries.

Table 2.1. Notable foodborne illness outbreaks (2008–2023): Pathogens, Contamination Sources, and Public Health Impacts.

Year	Country/ Region	Pathogen	Source of Contamination	Impact	Reference
2017	Korea	*Vibrio parahaemolyticus*	Squid	237 symptoms, 53 hospitalisations	Jung (2018)
2017-2019	Canada	*Salmonella* spp.	Frozen raw breaded chicken	68 cases	Kerr et al. (2024)
2021	China	*B. cereus*	Poor storage of rice noodles	198 cases	Li et al. (2023)
2022	Australia	*Campylobacter*	Duck liver	20 cases	Mc Allistar et al. (2023)
2023	Indonesia	*E. coli*	Fried chicken	68 cases	Iskandar et al. (2025)
2024	USA	*L. monocytogenes*	Deli-sliced meat	61 cases	*Sharma et al. (2025)*

2.1.3 Pathogenic mechanisms of *B. cereus*

2.1.3.1 Biofilm formation and food industry implications

B. cereus is a Gram-positive, spore-forming bacterium commonly associated with foodborne illnesses due to its ability to persist in diverse environments. Its growth is influenced by temperature and pH, with most strains thriving between 30–40°C, though psychotropic strains can grow at temperatures as low as 4°C, posing a risk to refrigerated foods (Rodrigo et al., 2021). The optimum pH range for growth is between 5.0 and 8.0, while acidic conditions (pH <5) significantly reduce proliferation and toxin production (Wang, Liu, et al., 2024)

One of the critical survival mechanisms of *B. cereus* in food environments is its ability to form biofilms. These structured microbial communities are enclosed in a self-produced extracellular matrix that adheres tightly to surfaces such as stainless steel and plastic. Biofilms not only enable persistence under adverse conditions but also significantly reduce the efficacy of cleaning and sanitization protocols (Ghosh et al., 2024). Strain-dependent variability in biofilm architecture and extracellular

polymeric substance (EPS) composition influences resilience. For instance, strain ATCC 10987 exhibits unique responses to matrix-degrading enzymes, indicating the complexity and heterogeneity of biofilm structures (Lim et al., 2021). This ability to form resilient biofilms directly contributes to the widespread and persistent contamination observed across diverse settings. Table 2.2 summarises major reported cases of *B. cereus* contamination across food and clinical settings, highlighting the persistence of this organism in dairy and starchy foods.

Table 2.2. Reported *B. cereus* contamination events in food.

Year	Study/Case	Country	Source of Contamination	Impact	Reference
2012	Rice outbreak in primary school	Malaysia	Nasi kuning	33 from 188 had upper gastrointestinal symptom	Mohammad & Jeffree (2016)
2021	Foodborne outbreak in middle school	China	Rice noodle	198 cases	Li et al. (2023)
2021	Outbreak in boarding school	Malaysia	Beef rendang	152 cases	Bujang et al. (2023)
2023	Outbreak in school	Uganda	Canteen food	267 cases	Namara et al. (2025)
2024	Outbreak in elementary school	Indonesia	Snacks	12 cases	Hadika et al. (2024)

Antibiotic stress, particularly from aminoglycosides, can induce certain *B. cereus* strains to transition into small colony variant (SCV) phenotypes. It is characterised by slower growth, enhanced toxin production, and elevated antimicrobial resistance (Frenzel et al., 2015). A growing body of evidence indicates that biofilm formation in *B. cereus* is intricately linked with its sporulation capacity, with both processes governed by shared genetic pathways. Central to this regulation are genes such as *SpoVG*, *Spo0A*, and *comER*, which coordinate the initiation of biofilm formation and spore development. The SinI–SinR circuit, along with matrix-associated genes *tasA* and *sipW*, further contributes to biofilm structural stability and maturation (Caro-Astorga et al., 2020; Lin et al., 2022). Notably, several natural agents including citral and terpinen-4-ol—have demonstrated dual-action capabilities by disrupting quorum sensing, inhibiting sporulation-associated gene expression, and reducing biomass accumulation, thereby offering promising strategies for biofilm-targeted interventions in food safety systems (Kalia et al., 2023).

Beyond developmental regulators, quorum sensing (QS) plays a pivotal role in synchronising biofilm formation, virulence expression, and stress adaptation in *B. cereus*. Multiple QS systems, including the PlcR–PapR and LuxS/AI-2 pathways, interact with global transcriptional regulators to coordinate community-level behaviours essential for biofilm maturation. Xu et al. (2025) demonstrated that disruption of these QS circuits using sub-inhibitory concentrations of hordenine significantly suppressed QS gene expression and reduced toxin production, highlighting the importance of QS signalling in maintaining structured biofilms. Importantly, QS pathways intersect with Spo0A-centred regulation. Spo0A functions as a master developmental switch linking QS signals with cellular decisions governing biofilm formation and sporulation. Xu et al. (2017) showed that Spo0A functions as a master developmental regulator essential for structured biofilm formation in *B. cereus* AR156, acting through the *spo0A, sinI and sinR* regulatory circuit to drive matrix production, extracellular fibre assembly, and cellular differentiation required for mature biofilm architecture. Together, these findings suggest that QS signalling and Spo0A-mediated developmental pathways are tightly interconnected, with QS cues modulating Spo0A-dependent cell-fate decisions that balance biofilm maturation and sporulation.

Environmental stressors such as nutrient limitation, oxidative stress, and antimicrobial exposure activate upstream regulators including AbrB, SpoVG, and ComER, modulating the phosphorylation and activation of Spo0A (Huang et al., 2021; Marathe et al., 2023). Once activated, phosphorylated Spo0A (Spo0A~P) simultaneously promotes biofilm matrix production and initiates sporulation, a dual role reinforced through the SinI–SinR system and matrix proteins TasA and SipW (Bianco et al., 2020; Lin et al., 2022). Mature biofilms therefore act not only as protective niches for vegetative cells but also as microenvironments conducive to spore formation, particularly under harsh food-processing conditions (Marmion et al., 2022). Figure 2.1 summarises the synergistic relationship between biofilm formation and sporulation in *B. cereus* under environmental stress.

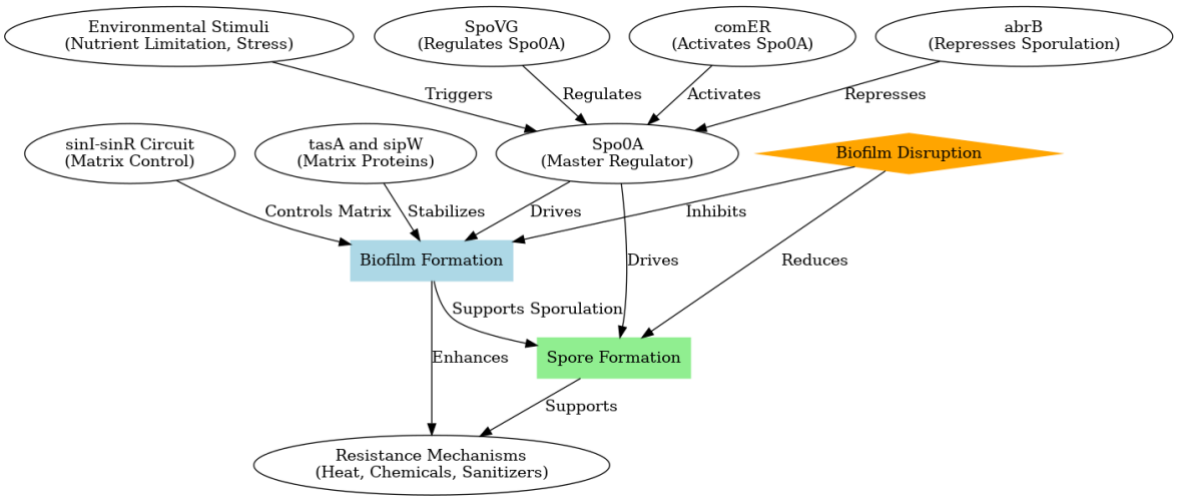

Figure 2.1. Regulatory network linking biofilm formation and sporulation in B. cereus. This figure was created using BioRender (https:/biorender.com).

Crucially, interference with QS signalling or Spo0A-centred regulatory pathways compromises biofilm integrity, disrupts sporulation efficiency, and reduces long-term persistence of *B. cereus* in food-related environments (Lin et al., 2022). In this context, natural compounds such as citral and terpinen-4-ol have emerged as promising dual-action agents capable of attenuating QS activity, downregulating sporulation-associated gene expression, and reducing biofilm biomass (Kalia et al., 2023; Almatroudi, 2024). Targeting the interconnected QS–Spo0A regulatory axis

therefore represents a strategic approach for mitigating *B. cereus* biofilms and enhancing food safety through integrated antimicrobial interventions.

2.1.3.2 Spore formation and thermal resistance

Spore formation in *B. cereus* serves as a key survival strategy, allowing the bacterium to endure extreme environmental conditions. Spores are highly resistant to heat, ultraviolet radiation, desiccation, and food preservatives such as nitrites, sulfites, and organic acids. This resistance is largely due to their specialized structure, including a thick spore coat, cortex, and inner membrane, which collectively protect the spore's core components (Zegeye et al., 2021).

Recent studies show that the environment in which spores are formed can influence how resistant they become. Kim et al. (2024) found that when *B. cereus* sporulates in media with higher NaCl concentrations, the resulting spores have lower heat resistance. They also observed that high-salt conditions reduce endospore hydrophobicity, membrane fluidity, and spore density. This suggests that salinity disrupts key structural and physicochemical features essential for maintaining spore robustness.

In addition, the location of the spores also affects their resistance. Studies have shown that spores embedded within biofilms possess greater resistance to heat treatments compared to their planktonic counterparts. The biofilm matrix acts as a physical barrier, shielding the spores from heat and sanitizers, thereby preserving their viability. This finding underscores the importance of biofilm control strategies in mitigating spore-mediated contamination in food processing environments (Liu et al., 2023).

Given the variability in strain behaviour and the global distribution of *B. cereus*, regulatory guidelines vary across regions. Table 2.3 presents selected international standards and regulatory thresholds for acceptable *B. cereus* levels in food.

Effective *B. cereus* control in food systems must integrate environmental monitoring, advanced cleaning technologies, and molecular detection methods for high-risk strains. Harmonisation of international standards remains a long-term

goal, yet practical progress has been made through the adoption of molecular surveillance tools in countries like China, Poland, Australia, New Zealand, America and within the EU

Table 2.3. International regulatory limits on *B. cereus* contamination

Region/Country	Threshold (CFU/g)	Details	Reference
European Union	$\leq 10^3$	Limit applies to ready-to-eat foods at end of shelf life to prevent toxin production.	Berthold-Pluta et al. (2019)
United States	$\geq 10^5$	The United States does not specify a regulatory limit for *B. cereus*; values shown reflect levels commonly associated with illness in outbreak investigations.	McDowell et al. (2023)
China	10^3–10^5	No official threshold: virulence genes are monitored using molecular tools.	Gao et al. (2018)
Australia/New Zealand	$\leq 10^3$	Regulatory agencies reject foods above this level.	FSANZ, (2013)
Poland	$\leq 10^3$	Emphasis on routine monitoring of processed food for spore-forming bacteria.	Berthold-Pluta et al. (2019)
Singapore	$< 10^2$	Microbiological standards for ready-to-eat-food must be less than this level.	Rusnan et al. (2020)

2.1.4 Limitations of synthetic preservatives and demand for natural alternatives

Traditional food preservation techniques such as chemical preservatives, thermal processing, and refrigeration have long been fundamental to maintaining food safety, extending shelf life, and preserving food quality. Chemical preservatives like nitrites, sulfites, and sorbates inhibit microbial growth and delay spoilage. Similarly, pasteurization and sterilization eliminate pathogens and enzymes, while refrigeration reduces microbial activity to prolong freshness (Ariyamuthu et al., 2022).

However, these methods are increasingly questioned due to health, regulatory, and sustainability concerns. Nitrites have been linked to carcinogenic nitrosamine formation, while sulphites can trigger allergic reactions in sensitive individuals (Shakil et al., 2022). Thermal treatments may degrade heat-sensitive nutrients (e.g., vitamin C) and alter flavour or texture, potentially reducing consumer appeal. Refrigeration, although effective, demands high energy use, which poses challenges in resource-limited or rural areas (Lisboa et al., 2024). Consumer preferences have shifted notably in recent years, favouring "clean label" products that exclude synthetic additives. This shift is driven by growing awareness of health impacts, environmental sustainability, and interest in minimally processed foods (Asioli et al., 2017). Natural preservation methods, particularly those involving plant-derived compounds, antimicrobial peptides, and non-thermal technologies like supercritical fluid (SFE) extraction are now widely explored as alternatives (Li et al., 2024).

Studies across various sectors support this trend. In the meat industry, polyphenol-rich plant extracts are being adopted to replace synthetic preservatives (Beya et al., 2021). In seafood, natural agents such as essential oils and chitosan extend shelf life while satisfying consumer demands for chemical-free preservation (Olatunde & Benjakul, 2018). While some natural preservatives may cause minor sensory changes, consumer acceptance generally remains high especially when the health and environmental benefits are communicated clearly. For example, propolis and spice extracts introduced slight variations in colour or flavour in meat products but did not significantly affect consumer acceptance when used at optimal

concentrations (Pobiega et al., 2019; Procopio et al., 2022). Natural preservatives sourced from plants (e.g., citral from lemongrass), animals (e.g., lysozyme, chitosan), and microbes (e.g., nisin) are often biodegradable, broadly antimicrobial, and less likely to promote resistance. Many also offer added antioxidant and sensory benefits, enhancing both food safety and quality (Barberis et al., 2018; Quinto et al., 2019; El-Saber Batiha et al., 2021). Figure 2.2 provides an overview of the classification of food preservation highlighting synthetic and the diversity of natural preservatives.

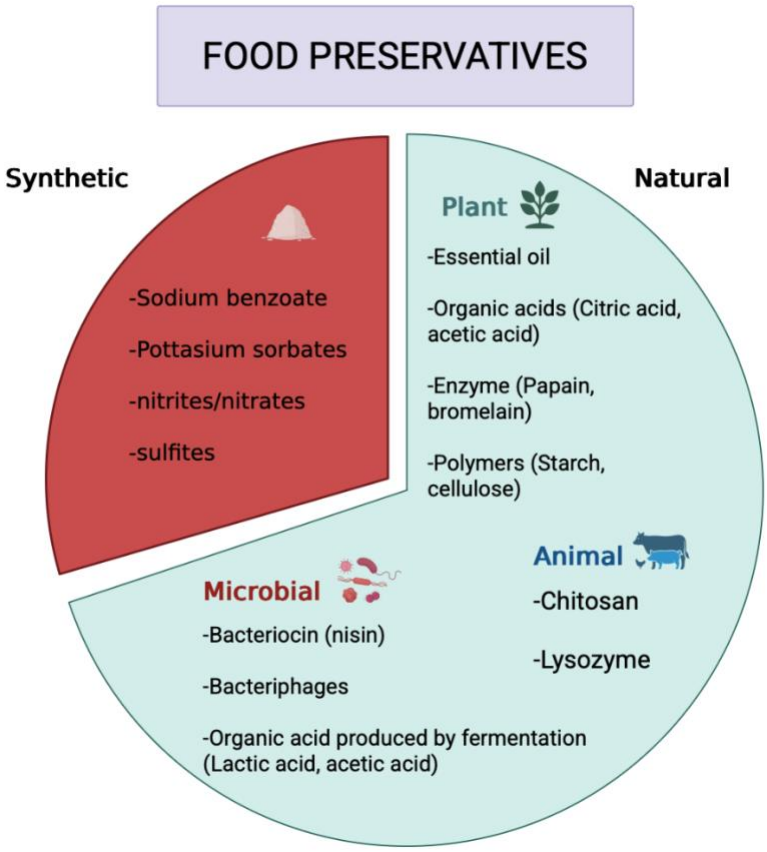

Figure 2.2. Classification of food preservatives. This figure was created using BioRender (https:/biorender.com).

The growing limitations of synthetic preservatives combined with shifting consumer demands for safe, natural, and sustainable food systems have accelerated research into natural alternatives Given these limitations and consumer trends, there is growing interest in plant-derived preservatives such as lemongrass, which

offers promising antimicrobial potential alongside cultural familiarity and wide availability in regions like Malaysia.

2.2 Lemongrass *(C. citratus)*: Botanical and Ethnopharmacological Background

2.2.1 Botanical description, distribution, and cultivation in Malaysia

C. citratus, (Figure 2.3), commonly known as lemongrass, is a perennial, aromatic grass belonging to the *Poaceae* family, which includes over 600 genera and 10,000 species (Aćimović et al., 2019). It grows in dense clumps, reaching up to 2 meters in height, with long, narrow, linear leaves and a rhizomatous root system. The plant releases a strong lemon-like fragrance due to the presence of citral, a key bioactive compound known for its potent antimicrobial properties (Kamaruddin et al., 2021; Shelar et al., 2023). Owing to these attributes, lemongrass is widely valued for its use in culinary applications, traditional herbal medicine, aromatherapy, and essential oil extraction (Ashaq et al., 2024).

Figure 2.3. Lemongrass farm in Beranang, Selangor, Malaysia

Although lemongrass is commonly believed to have originated in India and Sri Lanka, several sources suggest Malaysia as a possible native region due to its long-

standing use and cultivation (Majewska et al., 2019). Today, lemongrass is extensively cultivated in tropical and subtropical regions, including Southeast Asia, Central and South America, Africa, and the Indian Ocean islands (Wifek et al., 2016). In Malaysia, it is grown for both domestic and commercial reasons, particularly in the states of Johor, Pahang, and Negeri Sembilan. The plant thrives in warm, humid climates with well-drained soils and full sunlight exposure, and it is typically propagated through vegetative division of clumps rather than seeds (Shahrul, 2022).

Given Malaysia's favourable climate and established agricultural practices, the country has seen progressive expansion in lemongrass cultivation over recent years, both in terms of land area and production output. According to the Department of Agriculture Malaysia's Agrofood Statistics Reports, lemongrass cultivation has shown steady growth in both planted area and production volume from 2017 to 2023. In 2023 alone, the total planted area reached 2,002.6 hectares, with a production output of 23,089.1 metric tons and an average yield of 13.6 metric tons per hectare. Notably, the production value in 2023 was estimated at RM 85.6 million, reflecting a strong market demand for lemongrass as both a culinary and commercial crop. Table 2.4 presents a year-by-year breakdown of cultivation area, yield, production volume, and economic value over the seven-year period (KPKM, 2023).

Lemongrass is also widely recognized under various common names reflecting its cultural reach: "lemongrass" or "West Indian lemongrass" in English, "citronnelle" in French, "hierba limón" in Spanish, "capim-santo" in Portuguese, "xiang mao" in Chinese, and "serai" in Malay. This diversity in nomenclature signifies the plant's broad global relevance in culinary and medicinal contexts (Rojas-Sandoval, 2016).

Table 2.4. Lemongrass cultivation area, production volume, and economic value in Malaysia (2017–2023).

Year	Planted Area (ha)	Production Area (ha)	Average Yield (mt/ha)	Production (mt)	Production Value (RM '000)
2017	1 818.0	1 493.6	9.2	13 674.2	40 019.90
2018	1 594.4	1 297.3	9.8	12 767.7	29 365.75
2019	1 418.5	1 274.0	9.7	12 332.0	30 213.34
2020	1 695.3	1 673.1	9.5	15 818.0	36 381.39
2021	1 981.3	1 840.5	10.1	18 585.0	40 886.99
2022	1 715.8	1 532.8	10.7	16 404.7	37 687.95
2023	2 002.6	1769.5	13.6	23.980.1	85 568.26

2.2.2 Traditional culinary and medicinal uses of lemongrass

Lemongrass is a versatile herb widely used across Southeast Asian cuisines, particularly in Malaysia, Indonesia, and Thailand. Its distinctive citrus aroma and flavour enhance a variety of culinary preparations, including soups, curries, stir-fries, and marinades. Notably, it features prominently in signature regional dishes such as *tom yum* and *tom kha kai*, where its bruised stalks are simmered to infuse the broth with aromatic oils (Gaba et al., 2020). Beyond its culinary appeal, lemongrass is also traditionally consumed as a herbal infusion and often prepared with ginger or mint to aid digestion, relieve bloating, and alleviate symptoms of colds and flu (Kassahun et al., 2020).

The medicinal significance of lemongrass is equally notable. In traditional medicine systems across Southeast Asia, lemongrass has long been used as a folk remedy for multiple therapeutic purposes, including coughs, fever, gingivitis, headaches, leprosy, malaria, respiratory infections, and vascular disorders (Gaba et al., 2020). In Malaysia and Indonesia, a decoction made by boiling lemongrass is traditionally consumed to enhance blood circulation, particularly in the pelvic and uterine regions (Kassahun et al., 2020). In addition, the herb is widely used as a natural insect repellent and insecticide, particularly in rural communities where chemical alternatives are less accessible (Kassahun et al., 2020).

Recent ethnobotanical studies from India highlight lemongrass's broad-spectrum medicinal potential. Traditionally, it is used to treat gastrointestinal issues such as stomach aches, constipation, and diarrhoea. Its antipyretic and profuse sweating effects make it effective for reducing fevers by promoting perspiration (Mukherjee et al., 2024). The essential oil derived from lemongrass has demonstrated significant antimicrobial, antifungal, and anti-inflammatory activity, underscoring its importance across various traditional healing systems (Boukhatem et al., 2014).

2.3 Bioactive Properties of Lemongrass Essential Oil

2.3.1 Antimicrobial activity against foodborne pathogens

Lemongrass essential oil (LEO) demonstrates potent antimicrobial activity, primarily due to its high citral content a mixture of the isomer's geranial and neral. Citral disrupts bacterial cytoplasmic membranes, increases membrane permeability, and causes intracellular leakage, leading to rapid cell death (Valková et al., 2022). This activity is particularly effective against Gram-positive foodborne pathogens such as *B. cereus* and *Staphylococcus aureus*. Although clove oil demonstrated greater overall antimicrobial potency than citral in comparative assays, citral exhibited stronger inhibitory activity against the Gram-positive *B. cereus* than against the Gram-negative *E. coli* (Gutierrez-Pacheco et al., 2023). Its preferential efficacy against Gram-positive bacteria is attributed to their simpler cell wall structure, in contrast to the outer lipopolysaccharide layer of Gram-negative bacteria, which impedes the penetration of hydrophobic essential oil components (Budiati et al., 2018; Subramaniam et al., 2020).

In addition to inhibiting planktonic cells, citral has also been shown to suppress spore germination and vegetative outgrowth of *B. cereus*, further supporting its role as a natural preservative in high-risk foods (Wang, Rui, et al., 2024). In dairy applications, LEO has shown moderate antimicrobial activity against multidrug-resistant strains such as *E. coli*, *Staphylococcus* spp., and *Enterobacter*, although its effectiveness against *Klebsiella* remains limited (Yasir et al., 2022).

Mechanistically, citral exerts its effects through multiple cellular targets. It disrupts the cytoplasmic membrane (1), causes leakage of intracellular contents (2), interferes with DNA replication and transcription (3), and induces protein

denaturation (4). Enzymatic inhibition (5) and impaired protein synthesis (6). In Gram-negative bacteria, citral can penetrate the lipopolysaccharide layer or form pores in the outer membrane (7), allowing access to internal cellular components (Shi et al., 2016; Gutierrez-Pacheco et al., 2023). These cumulative actions explain the antimicrobial efficacy of LEO against a broad range of pathogens. The proposed cellular mechanisms are visually summarised in Figure 2.4.

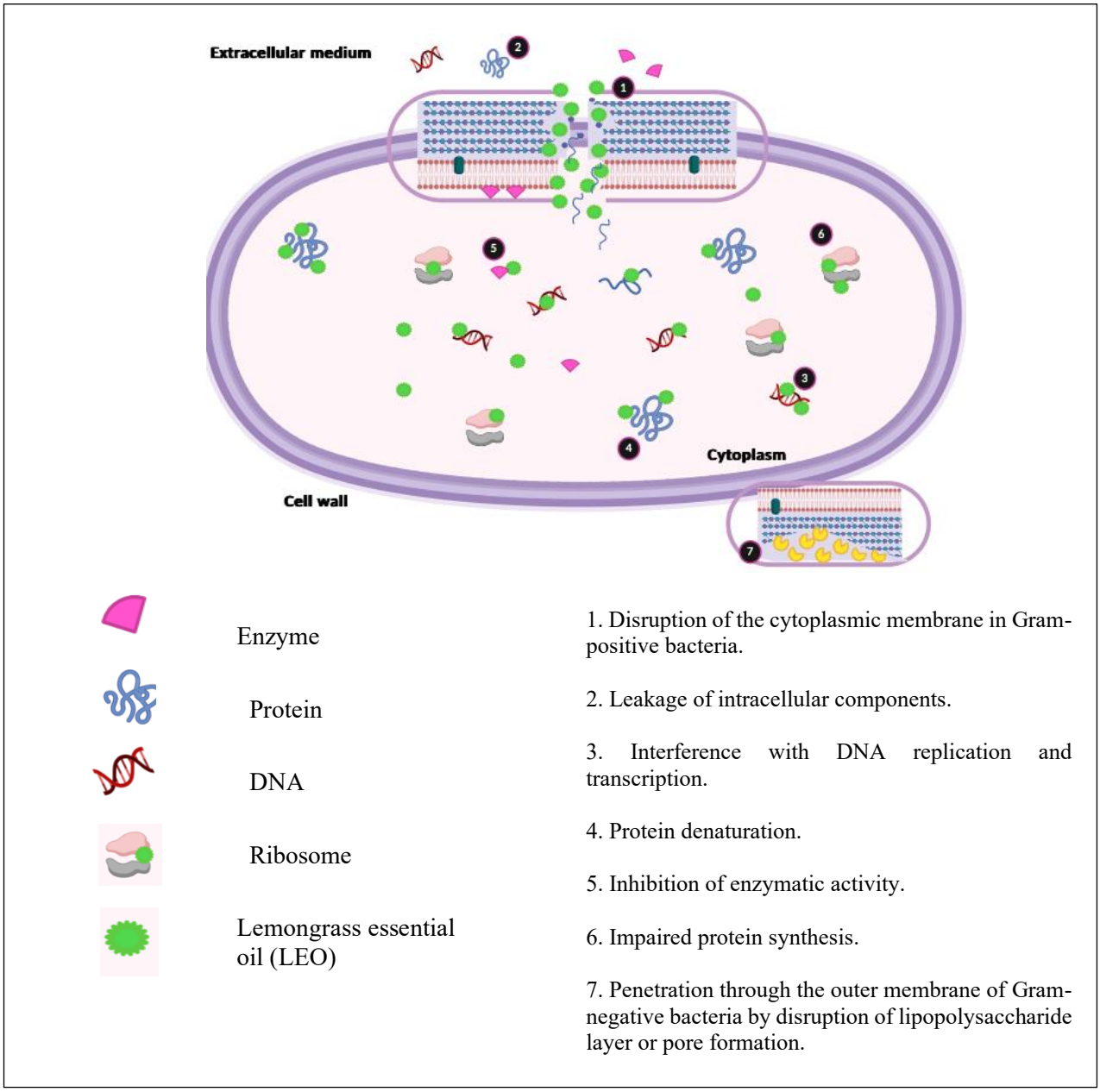

Figure 2.4. Proposed mechanism of antimicrobial action of LEO against Gram-positive and Gram-negative bacteria. This figure was created using BioRender (https:/biorender.com).

2.3.2 Anti-biofilm activity of lemongrass oil

Biofilm formation is a key survival strategy for many foodborne pathogens, including *B. cereus*, enabling persistence on food contact surfaces and resistance to sanitization. LEO, rich in citral, has shown promising anti-biofilm properties, including disruption of biofilm matrix structure and inhibition of quorum sensing signals essential for microbial communication and virulence (Mukarram et al.,

2021). These stage-specific anti-biofilm actions are schematically illustrated in Figure 2.5.

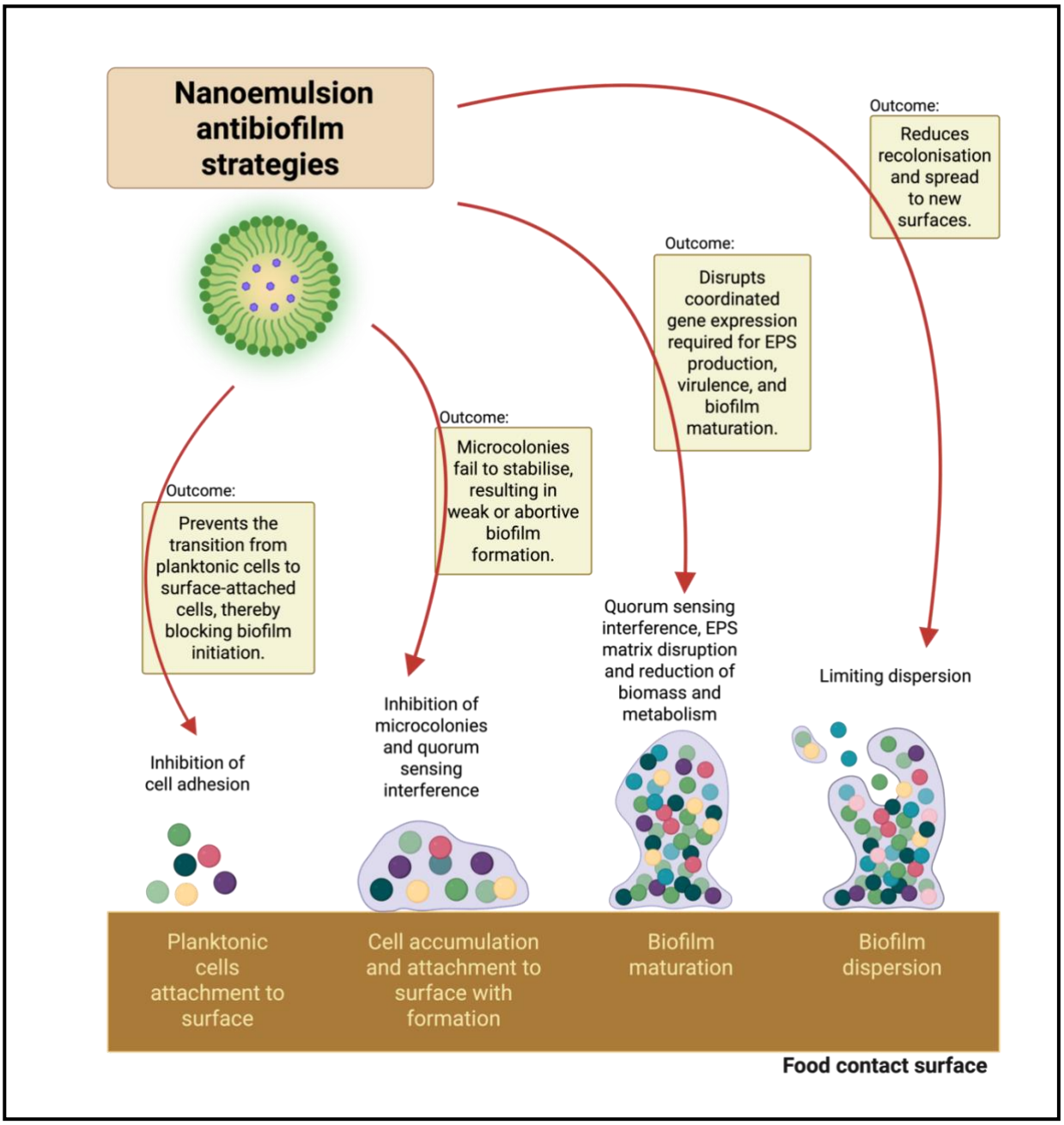

Figure 2.5. Schematic representation of nanoemulsion-mediated anti-biofilm strategies of lemongrass essential oil. This figure was created using BioRender (https:/biorender.com).

Studies have demonstrated that LEO can significantly inhibit early biofilm development and reduce biomass. Gao et al. (2020) reported that citral

concentrations as low as 0.0781% effectively inhibited the planktonic growth of *S. aureus*, *Candida albicans*, and *C. tropicalis*, while 0.3125% LEO disrupted dual-species biofilms by targeting biofilm structural integrity and signalling pathways. Supporting these findings, Karimou et al. (2024) further demonstrated the anti-biofilm potential of lemongrass extracts. In their study, aqueous extracts of lemongrass inhibited biofilm formation of *E. coli* by up to 50.09% and *Staphylococcus* spp. by up to 48.56%. Notably, the highest inhibition against *E. coli* (50.09%) and *Staphylococcus* spp. (48.56%) was observed with the aqueous lemongrass extract, whereas ethanolic extracts were generally less effective. Adukwu et al. (2012) further highlighted citral's potent activity, observing complete inhibition of *S. aureus* biofilm formation at concentrations as low as 0.060–0.125% (v/v). However, LEO was less effective in eradicating mature, established biofilms, indicating its role may be more preventive than curative. Interestingly, while citral alone showed strong inhibition zones (>8.6 cm), whole LEO sometimes performed better due to synergistic effects among minor components.

Combination treatments have also shown promise. Oliveira et al. (2010) reported that a mixture of *C. nardus* and lemongrass oils eliminated adhered cells in 240 h old biofilms after only 60 min of contact, demonstrating enhanced efficacy in complex matrices. Nonetheless, results can vary across species and concentrations. Leonard et al. (2010) showed that while certain essential oils reduced *Listeria monocytogenes* biofilm biomass, lemongrass oil was also associated with biofilm enhancement rather than inhibition. These contrasting findings emphasise the need for careful optimisation of essential oil selection and application in food systems, as inappropriate use may unintentionally promote biofilm development. Despite its generally higher efficacy against bacterial biofilms, fungal biofilms tend to be more resilient to LEO. Andrade-Ochoa et al. (2021) reported that fungi were more resistant compared to bacterial species, while Adebayo & Osuolale (2024) confirmed the antifungal efficacy of LEO against *Candida* species, further supporting its broad-spectrum application in food spoilage prevention.

Lemongrass oil's ability to interfere with biofilm development and quorum sensing offers a promising natural strategy for controlling *B. cereus* contamination in food

systems. Its preventative role is particularly valuable in environments prone to biofilm accumulation, such as dairy processing lines, packaging surfaces, and ready-to-eat food facilities.

2.3.3 Anti-spore effects and food preservation potential

Bacterial spores, particularly those of *B. cereus*, present significant challenges in food safety due to their resilience against conventional inactivation methods. Traditional approaches such as high-pressure processing, irradiation, and recent treatment cold plasma low pressure, are often effective but can compromise food quality and nutritional value (Kim et al., 2019; Mok et al., 2022; Valdez-Narvaez et al., 2024). Consequently, natural antimicrobials like LEO are gaining attention as safer, more sustainable alternatives.

In laboratory settings, LEO has demonstrated promising anti-bacterial spore activity. Schweitzer et al. (2022) reported that citral, the major component of LEO, significantly reduced the germination and outgrowth of *B. thuringiensis* spores by compromising membrane integrity and inhibiting early germination events.

2.4 Extraction and Characterisation of Lemongrass Essential Oil

2.4.1 Role and biosynthesis of citral in lemongrass

From a chemical perspective, citral is an acyclic monoterpene aldehyde that exists as a mixture of two geometric stereoisomers: geranial, the trans (E) isomer also known as citral A, and neral, the cis (Z) isomer referred to as citral B (Figure 2.6). Structurally, citral contains both hydrophobic and polar functional regions, a characteristic that may facilitate its interaction with microbial membranes and contribute to its antimicrobial activity. Consistent with this molecular structure, citral is predominantly lipophilic and highly volatile, being readily soluble in alcohols and oils but poorly soluble in water and most biological media. In addition, citral exhibits limited stability when exposed to light, which can lead to chemical degradation and a consequent reduction in bioactivity (Bailly, 2020).

Beyond its physicochemical characteristics and extraction sensitivity, citral also serves a distinct biological function within the plant. It functions as a secondary metabolite involved in defence mechanisms against herbivores, pathogens, and

environmental stressors (Li et al., 2025). Jiang et al. (2022) reported that citral from *Litsea cubeba* induced significant plant disease resistance activity at 68.20% of 500 µg/mL. Its presence contributes to the plant's chemical protection system while also playing a role in ecological interactions such as deterrence of herbivory and attraction of beneficial organisms (Hu et al., 2025).

In *C. flexuosus*, citral accumulates in specialized secretory cells within the leaf mesophyll, as demonstrated by histochemical staining. These cells serve as localized sites for citral storage, enabling high essential oil content without disrupting primary metabolic processes (Ganjewala et al., 2010). The high abundance of citral in lemongrass oil distinguishes it from many other aromatic plants and underpins its commercial value.

Chemically, citral is a mixture of two geometric isomers, geranial (trans-citral) and neral (cis-citral), both of which contribute to its characteristic aroma and biological activity (Solon et al., 2025). Biosynthetically, citral originates from the universal monoterpene precursor geranyl diphosphate (GPP), which is produced through the plastidial methylerythritol phosphate (MEP) pathway. In this pathway, the isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are condensed by geranyl diphosphate synthase to form GPP. Subsequently, GPP is converted to geraniol through the action of geraniol synthase, followed by oxidation mediated by alcohol dehydrogenases to yield the aldehydes geranial and neral, collectively referred to as citral. This biosynthetic sequence underpins the accumulation of citral in lemongrass tissues and explains its predominance among the plant's volatile constituents (Hu et al., 2025).

The concentration and composition of citral in lemongrass are strongly influenced by both genetic background and environmental conditions. At the cultivar level, Tomar et al. (2025) showed evidence of genotype-dependent differences in citral accumulation, reporting that cultivars such as CIM-Suwarna and Nima contained the highest citral proportions (>50%), whereas CIM-Shikhar and Pragati showed comparatively lower levels, highlighting strong genetic control over citral biosynthesis. In addition to genotype, plant developmental stage further affects oil

quality, as younger leaf tissues typically accumulate greater essential oil yields and higher citral concentrations than older or senescent tissues.

Recent work by Xu et al. (2025) demonstrated that radiation intensity significantly influences both oil yield and citral content in *C. citratus*, with leaf tissues responding more sensitively than stems. Increased light exposure enhanced monoterpene accumulation, including citral, whereas reduced radiation intensity led to lower oil yield and altered compositional profiles. Field-based evidence from Mwithiga et al. (2022) similarly demonstrated that agro-ecological conditions strongly affect citral concentration, with lemongrass grown in different ecological zones exhibiting substantial variation in citral content (54.80–71.45%). Notably, oils from upper and lower zones attained citral levels within the optimal range for commercial applications, whereas intermediate zones showed reduced citral proportions.

In addition to spatial environmental variation, harvest timing and plant maturity are critical determinants of oil quality. Costa et al. (2016) demonstrated that seasonal factors and leaf quality significantly influence secondary metabolite accumulation in *C. citratus*, with younger, high-quality leaves harvested during periods of optimal sunlight exhibiting superior phytochemical profiles. Complementing this, Krishna Veni et al. (2023) showed that extended harvesting intervals (approximately 100–110 days) maximised essential oil yield in *C. flexuosus*, reflecting the combined effects of biomass accumulation and developmental stage on oil production. Together, these findings indicate that light availability, temperature, soil conditions, water status, plant maturity, and harvest timing interact to regulate citral biosynthesis and stability, underscoring the importance of optimised agronomic and post-harvest practices for maximising citral yield and quality.

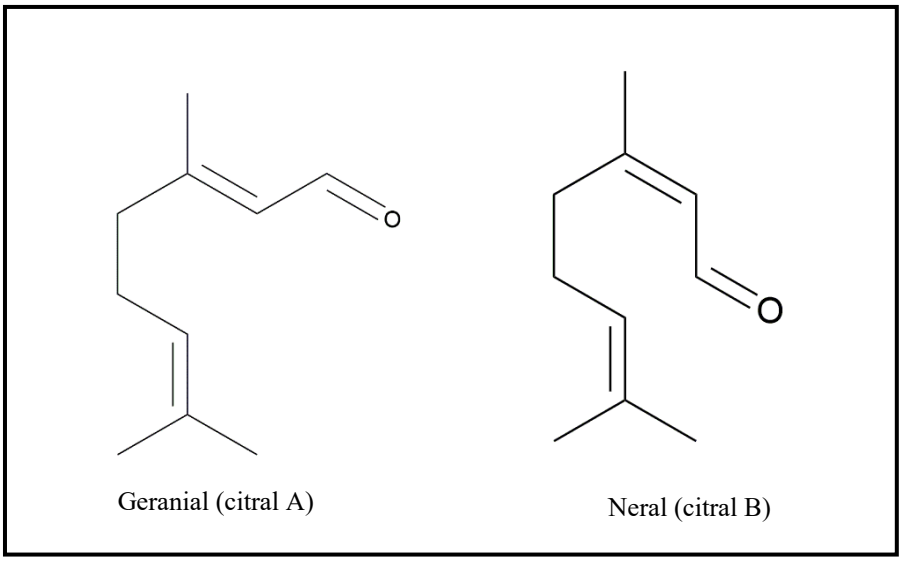

Figure 2.6. Molecular structure of citral, an acyclic monoterpene aldehyde composed of two geometric isomers: geranial (citral A) and neral (citral B).

2.4.2 Overview of conventional and modern extraction methods

The extraction of LEO has undergone significant advancements to improve yield, preserve bioactive compounds, and enhance sustainability. Among the traditional methods, steam distillation remains the most widely used due to its simplicity, low cost, and environmental friendliness. It is particularly favoured for large-scale production as it effectively extracts essential oils without altering product quality. However, steam distillation can be time-consuming and may cause degradation of heat-sensitive components (Machado et al., 2022). Soxhlet extraction, another conventional approach, uses solvents such as ethanol to extract oil from plant materials. This method generally achieves higher yields than steam distillation but is limited by its long extraction time and high solvent consumption, which raises environmental and safety concerns (Okonkwo & Ohaeri, 2019).

To address these limitations, modern extraction techniques have been developed. Microwave-assisted hydrodistillation (MAHD) utilizes microwave energy to heat plant material rapidly and evenly, improving extraction efficiency and preserving volatile constituents. It has been shown to significantly reduce processing time while yielding essential oils of similar quality than conventional methods (Ghazanfari et al., 2020). Another technique, ultrasound-assisted

extraction (UAE)**,** employs ultrasonic waves to disrupt plant cell walls, facilitating the release of intracellular oils. UAE has proven particularly effective in increasing extraction yields and reducing solvent usage (Shen et al., 2023).

In recent years, SFE using carbon dioxide (CO_2) has gained prominence as a green technology for essential oil extraction. Supercritical CO_2 is non-toxic, non-flammable, chemically inert, and readily recyclable, making SFE a more sustainable and environmentally friendly alternative to conventional solvent-based extraction methods and enabling efficient recovery of target compounds without harmful solvent residues (Herzyk et al., 2024). Importantly, the physicochemical properties of supercritical CO_2 are highly tunable, increasing extraction pressure enhances CO_2 density, thereby improving its solvating power and the solubility of non-polar and moderately lipophilic compounds, including essential oils and hydrophobic bioactives (Molino et al., 2019). Consistent with these advantages, Ashaq et al. (2024) demonstrated that optimised SFE conditions resulted in efficient extraction of LEO with minimal thermal degradation, yielding citral-rich extracts suitable for food and pharmaceutical applications. Figure 2.7 presents an example of a SFE machine used at Universiti Putra Malaysia (UPM), illustrating the typical setup employed for essential oil extraction.

Figure 2.7. Supercritical fluid extraction machine

The choice of extraction method affects the chemical profile and bioactivity of LEO. Parameters such as extraction time, temperature, solvent type, and plant material characteristics must be carefully optimized to meet industrial and therapeutic objectives. Recent studies emphasize that the application of modern, sustainable extraction techniques not only improves efficiency and safety but also enhances the functional properties of essential oil (Hedayati et al., 2025).

2.4.3 **Influence of the extraction technique on citral content and bioactivity**

The efficiency of extracting citral from lemongrass is strongly influenced by the extraction technique used, which also affects the oil's overall bioactivity. Citral, comprising the isomers neral and geranial, is a key component contributing to LEO's antimicrobial and antioxidant properties. Among the advanced techniques, ultrasonic-assisted extraction has been shown to produce high citral content, with Yuniarto et al. (2022) reporting a yield of 83.15%, while microwave-assisted hydrodistillation yielded 68.32% of citral. SFE remains one of the most effective methods, achieving up to 84.95% citral content likely due to its low thermal degradation and efficient penetration of plant matrices (Carlson et al., 2001). In contrast, traditional hydrodistillation methods yield significantly lower citral concentrations. For instance, Jaleel et al. (2017) reported a citral content of only 43.10%, which may be attributed to thermal decomposition during prolonged heating. Vacuum distillation offers a moderate yield, with Viktorova et al. (2020) reporting a citral concentration of 63.00%, suggesting that reduced pressure can minimize degradation but may not maximize extraction efficiency. These findings collectively highlight that advanced, non-thermal extraction methods such as supercritical fluid and ultrasonic-assisted techniques not only preserve citral content more effectively but also enhance the potential bioactivity of the essential oil.

2.4.4 Stability and physicochemical profile of the extracted oil

The stability of LEO, particularly its major active component citral, is influenced by environmental factors such as light, heat, oxygen, and moisture. These factors can lead to chemical degradation through oxidation, polymerization, isomerization, or hydrolysis, ultimately compromising the oil's therapeutic efficacy and sensory

attributes. Studies have shown that prolonged storage reduces citral content; for instance, Akinkunmi et al. (2016) reported a drastic decline in citral content from 84.32% in fresh LEO to 2.39% after nine years of storage, along with reduced antimicrobial activity. The deterioration occurs because the chemical constituents of essential oils are prone to various reactions, including oxidation, isomerisation, polymerisation, disproportionation, cyclisation, and dehydrogenation. Interestingly, new compounds were detected in the aged oil, indicating chemical changes over time. These findings highlight the need for proper storage such as protection from light and storage at low temperatures to preserve LEO's therapeutic quality (Rowshan et al., 2013; Turek & Stintzing, 2012).

Changes in citral content due to ageing and exposure to heat or light underscore the importance of controlled storage. Jenny et al. (2019) evaluated the physical stability of LEO-based microemulsions stored at 4°C and 60°C. The formulations maintained physical integrity over several months with no phase separation or creaming observed, though pH levels (4.1–5.1) suggested bacterial growth inhibition rather than fungal protection. Additionally, citral was shown to be more susceptible to degradation under acidic conditions. This is because low pH environments promote acid-catalyzed reactions such as isomerization and cyclization, leading to a breakdown of citral's chemical structure (Maswal & Dar, 2013).

Given citral's sensitivity to environmental degradation, ensuring the stability of essential oils is crucial for maintaining their bioactivity and shelf life. Nanoemulsions and encapsulation technologies have emerged as effective strategies to protect essential oils from heat, light, and oxidation. Their small droplet size and large surface area help entrap volatile compounds, reducing degradation. Ganosi et al. (2023) highlighted that such systems also enhance solubility and permeability, while extending shelf life. However, their effectiveness depends on formulation choices, particularly the type and concentration of surfactants and emulsifiers, which influence droplet size and system stability. Barradas & de Holanda e Silva (2020) noted, even under controlled storage, minor compounds may still degrade if the formulation is not fixed to the optimal standard.

Therefore, optimal design of encapsulation systems is essential to maximize the stability of citral-rich oils.

2.5 Advanced Formulations to Enhance Lemongrass Oil Efficacy

2.5.1 Nanoemulsion-based delivery enhancements for antimicrobial applications

The formulation and stability of LEO nanoemulsions are significantly influenced by the extraction method used to obtain the oil. Various studies have demonstrated that the choice of extraction technique not only affects citral content but also the physicochemical properties and bioactivity of the resulting nanoemulsion. Hebishy et al. (2022) employed SFE, which preserved a high citral concentration and produced nanoemulsions with higher antimicrobial activity and stability. In contrast, Ayoub et al. (2023) and Saada et al. (2020) used hydrodistillation and produced stable nanoemulsions with average droplet sizes of 65–80 nm, although they exhibited lower citral retention. Similarly, Ali et al. (2023) formulated nanoemulsions from steam-distilled LEO, which were effective against *E. coli* and *S. aureus*, while Bezerra et al. (2023) applied microwave-assisted hydrodistillation (MAHD), resulting in better citral preservation and sustained antimicrobial action, particularly against *L. monocytogenes*.

Compared to conventional LEO formulations, nanoemulsions offer several advantages. Due to their small droplet size and increased surface area, nanoemulsions enhance solubility and bioavailability of hydrophobic compounds like citral. They also improve stability under environmental stressors (e.g., light, heat, oxygen), reduce volatility, and facilitate controlled release, which helps maintain antimicrobial and antioxidant efficacy over time. Furthermore, nanoemulsified LEO demonstrates greater dispersion in aqueous systems and stronger antimicrobial effects at lower concentrations compared to bulk oil (Ayoub et al., 2023; Hebishy et al., 2022).

Additional studies further validate these findings. Noorbakhsh et al. (2025) reported that LEO nanoemulsions produced using ultrasound-assisted emulsification showed excellent antimicrobial efficacy with droplet sizes below

100 nm and high kinetic stability under storage conditions. Gago et al. (2019) emphasized the relevance of nanoemulsion systems for food preservation, noting enhanced retention of sensory qualities and delayed microbial spoilage when lemongrass nanoemulsions were applied to fresh produce surfaces. Gonzalez et al. (2021) demonstrated that nanoemulsions incorporated into edible coatings significantly reduced pathogen load in fresh-cut fruit, confirming their utility in food packaging systems. Meanwhile, Salvia-Trujillo et al. (2012) found that nanoemulsions prepared with optimized surfactant-to-oil ratios promoted superior in vitro bioaccessibility and delivery of citral compared to macroemulsion systems, further supporting their functional advantages.

The formation of extremely small droplets or globules markedly reduces the influence of gravitational forces while amplifying Brownian motion, which arises from constant collisions between droplets and surrounding water molecules (Preeti et al., 2023). This size-dependent behaviour enables nanoemulsion droplets to remain uniformly dispersed in the liquid phase, thereby preventing creaming or sedimentation during storage. In contrast to conventional macroemulsions, nanoemulsion systems generate substantially smaller droplet sizes, for which Brownian motion predominates over gravitational separation forces, resulting in enhanced gravitational stability in aqueous environments (Kumar et al., 2019). This sustained dispersion and increased droplet mobility enhance the frequency of interactions between nanoemulsion droplets and microbial cells, facilitating more effective delivery of bioactive compounds such as citral to microbial membranes (Lu et al., 2018). As illustrated in Figure 2.8, reduced droplet size enhances Brownian motion and promotes more frequent interactions between nanoemulsion droplets and microbial cells.

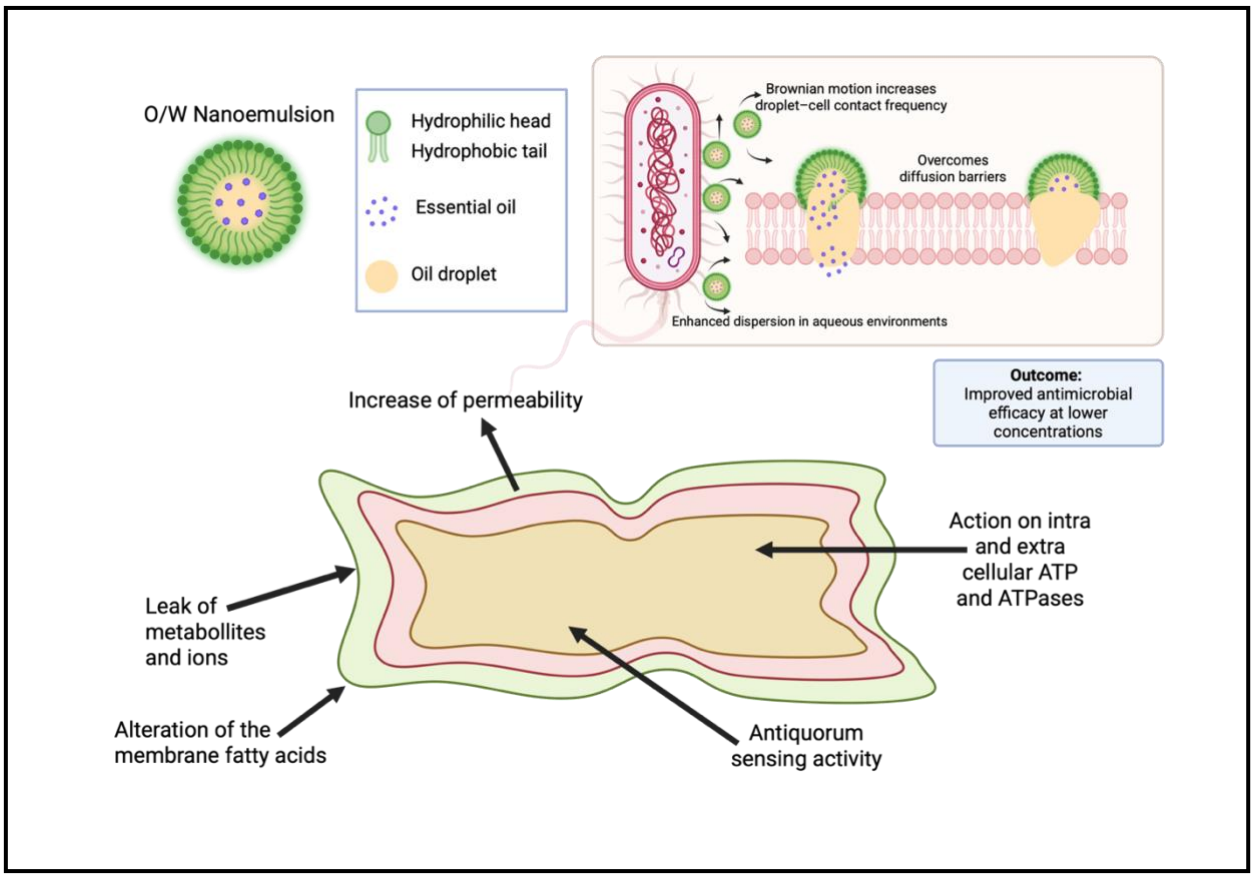

Figure 2.8. Schematic of nanoemulsion-mediated LEO delivery showing enhanced dispersion, improved droplet–cell interaction, and sustained citral release leading to increased antimicrobial activity. This figure was created using BioRender (https:/biorender.com).

Overall, nanoemulsion technology enhances the functional potential of LEO as a natural antimicrobial agent. Its improved dispersion, storage stability, and pathogen-targeting capabilities support its application in food preservation and safety, as well as in broader health and environmental contexts.

2.5.2 Toxicity, biocompatibility, and safety in food applications

Lemongrass essential oil (LEO), particularly its key compound citral, has demonstrated notable bioactivity alongside a generally favourable safety profile. Plata-Rueda et al. (2020) reported strong insecticidal effects of LEO, citral, and geranyl acetate against *Sitophilus granarius*, with low LD_{50} values ranging from 3.93 to 6.92 µg/insect and significant sublethal effects, including reduced respiration and mobility. In contrast, mammalian studies show low toxicity. Xavier

et al. (2022) found no adverse outcomes following a 2000 mg/kg oral dose in rats, classifying both LEO and citral under Category 5 (lowest toxicity) of the Globally Harmonized System (GHS).

Complementing these findings, Weshahi et al. (2025) reported strong antioxidant activity in LEO extracted from an Omani cultivar. Although specific cytotoxicity data in mammalian models were not provided, the isolated oil demonstrated significant cytotoxic potential in a brine shrimp lethality assay, achieving 100% mortality at 1000 µg/mL.

To ensure consumer safety, LEO concentrations in food systems must be carefully optimized. Balancing efficacy and biocompatibility are crucial, especially considering its cytotoxicity at elevated doses. Regulatory guidelines provide an essential framework for establishing safe application levels in food industries.

2.6 Applications of Lemongrass Essential Oil in Food Safety

2.6.1 As a natural preservative in food products (e.g., dairy, meat, grains)

Growing consumer demand for natural food preservatives has spotlighted LEO as a promising candidate, owing to its strong antimicrobial and antioxidant properties primarily driven by its citral content. Its application spans a variety of food matrices, including meat, tofu, dairy, beverages, and fresh produce. In tofu preservation, Hamad et al. (2019) demonstrated that a 20% water extract of lemongrass inhibited bacterial growth and extended shelf life by four days, while the essential oil component, although not antimicrobial in this context, helped maintain sensory qualities such as color, odor, and texture.

Advanced technologies such as encapsulation and nanoemulsion further enhance LEO's preservative effectiveness. Faheem et al. (2022) reported that nanoformulations of LEO combined with edible films (e.g., chitosan, alginate, or starch-based matrices) enhanced antimicrobial efficacy against pathogens like *E. coli*, *Salmonella*, and *L. monocytogenes*, while also improving the mechanical and barrier properties of packaging materials. In chicken meat preservation, Gan et al.

(2024) encapsulated LEO in bilayer liposomes and demonstrated significant delays in spoilage indicators, such as total volatile nitrogen and bacterial load, extending shelf life from 7 to 12 days at 4°C. The diverse applications of lemongrass essential oil nanoemulsions across food matrices and preservation strategies are schematically illustrated in Figure 2.9.

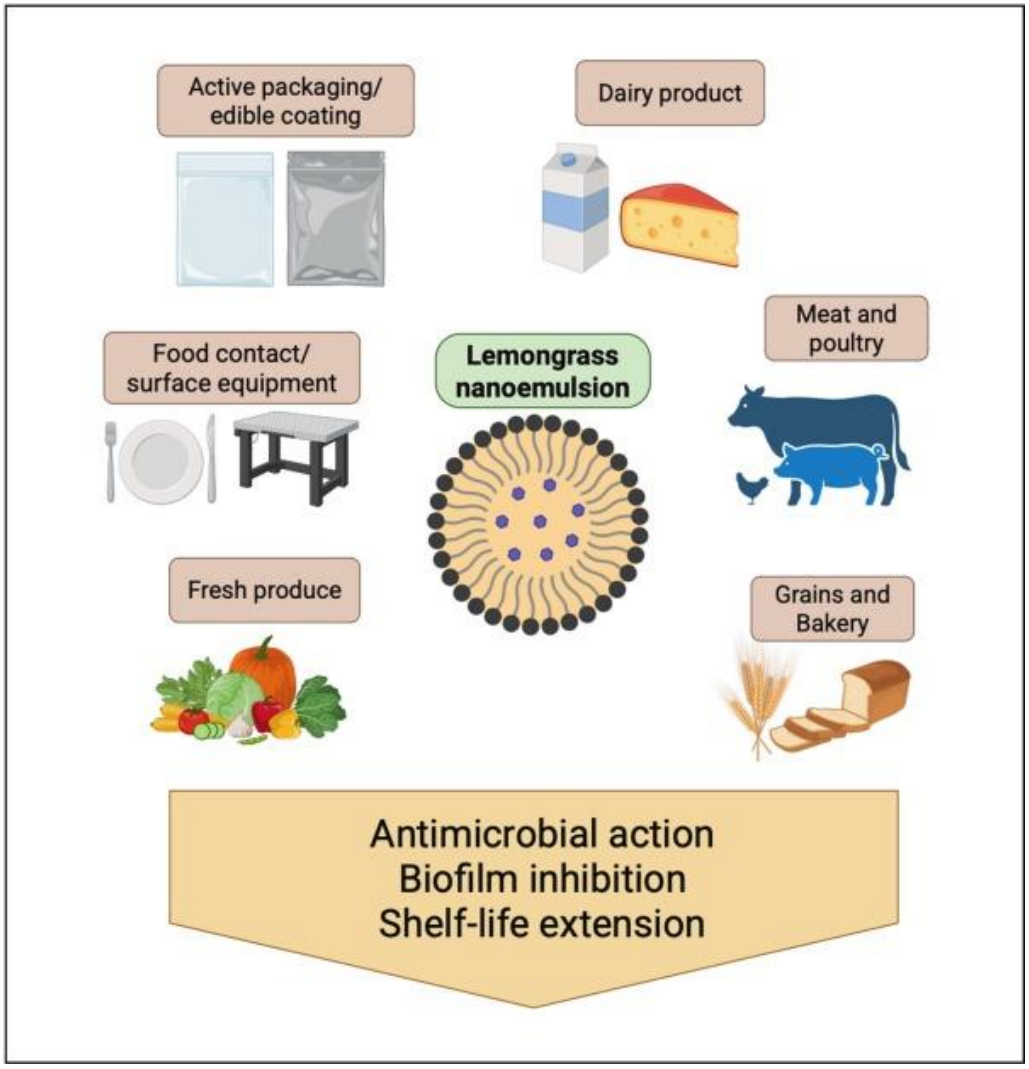

Figure 2.9. Schematic overview of the application of lemongrass essential oil nanoemulsions in food preservation and safety systems. This figure was created using BioRender (https:/biorender.com).

Additionally, Iranloye et al. (2024) emphasized LEO's role as a broad-spectrum antimicrobial agent capable of targeting a variety of spoilage organisms across food types, supporting its integration in ready-to-eat and minimally processed foods.

Applications have also extended to beverages and dairy, where lemongrass extract preserved freshness and inhibited microbial growth during storage.

Several studies have demonstrated the effective application of LEO nanoemulsions in food preservation, particularly in reducing microbial load and maintaining sensory quality. These studies span a range of food products, emulsifier types, and target microorganisms, as summarised in Table 2.5, highlighting their potential for food safety enhancement and shelf-life extension. Nevertheless, the performance of LEO can be affected by food composition and delivery methods, warranting further exploration into formulation technologies to optimize its use in industrial food preservation.

Table 2.5. Applications of lemongrass nanoemulsions in food preservation with antimicrobial impact across various food matrices.

Food Product	Target Microorganism	Emulsifier Type	LEO Concentration	Impact	Reference
Beef Burger	Total bacterial count (TBC)	Tween 80	1.0% and 1.5%	0.57 log reduction in TBC; improved sensory properties	Bakheet et al. (2024)
Chewy Candy	*Streptococcus mutans*, *Porphyromonas gingivalis*	Soy protein isolates and soy lecithin	Lemongrass to lemon ratio 1.85: 2.25.	85% inhibition of *S. mutans* and 77.20% inhibition of *P. gingivalis*; improved texture and color	Jayus et al. (2024)
Grape Berries	*S.* Typhimurium or *E. coli* O157:H7	Carnauba wax, Tween 80	2.0%	2 log reduction on *S. typhimurium* and 1 log reduction on *E. coli* O157:H7; maintained firmness and phenolic compound	Kim et al. (2014)
Pomegranate arils	*S.* Typhimurium or *E. coli* O157:H7	Flaxseed gum	800 ppm	4 log reduction in TPC; extended shelf life; maintained sensory attributes such as color, texture, and taste over 12 days of storage at 5 ± 1 °C.	Yousuf and Srivastava (2017)

2.6.2 Use in surface sanitization and active food packaging

Lemongrass extract and essential oil have demonstrated promising antimicrobial efficacy for surface sanitation in both healthcare and food industry contexts. Mathew et al. (2016) showed that lemongrass oil exhibited strong disinfectant properties against *E. coli* and *S. aureus* on floor surfaces, comparable to commercial Lysol disinfectant with a significant reduction in bacterial presence within just 10 minutes of application. Similarly, Sasi et al. (2020) assessed the sanitizing potential of lemongrass oil in meat processing environments and found it to significantly reduce total viable counts, coliforms, and yeast and mould loads on meat cutting boards ($p < 0.05$). Compared to other natural sanitizers like lemon juice and orange peel extract, lemongrass oil yielded the lowest microbial counts, suggesting its superior efficacy in reducing cross-contamination risks in hand hygiene applications.

Building on its versatility in antimicrobial applications, LEO has also gained substantial attention in the development of sustainable active packaging systems. Its potent antimicrobial and antioxidant properties make it well-suited for incorporation into biodegradable film matrices aimed at extending food shelf life and enhancing product safety. For example, Istiqomah et al. (2022) demonstrated that integrating LEO into a chitosan–*Dioscorea hispida* starch composite film significantly improved mechanical, thermal, and barrier properties while also exhibiting strong antibacterial activity against *E. coli*, *S. aureus*, *S.* Typhimurium, and *S. epidermidis*.

Molecular docking analysis further revealed that the active components in LEO interact with bacterial FtsA enzymes through hydrogen bonding and hydrophobic interactions, suggesting a specific antibacterial mechanism. Similarly, Silva et al. (2025) applied a chitosan–LEO emulsion as a coating on paperboard and found enhanced thermal stability, hydrophobicity, and microbial resistance, particularly against insect infestation in stored grain products. The functional performance of these coated systems improved with increasing LEO concentration, although

higher concentrations slightly affected cytotoxicity, indicating a need for optimal dosage balance.

In a separate study, Magri et al. (2025) developed LEO-loaded nanoemulsion films using gelatin as the carrier and reported extended shelf-life of perishable foods due to the controlled release of antimicrobial compounds. Jamroz et al. (2023) also confirmed this controlled-release profile in polylactic acid (PLA) based films, where the inclusion of LEO maintained antimicrobial efficacy without adversely impacting the sensory characteristics of food. Finally, Ruskova et al. (2023) highlighted the role of LEO in enhancing the multifunctionality of bio-based films, emphasizing its dual role as a natural preservative and an eco-friendly alternative to synthetic packaging additives. Collectively, these findings affirm that LEO not only improves the bioactivity of active packaging materials but also contributes to environmental sustainability in food preservation systems.

2.6.3 Regulatory status, consumer acceptability, and market potential

Lemongrass essential oil (LEO) is widely recognized by regulatory authorities and continues to see rising global demand, supporting its role as a natural preservative and flavoring agent in both food and consumer products. From a safety perspective, the Flavor and Extract Manufacturers Association (FEMA) expert panel has affirmed LEO (FEMA No. 2624) as generally recognized as safe (GRAS) for use as a natural flavoring ingredient, following comprehensive toxicological evaluation and constituent profiling (Rosol et al., 2023). Similarly, the European Chemicals Agency (ECHA) recognizes citral as the primary component of LEO and it is safe to use under specified concentrations in food, cosmetics, and household products, though it must be labeled for potential sensitization effects in some consumer goods. This dual recognition in the U.S. and EU enhances consumer confidence and regulatory clarity for manufacturers using LEO in natural formulations.

In terms of consumer perception, lemongrass oil is widely regarded as a clean-label, plant-based ingredient with antimicrobial, aromatic, and therapeutic benefits. Its integration into functional foods, beverages, and aromatherapy aligns with consumer preferences for natural and multifunctional additives. Rosol et al. (2023) noted its long-standing use in global cuisines, teas, and confectionery, with

increasing consumption observed in North America and Europe due to the popularity of Southeast Asian culinary trends.

The market potential of LEO is likewise promising. According to report from Global Market Insights in 2025, the global lemongrass oil market is expected to surpass USD 130 million by 2027, driven by rising demand across food, cosmetic, and pharmaceutical sectors. India plays a leading role in global supply. Singh et al. (2022) reported a significant increase in India's LEO exports from 80 280 kg in 1997–2002 to over 2.5 million kg in 2017–2020 generating substantial foreign exchange earnings. Key export destinations include the United States, Canada, Germany, and France. The cultivation of lemongrass also contributes to rural employment, crop diversification, and marginal land utilization, reinforcing its socio-economic importance. In summary, the regulatory recognition, positive consumer perception, and expanding market potential of LEO reflect its growing prominence in food safety applications.

2.7 Research Gaps and Rationale for the Current Study

Despite the broad recognition of LEO for its antimicrobial and antifungal properties, key research gaps remain. Most studies have focused on in vitro antimicrobial activity against planktonic cells, with limited application to real food matrices, especially those rich in fats or proteins. These complex systems may reduce the efficacy of LEO due to interactions that limit its bioavailability.

While LEO has shown antibiofilm effects against some pathogens, its specific performance against *B. cereus* biofilms in food environments remains under-investigated. Additionally, there is no existing study evaluating the use of SFE-derived LEO nanoemulsions despite their potential for higher citral purity and thermal stability in targeting *B. cereus* contamination in food.

Mechanistically, although citral disrupts bacterial membranes and impairs metabolic activity, how SFE-derived LEO or its nanoemulsions affect *B. cereus* membrane integrity or biofilm formation remains unclear. Clarifying these pathways is essential for developing effective food preservation strategies.

Further gaps include limited long-term stability data for nanoemulsions, unknown sensory impacts in real food systems, and uncertainties around optimal dosing and safety thresholds for chronic human exposure. Moreover, consumer acceptability of LEO-preserved foods has not been well characterized.

2.8 Copyright information

Part of this study has been submitted to Food Chemistry journal and the Online Statement of Contribution form is attached in Appendix I

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Chapter 3. Supercritical Lemongrass Extraction: A Natural Defence Against *Bacillus cereus* in Food systems

3.1 Introduction

Lemongrass (*Cymbopogon citratus*) is a tropical herb widely used in culinary, pharmaceutical, and food preservation applications due to its essential oil, which is rich in bioactive compounds primarily citral, a mixture of geranial and neral. The method of essential oil extraction plays a crucial role in determining both the yield and the chemical stability of the extract. Traditional extraction methods such as steam distillation and hydrodistillation are still widely used, however, they often result in the degradation of thermolabile compounds due to prolonged exposure to high temperatures (Pheko-Ofitlhile & Makhzoum, 2024). In contrast, supercritical fluid extraction (SFE) using carbon dioxide (CO_2) has emerged as a superior green technology that enhances extraction efficiency while preserving heat-sensitive volatiles. By enabling selective and adjustable extraction through the manipulation of pressure and temperature, SFE has been widely reported to produce highly enriched extracts and improved extraction efficiency for volatile compounds compared with conventional techniques (Herrero et al., 2010). Notably, the efficiency of SFE is highly dependent on the optimisation of extraction parameters, particularly pressure and temperature, which govern the density and solvating power of CO_2. For example, Shyaula et al. 2024 emphasized that maintaining optimal conditions (e.g., 150–200 bar at 40–50°C) is critical to maximizing yield and preserving citral integrity during extraction.

With high quality extracts containing citral obtained through optimized SFE, the practical application of lemongrass essential oil (LEO) in food preservation has gained increasing attention, particularly for combating foodborne pathogens such as *S. aureus*, *E. coli* and *B. cereus*. *B. cereus* is a spore-forming, heat-resistant pathogen frequently associated with foodborne illnesses, particularly in dairy and starch-rich foods. Its ability to survive pasteurization and form biofilms makes it a persistent challenge to food safety. To combat this, various plant-based antimicrobials have been investigated. Extracts from *Inula britannica*, *Polygonatum sibiricum* and *Rhus coriaria* (sumac) have demonstrated inhibitory effects against *B. cereus* in food applications, especially in dairy matrices, contributing to microbial control and improved shelf life (Alsamri et al., 2021; Fei

et al., 2024; Ibadullayeva et al., 2024; Zannou et al., 2025). In addition to its efficacy against *B. cereus*, lemongrass has shown strong antimicrobial activity against a broad range of foodborne pathogens such as *L. monocytogenes*, *S. aureus*, and *E. coli*, highlighting its versatility as a natural preservative (Gao et al., 2020). More specifically, LEO has been shown to inhibit *B. cereus* effectively, particularly when formulated as an emulsion (Mohd Daud et al., 2025). Emulsification enhances the oil's dispersibility, bioavailability, and membrane interaction, thereby boosting its antimicrobial performance in aqueous food systems (Mendes et al., 2020). Oil-in-water emulsions, especially those produced by high-shear homogenization, have been reported to be significantly more effective than crude extracts in inhibiting *B. cereus* growth (Moghimi et al., 2016; Salvia-Trujillo et al., 2017).

Although SFE is known to produce high-quality citral-rich lemongrass oil and emulsification has been shown to enhance its antimicrobial efficacy in food systems, no study to date has evaluated the application of SFE-extracted lemongrass oil in emulsion form specifically against *B. cereus* in real food matrices. Most existing studies focus on either the extraction method or the emulsion formulation independently. Therefore, this study addresses this gap by exploring the combined potential of SFE and emulsification for natural food preservation.

3.2 Materials and methods

3.2.1 Sample

Two varieties of raw lemongrass were purchased from a farmer in Beranang, Selangor, Malaysia and the plant taxonomic identification was verified by the Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) under the voucher specimen number of MFI 0217/21for lemongrass Peha ayam and MFI 0217/21 for lemongrass Gajah. Citral, (Sigma-aldrich CAS number: 5392-40-5, MO, USA) a pure compound, was utilized as a benchmark to evaluate and compare its activity against lemongrass extract.

3.2.2 Extract preparation

Lemongrass leaves and stems were chopped to an average of 1 cm in size, dried in a forced air convection oven at 50˚C for 24 h and were ground through a dry blender until the size was not less than 100 µm in size.

3.2.3 Supercritical fluid extraction

Approximately 170 g of the dried, ground lemongrass was loaded into a supercritical fluid extractor (SFE420-50-200, Feyecon Development and Implementation B.V., Netherlands). Carbon dioxide was used as the extraction solvent at an average flow rate of 1.8–2.0 kg/h. Extractions were performed under four pressure conditions, 85, 100, 200, and 300 bar and each maintained at a constant temperature of 40 °C. Each extraction run lasted for 7 hours. The resulting stock solutions were stored at –20 °C for up to 24 months.

3.2.4 Yield of lemongrass

Extraction yield was expressed as the mass of extract collected per gram of dried sample (mg/g) to facilitate interpretation, as SFE using CO_2 does not involve collection of an extract solvent volume. The yield was calculated as:
Yield (mg/g) = [(Mass of extracted (g)) X 1000] / Initial dry sample mass (g)).

3.2.5 Identification and quantification of active compounds in lemongrass extract using Gas chromatography-mass spectrometry (GC-MS)

LEO extract was dissolved in High Performance Liquid Chromatography (HPLC) grade ethanol (R & M Marketing, Essex, UK) to yield a 5 mg/mL aliquot of the extract injected into a QP2010 Ultra gas chromatograph-mass spectrometer (Shimadzu Corporation, Kyoto, Japan) with a BP5MS column (30 m × 0.25 mm × 0.25 µm) for compound separation. Helium was used as the carrier gas at a flow rate of 3 mL/min. The oven injector temperature was 250°C; source temp 200°C. The oven temperature was ramped up to 300°C at 15°C/min to and held for 10 min. The peaks were analysed by comparing their retention times and mass fragments

patterns with standard spectra available in the Shimadzu GC-MS NIST/ Wiley library.

3.2.6 Solvent preparation

3.2.6.1 Ethanol

To prepare the ethanol-based stock solution, 0.05 g of lemongrass or citral extract was dissolved in 1 mL of ethanol. The solution was left to evaporate at room temperature until the final volume was reduced to 0.25 mL, resulting in a concentration of 200 mg/mL. Before use, the solution was vortexed (Scilogex, Germany) thoroughly to ensure homogeneity and stored at 4°C.

3.2.6.2 Water

For aqueous preparations, the extract was weighed and dissolved in sterilized distilled water to obtain a final concentration of 10% (w/v). The solution was mixed by vortex (Scilogex, Germany) to ensure complete dispersion before being used in antimicrobial assays and stored at 4°C.

3.2.7 Emulsion formation

A sodium alginate aqueous solution was made by dissolving 2% sodium alginate (w/w) (Ajax Finechem, Auckland, New Zealand) in miliQ water at 70°C with continuous overnight stirring until it was completely dissolved. Then, it was left to cool at 21°C. The primary emulsion was formed by mixing the sodium alginate aqueous solution with 2% of lemongrass extract or citral and Tween 80 with a laboratory blender (T-25 digital Ultraturrax IKA, Staufen, Germany Ultraturrax) at 13 500 rpm for 2 to 3 min with 30 second intervals. The volume of Tween 80 used was 1:3 ratio of extract and Tween 80 following methodology proposed by (Gago et al., 2019).

3.2.8 Bacterial Cultures

Four bacterial isolates commonly associated with foodborne illnesses were selected for preliminary antimicrobial screening. These included food-derived isolates which are two Gram-positive bacteria: *B. cereus* IF2201 and *S. aureus* IF2202, and two Gram-negative bacteria: *E. coli* IF2203 and *S.*Typhimurium IF2204. All strains were maintained on Mueller Hinton Agar (MHA) (Bacto™, Becton, Dickinson and Company, USA) and subcultured regularly. For experimental use, a loopful of overnight culture was inoculated into Mueller Hinton Broth (MHB) (Bacto™, Becton, Dickinson and Company, USA) and incubated overnight at 37°C to obtain active cultures.

The initial antimicrobial activity of lemongrass and citral extracts was tested against these standard foodborne pathogens to assess their broad-spectrum potential. Based on the preliminary results, *B. cereus* was selected for further investigation due to its importance in food safety, notable resistance profile, and the strong antimicrobial activity exhibited by both lemongrass and citral against this pathogen.

3.2.8.1 B. cereus Isolates

Three *B. cereus* isolates were used for the main antimicrobial application study: one reference isolate (*B. cereus* ATCC 14579) originated from a cowshed, and two food-derived isolates, *B. cereus* P4 (from potato) and *B. cereus* M2 (from milk). All isolates were kindly provided by the Food Microbiology Laboratory at Massey University. Each strain was cultured in Brain Heart Infusion (BHI) broth (Bacto™, Becton, Dickinson and Company, USA) and incubated overnight at 30°C before use.

These isolates were selected for their practical relevance in food systems, allowing the evaluation of the antibacterial efficacy of lemongrass and citral emulsions under conditions simulating real-world food contamination.

3.2.9 Disk diffusion

Disk diffusion assays were performed in accordance with the general principles outlined by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing, with modifications to accommodate plant extract application (Weinstein et al., 2021). A 200 mg/mL of lemongrass extract was tested against Gram-positive and Gram-negative foodborne pathogens using disk diffusion. Each bacterium was grown in Muller Hinton broth (MHB) at 37°C overnight. 25 µL of standardized inoculum 6-8 $\log_{10}$ CFU/mL of each foodborne pathogen were evenly spread on an MHA agar using sterile cotton swabs. Sterile commercial disks (diameter 6 mm) were aseptically placed onto the agar plates using sterile forceps and 10 µL of lemongrass extract (200 mg/mL) was pipetted onto each disk. Plates were labelled accordingly and were incubated at 37°C for 24 h. Any clear zone presence surrounding the disc were observed and recorded.

3.2.10 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Conentration (MBC)

The minimum inhibition concentration (MIC) was performed in a 96-well microtiter plate using two-fold serial dilutions. Lemongrass or citral emulsions with a concentration of 2.00% (v/v) were mixed and two-fold dilutions were prepared in Brain Heart Infusion (BHI) broth containing the inoculum. Column 12 was filled with the highest concentration of lemongrass or citral emulsions (1.00% (v/v)), while column 3 was filled with the lowest concentration of Lemongrass or citral emulsions (0.02% (v/v)). Column 1 served as the negative control (broth only, no inoculum) and column 2 served as the positive control (broth with inoculum only, no antimicrobial agent). The MIC was determined as the lowest concentration of antimicrobial agent that inhibits microbial growth, and the absorbance was measured using a microtiter plate reader (Varioskan ^{TM} LUX, UK) with reading <0.05 change in optical density $(OD)_{590}$ after incubating the sample for 24 h at 30°C. The minimum bactericidal concentration (MBC) was determined by sub-culturing 10 µL of the suspension from each well in the microtiter plate (including the positive and negative controls) onto Mueller–Hinton agar (MHA)

plates. The plates were then incubated at 30°C for 12–24 h. MBC was defined as the lowest concentration of antimicrobial agent that resulted in no bacterial colony growth on MHA following incubation. MIC and MBC values were expressed as percentage concentration (% v/v). All assays were performed in triplicate.

3.2.11 Application of lemongrass emulsion in food systems

3.2.11.1 Rice and Soy milk

1 L Organic rice milk and soy milk (Macro brand) were purchased from a local supermarket (Woolworth, Broadway, Palmerston North). *B. cereus* isolates (ATCC 14579, P4, and M2) were cultured overnight in Brain Heart Infusion (BHI) broth at 30°C. After incubation, 5 mL of each bacterial culture was transferred to sterile Eppendorf tubes and centrifuged at 5 000 × *g* for 10 min (Sigma® 6–16, John Morris Scientific Ltd., New Zealand). The cell pellets were washed twice with sterile distilled water via vortex mixing for 30 s followed by centrifugation (5,000 × *g* for 5 min). The final pellets were resuspended in a sterile saline water. To confirm that the culture reached 6 $\log_{10}$ CFU/mL, optical density (OD) measurement was performed using spectrophotometry (Varioskan™ Lux, Thermo Fisher Scientific, Massachussetts, USA) at 600 nm. A correlation curve between OD600 and CFU/mL was established, allowing the estimation of bacterial concentration based on absorbance readings.

To prepare the inoculated food samples, 1 mL of the washed bacterial suspension was added to 10 mL of either rice milk or soy milk to achieve an initial bacterial load of approximately 6 $\log_{10}$ CFU/mL. The inoculated food samples were then left at room temperature (21°C ± 2) for 30 min to allow bacterial adaptation before proceeding with the next experimental step. Subsequently, 0.1 mL of lemongrass or citral emulsion at varying concentrations (0.25–2.00% (v/v)) was added to the inoculated milk samples. The mixtures were incubated at 30°C for 1 h to allow antimicrobial interaction. After incubation, samples were centrifuged at 12,000 × *g* for 5 min at 4°C. The pellets were washed once with 0.85% sterile saline, mixed by vortex, and re-centrifuged (5,000 × *g*, 5 min). The final pellets were resuspended in sterile saline water for viable count analysis. Serial dilutions were plated on Tryptic Soy Agar (TSA; Difco™, Becton Dickinson, USA) and incubated at 30°C

for 24 h. Colonies were counted, and bacterial concentrations were calculated using the formula: CFU/mL = (Number of colonies × dilution factor) / volume plated. The detection limit was 1 $\log_{10}$ CFU/mL. Control samples (samples without emulsion) were run in parallel.

3.2.11.2 Milk protein concentrate (MPC)

Gamma sterilised milk protein concentrate (MPC) powder was obtained from Fonterra Co-operative Group, Palmerston North, New Zealand. Solutions of MPC were prepared at concentrations of 5%, 10%, and 15% (w/v) by dissolving the appropriate amount of MPC powder in sterilized Milli-Q water under constant stirring until fully dissolved.

The inoculum of *B. cereus* was prepared as described for rice and soy milk. To the 10 mL of MPC samples, 1 mL of the washed bacterial suspension was added to achieve an initial bacterial load of approximately 6 $\log_{10}$ CFU/mL. The inoculated milk samples were then left at room temperature for 30 min to allow bacterial adaptation before proceeding with the next experimental step. The bacterial inoculation, emulsion treatment, incubation, and microbial enumeration steps were performed as described in Section 3.2.11.1.

3.2.12 Statistical analysis

All experiments were performed in triplicate. Data for application of lemongrass and citral emulsions in soy milk and rice milk against *B. cereus* isolates were analyzed by two-way analysis of variance (ANOVA) and other data were analayzed by one-way ANOVA (IBM SPSS version 29) and, where necessary, the Tukey test ($\alpha = 0.05$) or t-test ($\alpha = 0.05$). All results were expressed as mean ± standard deviation.

3.3 Results

3.3.1 Yield collected

The yield data (Table 3.1) indicates that Lemongrass G consistently produced a higher yield compared to lemongrass PA at all pressure levels tested. Both varieties of lemongrass generally demonstrated a positive relationship between pressure and yield, where increasing pressure resulted in higher yield. However, an exception was observed in lemongrass G at 200 bar, where the yield dropped noticeably before increasing significantly at 300 bar. In contrast, lemongrass PA showed a steady increase in yield with rising pressure, reflecting a more consistent trend.

Table 3.1. Yield collected of two different lemongrasses at different pressures level.

Pressure (bar)	Peha ayam (PA)			Gajah (G)		
	Sample (g)	Extract collected (g)	Yield collected (mg/g)	Sample (g)	Extract collected (g)	Yield collected (mg/g)
85	172.80	0.13	0.75	172.56	1.32	7.65
100	185.31	0.54	2.91	167.76	1.25	7.45
200	184.45	0.79	4.28	181.22	0.83	4.58
300	185.22	1.51	8.15	182.51	2.10	11.50

Figures 3.2 and 3.3 (supplementary data) illustrate the effect of varying pressures on the physical form of lemongrass extracts from the Gajah (G) and Peha Ayam (PA) varieties at a constant temperature of 40°C. For both varieties, at lower pressures of 85 bar and 100 bar, the extracts appeared in oil form. However, as the pressure increased to 200 bar and 300 bar, the extracts transitioned to a wax-like form. This consistent trend in both lemongrass G and PA suggests that higher pressures promote the extraction of heavier, waxy compounds, whereas lower pressures favour the extraction of lighter, oil-based components.

Due to these observations, only extracts obtained at 85 bar and 100 bar were selected for further analysis. The oil form of the extracts at these pressures was preferred, as it allowed for easier dissolution in solvents and simpler handling

during subsequent analysis. In contrast, the wax form observed at 200 bar and 300 bar presented challenges in solubility and sample preparation, which could compromise the consistency and reliability of the analysis. Therefore, higher-pressure extracts were excluded to ensure uniformity and accuracy in the experimental results.

3.3.2 Composition of active compounds by Gas Chromatography Mass Spectrometry (GCMS) analysis

The citral content in Table 3.2, calculated as the sum of neral and geranial, varied significantly between the two lemongrass varieties and under different pressures. Lemongrass (PA) showed notably higher citral abundance than lemongrass (G) under both supercritical CO_2 extraction conditions. Specifically, PA extracted at 85 bar exhibited the highest citral content, comprising 25.25% neral and 46.44% geranial (total citral = 71.69%). When pressure increased to 100 bar, total citral in lemongrass PA decreased substantially to 30.44%. In contrast, lemongrass (G) demonstrated considerably lower citral abundance, with total citral of 14.45% at 85 bar and 13.76% at 100 bar. Overall, lemongrass PA consistently yielded more citral-rich extracts than lemongrass G. While lower pressure (85 bar) was associated with higher citral abundance in lemongrass PA, the pressure effect in lemongrass G was comparatively minimal, indicating that the relationship between pressure and citral extraction efficiency may be variety-dependent within the tested range (85–100 bar).

Table 3.2. Composition of bioactive components in lemongrass extract (Gajah and Peha Ayam).

This No.	Name of compound	Standard		Supercritical fluid condition							
		Citral		PA 85’		PA 100’		G 85’		G 100’	
		RT	RPA (%)	RT	RPA (%)	RT	RPA %	RT	RPA %	RT	RPA %
1	Neral	22.179	44.08	22.285	25.25	22.202	11.05	22.198	5.17	22.182	4.83
2	Geraniol	-	-	22.859	4.18	22.844	14.25	22.815	2.15	22.814	2.61
3	Geranial	23.561	45.98	23.740	46.44	23.610	19.39	23.608	9.28	23.573	8.93
4	Caryophyllene			30.497	2.11	30.474	1.45	30.481	1.64	nd	nd
5	Cis-.alpha.-Bergamotene			31.147	1.00	nd	nd	31.148	2.46	nd	nd
6	Geranial diethylacetal			31.262	0.75	31.230	2.10	nd	nd	nd	nd
7	Germacrene D			33.160	1.10	nd	nd	33.167	1.45	34.549	1.52
8	Bulnesene <alpha>			34.221	0.79	nd	nd	nd	nd	nd	nd
9	Muurolene <gamma>			34.557	0.81	34.537	1.36	34.562	2.64	nd	nd
10	Endo-1-bourbonanol			37.095	2.29	37.075	1.79	37.107	5.44	37.087	5.07
11	Junipercamphor			38.864	7.75	38.854	15.81	38.893	13.96	38.856	15.94
12	Viridiflorol			40.228	0.83	40.212	2.69	-	-	40.221	3.59
13	Intermedeol			40.444	1.42	40.426	3.24	40.461	2.99	40.437	3.32
14	Eudesm-7(11)-en-4-ol			41.871	1.29	41.849	2.48	nd	nd	nd	nd
15	Citronellol			nd	nd	21.582	5.88	nd	nd	nd	nd
16	6-methyl-5-hepten-2-one			nd	nd	24.006	1.33	nd	nd	nd	nd
17	Geranic acid			nd	nd	27.953	3.27	nd	nd	nd	nd
18	Cryptomeridiol			nd	nd	nd	nd	44.728	0.87	45.603	2.06
19	Longifolol			nd	nd	34.139	1.36	nd	nd	nd	nd
20	Farnesal<2Z, 6Z->			nd	nd	43.402	1.47	nd	nd	nd	nd

Continuation of **Table 3.2.** Composition of bioactive components in lemongrass extract (Gajah and Peha Ayam).

21	Kessane	nd	nd	46.189	2.16	46.295	11.08	46.266	20.81
22	(Z)-3,7-Dimethylocta-2,6-dien-1-yl palmitate	nd	nd	73.670	2.58	nd	nd	nd	nd
23	Geranyl palmitoleate	nd	nd	77.870	2.26	nd	nd	nd	nd
24	Manool	nd	nd	90.712	3.24	nd	nd	nd	nd
25	Beta elemene	nd	nd	nd	nd	29.238	0.96	nd	nd
26	(E)-.beta.-Famesene	nd	nd	nd	nd	31.990	1.45	nd	nd
27	Selinene <alpha>	nd	nd	nd	nd	33.277	0.91	nd	nd
28	Bisabolene <beta->	nd	nd	nd	nd	34.247	1.65	nd	nd
29	Cadinene <delta->	nd	nd	nd	nd	34.916	1.23	nd	Nd
30	Elemol	nd	nd	nd	nd	35.968	1.21	nd	nd
31	Alpha.-eudesmol	nd	nd	nd	nd	38.324	7.07	nd	nd
32	Guaiol	nd	nd	nd	nd	39.501	5.37	39.484	5.39
33	Agarospirol	nd	nd	nd	nd	39.808	3.92	39.785	3.09
34	Rosifoliol	nd	nd	nd	nd	40.102	1.36	44.723	1.61
35	Muurolol	nd	nd	nd	nd	40.234	3.75	nd	nd
36	Farnesol<2Z, 6Z->	nd	nd	nd	nd	42.686	4.36	42.668	5.00
37	Oplopanone	nd	nd	nd	nd	43.417	1.97	43.393	2.28
38	Eudesmol<5-epi-7-epi-alpha->	nd	nd	nd	nd	nd	nd	38.304	6.52
39	Kolavenol	nd	nd	nd	nd	nd	nd	60.480	1.73

Continuation of **Table 3.2.** Composition of bioactive components in lemongrass extract (Gajah and Peha Ayam).

40	Lup-20(29)-en-3-one	nd	nd	nd	nd	nd	nd	90.718	2.36
41	2,3-epoxygeranial	nd	nd	nd	nd	nd	nd	nd	nd
42	2,3-epoxyneral	nd	nd	nd	nd	nd	nd	nd	nd
43	Selin-6-en-4.alpha.-ol	nd	nd	nd	nd	nd	nd	nd	nd

RT: Retention time; RPA: Relative peak area; nd: not detected

3.3.3 Disk diffusion assay

The antibacterial activity of lemongrass extracts (G and PA) varieties at 85 bar and 100 bar was assessed against four bacterial strains: *B. cereus*, *S. aureus* (Gram-positive), and *E. coli*, *Salmonella* spp (Gram-negative). (Table 3.3). Lemongrass extracts (G and PA) varieties showed stronger antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria. Lemongrass PA extracts demonstrated larger zones of inhibition, particularly against *B. cereus* (12 mm at 85 bar, 15 mm at 100 bar) and *S. aureus* (9.5 mm and 9 mm). Lemongrass G extracts showed moderate inhibition, with smaller zones. For Gram-negative bacteria, only weak antibacterial activity was observed against *E. coli*, while no activity was detected against *Salmonella.*

Table 3.3. Zone of inhibition of lemongrass extract (Gajah and Peha Ayam) at 85 bar and 100 bar against Gram-positive and Gram-negative bacteria.

Bacteria	Lemongrass Extract	Zone of Inhibition (mm)	Lemongrass Extract	Zone of Inhibition (mm)
B. cereus	G 85	10.0 ± 0.7	PA 85	12.0 ± 0.0
	G 100	7.0 ± 0.0	PA 100	15.0 ± 0.0
S. aureus	G 85	7.0 ± 0.0	PA 85	9.5 ± 0.0
	G 100	7.5 ± 0.5	PA 100	9.0 ± 0.0
E. coli	G 85	NI	PA 85	7.5 ± 0.7
	G 100	6.0 ± 0.0	PA 100	6.0 ± 0.0
Salmonella	G 85	NI	PA 85	NI
	G 100	NI	PA 100	NI

*NI: No inhibition; All zone inhibitions include the size of disk 6 mm

3.3.4 Antimicrobial activity

Since the antibacterial activity of lemongrass extracts was primarily effective against Gram-positive bacteria, further evaluation focused on *B. cereus*. As shown in Table 3.4, the MIC and MBC results revealed significant differences in antibacterial potency between lemongrass (G and PA) extracts. The lemongrass PA extract at 85 bar demonstrated the strongest antibacterial effect, with the lowest MIC (0.313% (v/v)) and MBC (0.625% (v/v)) values, indicating potent inhibitory and bactericidal activity. In contrast, lemongrass PA extract at 100 bar and both lemongrass G extracts (85 bar and 100 bar) exhibited MIC and MBC values above 2.500% (v/v), reflecting much weaker effects. Therefore, the lemongrass PA extract at 85 bar pressure was selected for subsequent experiments to further investigate its antibacterial potential against *B. cereus*.

Table 3.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of lemongrass extracts (Gajah and Peha ayam) against *B. cereus*

Bacteria	Lemongrass Extract	MIC (% v/v)	MBC (% v/v)
B. cereus	G 85	2.500	> 2.500
	G 100	2.500	> 2.500
	PA 85	0.313	0.625
	PA 100	> 2.500	> 2.500

The MIC results indicate that both lemongrass and citral emulsions demonstrated superior antibacterial activity against *B. cereus* isolates from food compared with the reference isolate ATCC 14579 originally isolated from a cowshed (Table 3.5). For lemongrass extracts, the emulsion achieved the lowest MIC value (0.125% (v/v)) compared to the water extract (0.630% (v/v)), highlighting its enhanced effectiveness. Similarly, the citral emulsion also showed strong antibacterial activity with an MIC of 0.125% (v/v) but was more effective than the water extract (0.160% (v/v)). MIC values were consistent across all three *B. cereus* strains. Therefore, MIC results are presented by strain for clarity, although no strain-specific differences were observed. In addition to its antibacterial potency, the emulsion was formulated using Tween 80 and sodium alginate, both of which are safe, edible, and commonly used food-grade ingredients. Therefore, the emulsion system was selected for further application in food systems due to its effectiveness and suitability for food use.

Table 3.5. Minimum Inhibitory Concentration (MIC) of lemongrass and citral against *B. cereus* isolated from food samples (ATCC 14579, P4 and M2)

Sample	**Minimum inhibitory concentration (MIC) (% v/v) of *B. cereus***		
	ATCC 14579	**P4**	**M2**
Lemongrass extract (Water)	0.630	0.630	0.630
Lemongrass emulsion	0.125	0.125	0.125
Citral extract (Water)	0.160	0.160	0.160
Citral emulsion	0.125	0.125	0.125

3.3.5 Application in food systems

Table 3.6 shows the antibacterial effect of lemongrass and citral emulsions at varying concentrations (0.25%, 0.500%, 1.00% and 2.00%) (v/v) in soy milk against *B. cereus* isolates (P4, M2, and ATCC 14579). Both emulsions demonstrated a concentration-dependent reduction in bacterial counts across all isolates. At lower concentrations (0.25% and 0.50%) (v/v), moderate reductions were observed, but the effect became more significant at higher concentrations. Complete inhibition (<1.00 $\log_{10}$ CFU/mL) was achieved for M2 and ATCC 14579 isolates at 2.00% (v/v) concentration for both emulsions. However, P4 showed slightly more resistance, requiring 2.00% (v/v) concentration to reduce the bacterial count to 1.00 log CFU/mL, with no complete inhibition observed. Notably, lemongrass emulsion at 2.00% (v/v) resulted in a reduction from 7.57 $\log_{10}$ CFU/mL to 1.00 $\log_{10}$ CFU/mL for P4, while citral emulsion achieved a similar reduction.

Table 3.7 shows the antibacterial effects of lemongrass and citral emulsions in rice milk against *B. cereus* isolates (P4, M2, and ATCC 14579), with reductions expressed in log values. Both emulsions exhibited concentration-dependent bacterial reduction. For the P4 isolate, at 2.00% (v/v) concentration, lemongrass emulsion achieved a 3.80 log reduction, while citral emulsion showed a 3.60 log reduction. For the M2 isolate, both emulsions at 2.00% (v/v) completely inhibited growth, resulting in more than a 6.54 log reduction**.** Similarly, for the ATCC 14579 isolate, both emulsions at 2.00% (v/v) led to complete inhibition with over a 6.26 log reduction. At lower concentrations (0.25% and 0.50%) (v/v), reductions were modest, with less than 1 log reduction observed. These results indicate that while both emulsions are effective at higher concentrations, the P4 isolate is more resistant compared to M2 and ATCC 14579.

These findings were supported by statistical analysis, which revealed that concentration had a highly significant effect on the bacterial count (CFU/mL) ($p < 0.001$). Additionally, a significant interaction between concentration of emulsion

and *B. cereus* isolate type ($p < 0.001$) confirmed that the antibacterial effect varied depending on the *B. cereus* isolate. However, no significant differences ($p > 0.05$) were detected between the different emulsion treatments (lemongrass and citral).

Table 3.6. Application of lemongrass and citral emulsions in soy milk against *B. cereus* isolates.

Isolates	**P4**				**M2**				**ATCC 14579**			
Concentration (v/v)	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**
Control	7.57 ± 0.38^{Aa}				7.54 ± 0.28^{Aa}				7.15 ± 0.21^{Aa}			
Lemongrass	$7.20^{Bb} \pm 0.27$	$6.30^{Bb} \pm 0.00$	$7.14^{Bb} \pm 0.03$	$1.00^{Cc} \pm 0.00$	$6.94^{Bb} \pm 0.83$	$7.33^{Bb} \pm 0.08$	$7.11^{Bb} \pm 0.05$	$<1.00^{Cc} \pm 0.00$	$6.30^{Bb} \pm 0.00$	$6.3^{Bb} \pm 0.18$	$5.48^{Bb} \pm 0.50$	$<1.00^{Cc} \pm 0.00$
Citral	$6.33^{Bb} \pm 0.35$	$6.48^{Bb} \pm 0.00$	$6.87^{Bb} \pm 0.16$	$1.00^{Cc} \pm 0.00$	$7.23^{Bb} \pm 0.07$	$7.29^{Bb} \pm 0.05$	$7.34^{Bb} \pm 0.08$	$<1.00^{Cc} \pm 0.00$	$6.48^{Bb} \pm 0.00$	$6.54^{Bb} \pm 0.03$	$6.11^{Bb} \pm 0.05$	$<1.00^{Cc} \pm 0.00$

Results in Log CFU/mL. Different uppercase letters within the same column indicate significant differences between concentration and emulsion treatments ($p < 0.05$). Different lowercase letters within the same row indicate significant differences between concentrations and isolates ($p < 0.05$), as determined by Two-Way ANOVA followed by Tukey's post-hoc test.

Table 3.7. Application of lemongrass and citral emulsions in rice milk against *B. cereus* isolates.

Isolate	**P4**				**M2**				**ATCC 14579**			
Concentration (v/v)	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**
Control	6.75 ± 0.05^{Aa}				6.54 ± 0.28^{Aa}				6.26 ± 0.24^{Aa}			
Lemongrass	$6.19^{Aab} \pm 0.21$	$6.47^{Ab} \pm 0.16$	$5.01^{Bc} \pm 0.42$	$2.95^{Cd} \pm 0.18$	$5.67^{Aab} \pm 0.18$	$6.19^{Ab} \pm 0.32$	$5.24^{Bc} \pm 0.14$	$<1.00^{Cd} \pm 0.00$	$6.38^{Aab} \pm 0.09$	$5.05^{Ab} \pm 0.50$	$5.19^{Bc} \pm 0.19$	$<1.00^{Cd} \pm 0.00$
Citral	$6.24^{Aab} \pm 0.06$	$5.98^{Ab} \pm 0.71$	$4.08^{Bc} \pm 1.05$	$3.15^{Cd} \pm 0.11$	$5.85^{Aab} \pm 0.00$	$6.65^{Ab} \pm 0.05$	$4.70^{Bc} \pm 0.01$	$<1.00^{Cd} \pm 0.00$	$6.60^{Aab} \pm 0.21$	$5.27^{Ab} \pm 0.31$	$3.63^{Bc} \pm 0.89$	$<1.00^{Cd} \pm 0.00$

Results in Log CFU/mL. Different uppercase letters within the same column indicate significant differences between concentration and emulsion treatments ($p < 0.05$). Different lowercase letters within the same row indicate significant differences between concentrations and isolates ($p < 0.05$), as determined by Two-Way ANOVA followed by Tukey's post-hoc test.

The antibacterial activity of lemongrass and citral emulsions at various concentrations (0.25%–2.00%) (v/v) was evaluated against *B. cereus* ATCC 14579 (Table 3.8), isolate P4 (Table 3.9), and isolate M2 (Table 3.10) under different MPC concentrations (5%, 10%, and 15%) (w/v). Both emulsions demonstrated a clear concentration-dependent reduction in bacterial counts, with higher concentrations resulting in more significant inhibition. Complete inhibition (<1.00 $\log_{10}$ CFU/mL) was achieved at the 2.00% (v/v) concentration for both emulsions across all MPC levels in *B. cereus* ATCC 14579 and isolate M2. In contrast, isolate P4 exhibited greater resistance, requiring higher concentrations to achieve comparable reductions. At lower concentrations, moderate reductions were observed, with no notable difference in effectiveness between lemongrass and citral emulsions. Statistical analysis using One-Way ANOVA indicated that MPC concentration had no significant effect on bacterial reduction ($p > 0.05$), suggesting that MPC concentration alone did not independently influence the antibacterial performance of the emulsions.

Table 3.8. Effect of lemongrass and citral emulsions in different MPC concentrations on *B. cereus* ATCC 14579

Isolate	**ATCC 14579**											
MPC Concentration (w/v)	**5%**				**10%**				**15%**			
Concentration of emulsion (%) (v/v)	**0.25**	**0.50**	**1.00**	**2.00**	**0.25**	**0.50**	**1.00**	**2.00**	**0.25**	**0.50**	**1.00**	**2.00**
Control	6.15 ± 0.21^{A}				6.15 ± 0.21^{A}				6.30 ± 0.00^{A}			
Lemongrass	$5.24^{B} \pm 0.34$	$5.32^{C} \pm 0.28$	$<1.00^{D} \pm 0.00$	$<1.00^{D} \pm 0.00$	$5.30^{B} \pm 0.00$	$4.95^{C} \pm 0.00$	$<1.00^{D} \pm 0.00$	$<1.00^{D} \pm 0.00$	$6.10^{B} \pm 0.17$	$4.63^{C} \pm 0.46$	$<1.00^{D} \pm 0.00$	$<1.00^{D} \pm 0.00$
Citral	$5.15^{A} \pm 0.21$	$4.89^{B} \pm 0.83$	$1.15^{C} \pm 0.22$	$<1.00^{D} \pm 0.00$	$6.15^{A} \pm 0.21$	$4.91^{B} \pm 0.19$	$1.43^{C} \pm 0.51$	$<1.00^{D} \pm 0.00$	$6.20^{A} \pm 0.17$	$5.96^{B} \pm 0.07$	$1.65^{C} \pm 0.50$	$<1.00^{D} \pm 0.00$

Results in Log CFU/mL. Different uppercase letters within the same column and row indicate significant differences between concentration treatments ($p < 0.05$) as determined by One-Way ANOVA followed by Tukey's post-hoc test. Additionally, an independent samples t-test showed no significant difference between the two treatment groups of emulsion ($p > 0.05$).

Table 3.9. Effect of lemongrass and citral emulsions in different MPC concentrations on *B. cereus* P4

Isolate	**P4**											
MPC Concentration (w/v)	**5%**				**10%**				**15%**			
Concentration of emulsion (v/v)	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**
Control	6.72 ± 0.28^{A}				6.37 ± 0.21^{A}				6.93 ± 0.42^{A}			
Lemongrass	$5.89^{A} \pm 0.78$	$5.19^{A} \pm 1.00$	$4.67^{B} \pm 0.01$	$2.41^{C} \pm 0.42$	$6.42^{A} \pm 0.37$	$6.55^{A} \pm 0.17$	$4.91^{B} \pm 0.51$	$2.22^{C} \pm 0.30$	$6.32^{A} \pm 0.24$	$6.62^{A} \pm 0.07$	$2.20^{B} \pm 0.35$	$1.65^{C} \pm 0.50$
Citral	$5.67^{A} \pm 0.59$	$5.59^{A} \pm 0.55$	$3.60^{B} \pm 0.43$	$1.28^{C} \pm 0.49$	$6.10^{A} \pm 0.54$	$6.37^{A} \pm 0.09$	$3.50^{B} \pm 0.71$	$1.63^{C} \pm 0.21$	$6.38^{A} \pm 0.10$	$6.58^{A} \pm 0.02$	$3.70^{B} \pm 0.27$	$1.47^{C} \pm 0.00$

Results in Log CFU/mL. Different uppercase letters within the same column and row indicate significant differences between concentration treatments ($p < 0.05$) as determined by One-Way ANOVA followed by Tukey's post-hoc test. Additionally, an independent samples t-test showed no significant difference between the two treatment groups of emulsion ($p > 0.05$).

Table 3.10. Effect of lemongrass and citral emulsions in different MPC concentrations on *B. cereus* M2

Isolate	**M2**											
MPC Concentration (w/v)	**5%**				**10%**				**15%**			
Concentration of emulsion (v/v)	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**	**0.25%**	**0.50%**	**1.00%**	**2.00%**
Control	6.91 ± 0.21^{A}				7.13 ± 0.16^{A}				6.57 ± 0.51^{A}			
Lemongrass	$5.90^{A} \pm 0.85$	$6.10^{A} \pm 0.13$	$<1.00^{B} \pm 0.00$	$<1.00^{C} \pm 0.00$	$6.84^{A} \pm 0.06$	$6.16^{A} \pm 0.05$	$<1.00^{B} \pm 0.00$	$<1.00^{C} \pm 0.00$	$6.77^{A} \pm 0.07$	$6.22^{A} \pm 0.07$	$3.65^{B} \pm 0.50$	$<1.00^{C} \pm 0.00$
Citral	$6.11^{A} \pm 0.37$	$5.66^{A} \pm 0.42$	$<1.00^{B} \pm 0.00$	$<1.00^{C} \pm 0.00$	$6.82^{A} \pm 0.13$	$6.12^{A} \pm 0.05$	$2.93^{B} \pm 0.81$	$<1.00^{C} \pm 0.00$	$6.83^{A} \pm 0.10$	$6.23^{A} \pm 0.05$	$3.60^{B} \pm 0.43$	$<1.00^{C} \pm 0.00$

Results in Log CFU/mL. Different uppercase letters within the same column and row indicate significant differences between concentration treatments ($p < 0.05$) as determined by One-Way ANOVA followed by Tukey's post-hoc test. Additionally, an independent samples t-test showed no significant difference between the two treatment groups of emulsion ($p > 0.05$).

3.4 Discussion

The yield of lemongrass G was consistently higher than that of lemongrass PA (Table 3.1), likely due to its physical characteristics. Lemongrass G possesses longer leaves and thicker stems (Figure 3.1 in supplementary material), contributing to a greater amount of extractable biomass. In both varieties, yield increased proportionally with pressure, aligning with findings by Kavoura et al. (2019) and Soto-Armenta et al. (2019), who reported that higher pressures enhance extraction efficiency in SFE systems. This effect is attributed to the increased solvent strength of supercritical CO_2 at higher pressures, allowing it to dissolve and extract more compounds from plant materials. For example, Majdoub et al. (2019) observed that wild carrot extract yield rose from 1.17% to 2.99% as pressure increased from 100 to 300 bar, while Wang et al. (2012) reported optimal yield of 1.82% at 294 bar for *Cyperus rotundus* Linn.

In addition to yield, pressure also influenced the physical form of the extracts. At lower pressures (85 and 100 bar), the extracts appeared as oils, whereas higher pressures (200 and 300 bar) produced wax-like forms (Figure 3.2 and Figure 3.3 in supplementary material). This shift occurs because lower pressures favour the extraction of light, volatile compounds such as essential oils, while higher pressures enable CO_2 to dissolve heavier, less volatile substances like waxes and resins. This phenomenon has also been documented in the extraction of grape seeds, where Ramazanov and Shakhbanov (2020) demonstrated the ability to selectively obtain oil or wax rich fractions by adjusting pressure levels during SFE.

Chemical analysis by GCMS (Table 3.2) revealed that citral content (defined as the sum of neral and geranial) varied across varieties and pressure conditions. Notably, the effect of extraction pressure on citral abundance differed between lemongrass varieties. In lemongrass PA, extraction at 85 bar produced substantially higher total citral compared with 100 bar, suggesting that this lower pressure condition favoured selective extraction of citral-rich volatiles within the tested range. In contrast, lemongrass G exhibited relatively stable citral abundance across pressures, indicating reduced pressure sensitivity under these conditions. This variety-dependent response may reflect differences in chemotype and plant matrix

characteristics, alongside pressure–temperature-dependent changes in CO_2 density and solvating power, which influence the relative solubility and co-extraction of volatile versus heavier constituents during SFE (Benomari et al., 2020; Uwineza & Waśkiewicz, 2020). As pressure increases, CO_2 density and solvating power rise, potentially increasing co-extraction of non-citral constituents and thereby diluting proportional citral content in the essential oil fraction (Baser & Buchbauer, 2009).

Importantly, citral recovery under SFE is frequently optimised within defined pressure windows rather than following a linear relationship with pressure. While CO_2 reaches supercritical conditions above 74 bar, extraction performance remains governed by the pressure–temperature dependence of CO_2 solvent properties, which shape selectivity and extraction composition (Majewska et al., 2019). Marongiu et al. (2006) reported optimal citral extraction at 90 bar and 50°C, where citral represented > 68% of the essential oil and extraction yield reached 0.65%. Similarly, Carlson et al. (2001) demonstrated that both extraction yield and essential oil composition vary significantly across pressure–temperature combinations, with the highest yields observed at 90 bar at 23°C and 120 bar at 40°C. Collectively, these findings support the view that optimal SFE conditions may be variety-specific and require tailored optimisation, with the present results indicating 85 bar as the most citral-favourable condition for lemongrass PA within the tested range.

Across conditions, lemongrass PA generally yielded higher citral concentrations than lemongrass G, particularly at 85 bar. This difference may relate to compositional and anatomical variation between varieties, given that citral is primarily concentrated in leaf tissues. Furthermore, the marked reduction in citral abundance at higher pressure in lemongrass PA suggests either (i) reduced selectivity due to increased co-extraction of non-target constituents, and/or (ii) partial losses of citral during high-pressure extraction and recovery. Although extraction temperature was held constant, citral is volatile and can be sensitive to processing conditions, and extraction parameter optimisation remains critical for maintaining volatile retention (Aytac et al., 2018). Recent reviews similarly emphasise that pressure plays a key role in determining essential oil composition

and the preservation of volatile compounds, particularly within CO_2-based extraction systems (Ashaq et al., 2024; Okpo & Edeh, 2023).

The influence of citral content on biological activity was also reflected in the antimicrobial assays. Among the tested extracts, lemongrass PA at 85 bar exhibited the strongest antimicrobial activity, particularly against Gram-positive bacteria such as *B. cereus* and *S. aureus* (Table 3.3 and Table 3.4). This extract showed the largest inhibition zones and the lowest MIC and MBC values, indicating strong bactericidal activity. The superior performance of lemongrass PA 85 may be linked to its higher citral content, supporting the well-documented role of citral as a key antimicrobial agent in LEO. Recent studies have demonstrated citral's effectiveness in disrupting bacterial membranes and inhibiting critical biosynthetic pathways in Gram-positive pathogens particularly pathways involved in cell envelope formation, including fatty acid biosynthesis and peptidoglycan biosynthesis, thereby compromising membrane integrity and bacterial survival (Gao et al., 2020; Kabotso et al., 2022). Furthermore, this observation is consistent with findings by Subramaniam et al. (2020), who reported that LEO exhibits greater antimicrobial activity against Gram-positive bacteria compared to Gram-negative strains, due to differences in cell wall structure and permeability. These findings support the conclusion that lemongrass PA extracted at 85 bar represents the most effective extract for antimicrobial applications, particularly for targeting Gram-positive pathogens.

The MIC results in Table 3.5 indicate that emulsions of both lemongrass and citral demonstrated enhanced antimicrobial activity against *B. cereus* compared to their respective water-based crude extracts. Specifically, the MIC of lemongrass emulsion (0.125%) was five times lower than that of lemongrass extract (0.630% (v/v)), while the citral emulsion (0.125% (v/v)) exhibited an MIC that was 1.28 times lower than the citral extract (0.160% (v/v)). These results suggest that emulsification significantly improves the efficacy of the active compounds. The enhanced performance of emulsions can be attributed to their ability to better dispersion of hydrophobic compounds like citral in aqueous environments, increasing their interaction with bacterial membranes. This is consistent with the findings of Alarcón-Moyano and Matiacevich (2019), who reported that emulsions

containing lemongrass and citral showed significant antimicrobial effects regardless of encapsulating agents used, reinforcing their potential as natural antimicrobial additives. Moreover, emulsions offer improved stability and control over the release of volatile compounds, making them particularly suitable for food preservation and other practical applications. Recent work by Jiang et al. (2020) demonstrated that emulsions not only improved the dispersibility of essential oils but also provided sustained antimicrobial activity and better performance during storage when applied to food systems.

The antimicrobial effectiveness of lemongrass and citral emulsions at concentrations ranging from 0.25% (v/v) to 2.00% (v/v) against *B. cereus* isolates (P4, M2, and ATCC 14579) demonstrated clear isolate-dependent responses. Among the three, isolates, P4 consistently showed greater resistance, requiring a 2.00% concentration to achieve a 1.00 $\log_{10}$ CFU/mL reduction, while M2 and ATCC 14579 reached complete inhibition (<1.00 $\log_{10}$ CFU/mL) at the same concentration (Table 3.6). Both lemongrass and citral emulsions exhibited concentration-dependent activity, with higher concentrations leading to stronger bacterial reduction across all isolates. Interestingly, *B. cereus* exhibited greater resistance in rice milk (Table 3.7) compared to soy milk (Table 3.6), which may be linked to its preference for starchy environments. Ingredient analysis (Table 3.11 in supplementary material) shows that rice milk contains higher carbohydrate content than soy milk, potentially providing more nutrients and protective effects that support *B. cereus* survival. This is supported by studies showing that rice and other carbohydrate-rich foods offer favorable growth conditions for *B. cereus* due to the presence of resistant spores and high starch content that facilitate survival and toxin production (Leong et al., 2023; Navaneethan & Effarizah, 2021).

Based on the results in Tables 3.8 to 3.10, the antimicrobial efficacy of lemongrass and citral emulsions against *B. cereus* was influenced by multiple factors, including concentration, isolate type, and possibly the surrounding matrix. Both emulsions showed a consistent concentration-dependent response, with greater reductions in bacterial counts at higher emulsion concentrations. Complete inhibition (<1.00 $\log_{10}$ CFU/mL) was achieved at 2.00% (v/v) concentration for *B. cereus* ATCC 14579 and isolate M2, while isolate P4 exhibited greater resistance,

maintaining higher bacterial counts under the same treatment conditions. This isolate-dependent response reflects the normal strain-dependent variability typical of prokaryotes, resulting in differences in susceptibility to antimicrobial compounds (Lim et al., 2021).

In addition to these factors, droplet size may contribute to the observed antibacterial performance. Smaller nanoemulsion droplets provide a larger surface area for contact with bacterial cells, potentially enhancing the transfer of lipophilic antimicrobial compounds (citral) to the bacterial membrane (Lu et al., 2018). Moreover, smaller droplets exhibit greater Brownian motion, which helps maintain a homogeneous dispersion in the medium and increases droplet–cell encounter frequency, thereby supporting antimicrobial action. Conversely, increases in droplet size due to coalescence or aggregation may reduce dispersion stability and limit active compound bioavailability, which may help explain the decline in antibacterial efficacy observed during extended storage.

Finally, protein concentration in the medium adjusted through different levels of milk protein concentrate (MPC) did not significantly influence antibacterial outcomes ($p > 0.05$). This is supported by Safitri et al. (2022), who found that increasing whey protein isolate concentrations significantly influenced viscosity and antioxidant properties but had no significant effect on antibacterial activity against *E. coli* and *Lactobacillus casei*, suggesting the dominance of other factors like emulsion concentration and active compound properties. This finding is further supported by Xie et al. (2025), who demonstrated that while soy protein isolates influenced emulsion stability, it did not significantly alter the antimicrobial efficacy of tea tree oil emulsions, which remained concentration-dependent based on the active compound itself.

3.5 Conclusion

This study highlights that the extraction yield, chemical composition, and antimicrobial efficacy of lemongrass are strongly influenced by variety, pressure, formulation, and bacterial strain. Lemongrass G exhibited higher overall extraction yields, likely reflecting its greater extractable biomass, whereas lemongrass PA

showed higher citral content, particularly at 85 bar. However, the effect of pressure on citral abundance was variety-dependent, lower pressure favoured higher citral content in lemongrass PA, while citral levels in lemongrass G were relatively stable across the tested pressures. This pattern likely reflects pressure-dependent changes in CO_2 solvating power and co-extraction of non-volatile constituents at higher pressure, which can dilute proportional citral content in the extract. Citral-rich extracts, especially lemongrass PA at 85 bar, showed the strongest antimicrobial activity, notably against Gram-positive bacteria, reinforcing citral's role as a key antimicrobial component. Emulsions of lemongrass and citral outperformed crude extracts due to better dispersion and stability, and both demonstrated clear concentration-dependent inhibition. The antimicrobial effectiveness was also isolate-dependent, with isolate P4 being the most resistant. Notably, *B. cereus* showed greater resistance in rice milk than soy milk, likely due to the protective effect of higher carbohydrate content. Despite variations in milk protein concentrate levels, protein concentration did not significantly affect antibacterial outcomes, affirming concentration and formulation as the primary factors influencing antimicrobial activity. For future research, exploring nanoemulsion systems may offer even greater antimicrobial efficiency, due to their smaller droplet sizes, enhanced bioavailability, and improved delivery of essential oil components making them promising candidates for food preservation and pharmaceutical applications.

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3.7 Copyright information

Parts of this study is intended to be submitted to a journal for publication and the Online Statement of Contribution form is attached in Appendix II.

3.8 References

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3.9 Supplementary material

Figure 3.1. Morphological comparison of two lemongrass varieties. Lemongrass Gajah with an average stem circumference of 8.5 cm measured 1 cm above the root (A) and lemongrass Peha Ayam with an average stem circumference of 5.5 cm (B) at the same measurement point.

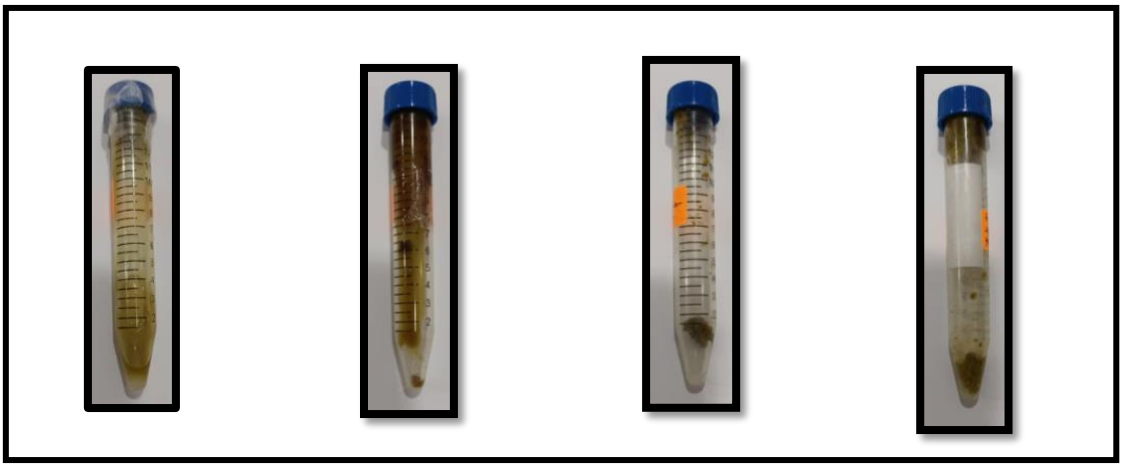

Figure 3.2. Effect of different pressures, 85 bar (A); 100 bar (B); 200 bar (C) and 300 bar (D) on the physical form of lemongrass (G) extract at 40°C.

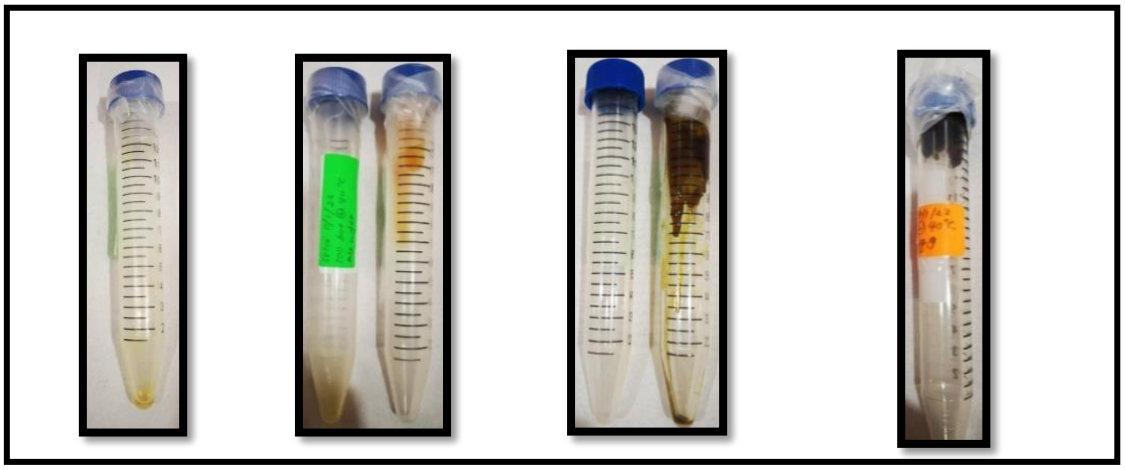

Figure 3.3. Effect of different pressures, 85 bar (A); 100 bar (B); 200 bar (C) and 300 bar (D) on the physical form of lemongrass (PA) extract at 40°C.

Table 3.11. Nutritional composition of soy milk, rice milk, and milk protein concentrate (MPC).

	Soy milk (g/100mL)	**Rice milk (g/100mL)**	**MPC (% m/m)**
Protein	3.0	0.7	85.01
Fat	3.8	1.4	1.37
Carbohydrates	4.0	10.7	-
Lactose	nd	nd	2.35

Preface

In Chapter 3, the potential of supercritical fluid extraction (SFE) for obtaining high citral content from lemongrass was highlighted, with emphasis on the role of optimal pressure and temperature conditions. Among the two varieties tested, lemongrass PA extracted at 85 bar and 40°C yielded the highest citral concentration and demonstrated the highest antibacterial activity. This optimal condition and variety were therefore selected for all subsequent experimental chapters. The chapter also compared the antibacterial efficacy of raw extracts to emulsion forms of lemongrass in food system applications. Building on these findings, Chapter 4 further investigates the nanoemulsion formulations of lemongrass extract and citral standard, alongside nisin as the established food preservatives widely used in the food industry. This chapter focuses on their antibacterial activity against *B. cereus*, particularly their effects on planktonic cells, biofilm control, and biofilm prevention, offering deeper insight into their potential application in food safety.

Chapter 4. Lemongrass nanoemulsion, citral nanoemulsion and nisin: antibacterial strategies against planktonic and biofilm *Bacillus cereus.*

4.1 Introduction

Bacillus cereus is a Gram-positive, spore-forming bacterium widely recognized for its role in foodborne illnesses and spoilage of processed foods, particularly dairy, rice, and meat products (Cayemitte et al., 2022). It produces heat-resistant endospores and various enterotoxins that can cause diarrhoea or emesis, posing serious health risks to consumers (Gharib et al., 2020). In food processing environments, *B. cereus* exists in two phenotypic states: planktonic (free-living) cells and biofilm-associated cells. Planktonic cells are metabolically active and susceptible to conventional antimicrobial agents. However, once attached to surfaces, *B. cereus* forms biofilms structured communities of cells embedded in extracellular polymeric substances (EPS) that confer enhanced resistance to cleaning, disinfection, and host defenses (Park et al., 2019).

Biofilm formation on food contact surfaces such as stainless steel, plastic, and rubber increases the likelihood of persistent contamination and cross-contamination during food handling and storage. The resilience of *B. cereus* biofilms is attributed to reduced diffusion of antimicrobials through the EPS, altered metabolic states of sessile cells, and the presence of dormant persister populations (Crabbe et al., 2019). These characteristics complicate control using conventional chemical sanitizers or preservatives, which may also pose regulatory, toxicological, or consumer acceptance concerns.

In response to these limitations, there is growing interest in plant-derived antimicrobials as safer, natural alternatives to synthetic preservatives. Lemongrass (*Cymbopogon citratus*) essential oil and its dominant constituent citral (a mixture of geranial and neral isomers) have demonstrated broad-spectrum antimicrobial activity against a variety of foodborne pathogens (Fernandes et al., 2022). However, essential oils in their native form often exhibit volatility, poor water solubility, and instability in food matrices (Zhu et al., 2021). Nanoemulsion-based formulations have emerged as a promising strategy to overcome these limitations by enhancing dispersion, protecting active components, and improving interactions with microbial membranes (Garcia et al., 2022; McClements et al., 2021).

While lemongrass and citral nanoemulsions have been shown to inhibit microbial growth in various models, comparative data on their efficacy against both planktonic and biofilm states of *B. cereus* are limited. Furthermore, the use of nisin, a well-characterized, Generally Recognized as Safe (GRAS) antimicrobial peptide widely used in the food industry as a comparator provides a valuable benchmark to assess the potential of plant-based nanoformulations (Ibrahim, 2019). Therefore, this study aimed to evaluate the antibacterial and antibiofilm efficacy of lemongrass and citral nanoemulsions in comparison with nisin across multiple *B. cereus* isolates. The findings offer new insights into the applicability of nanoemulsified plant extracts as effective, natural antimicrobials for controlling both planktonic and biofilm forms of *B. cereus* in food.

4.2 Materials and methods

4.2.1 Sample

Raw lemongrass (Variety of Peha Ayam) was purchased from farmer in Beranang, Selangor, Malaysia and the plant taxonomic identification was verified in Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) under the voucher specimen number of MFI 0217/21. A commercial sample of citral, (Sigma-aldrich CAS number: 5392-40-5, MO, USA) a pure compound, is utilized as a benchmark to evaluate and compare its activity against lemongrass extract.

4.2.2 Extract Preparation

Lemongrass extracts used in this chapter were prepared as described in Chapter 3, Section 3.2.2 (Extract preparation).

4.2.3 Supercritical Fluid Extraction

Supercritical fluid extraction procedures and operating parameters were conducted as described in Chapter 3, Section 3.2.3 (Supercritical fluid extraction) with the modification that only the 85 bar pressure condition was used in this chapter.

4.2.4 Nanoemulsion Formation

A 2.0% (w/w) sodium alginate solution was prepared by dissolving sodium alginate (Ajax Finechem, Auckland, New Zealand) in Milli-Q water at 70°C, with continuous stirring overnight to ensure complete dissolution. The solution was then allowed to cool to room temperature (approximately 21°C). To form the primary emulsion, the sodium alginate solution was blended with 2.0% lemongrass extract and Tween 80 using a high-shear laboratory homogenizer (T-25 Digital ULTRA-TURRAX, IKA, Staufen, Germany) at 13,500 rpm for 2 to 3 minutes with 30-second intervals. The volume ratio of extract to Tween 80 was maintained at 1:3, as described by Gago et al. (2019). The resulting coarse emulsions were further processed using a microfluidizer (M-110P, Microfluidics, USA) at 150 MPa for three cycles to produce the nanoemulsions. To maintain thermal stability, the microfluidizer's external coil was immersed in ice, ensuring the outlet temperature remained at approximately 10°C. The final nanoemulsions were collected in capped plastic tubes and stored at 4°C in the dark to prevent degradation.

4.2.5 Bacterial Strains and Culture Conditions

Three *B. cereus* isolates were used in this study: a reference isolate (*B. cereus* ATCC 14579) and food isolates obtained from potato (P4) and milk (M2). All isolates were provided by the Food Microbiology Laboratory, Massey University. Prior to each experiment, frozen stock cultures (stored at –80°C) were revived in brain heart infusion (BHI) broth (Bacto™, Becton, Dickinson and Company, USA) and incubated at 30°C for 18–24 h. The cultures were then streaked onto tryptone soy agar (TSA) plates (Difco™, Becton, Dickinson and Company, USA) and incubated under the same conditions. A single colony from each plate was transferred into 5 mL of fresh BHI broth and incubated at 30°C for another 18–24 h to serve as the working culture.

Following incubation, cultures were centrifuged (Sigma® 6–16, John Morris Scientific Ltd., New Zealand) at 5,000 × *g* for 10 min at room temperature (21 ± 2°C). The resulting cell pellets were washed with 0.85% sterile saline and resuspended to achieve a final concentration of 6 $\log_{10}$ CFU/mL. To verify cell density, optical density (OD) at 600 nm was measured using a spectrophotometer (Varioskan™ Lux, Thermo Fisher Scientific, Massachusetts, USA). A standard

calibration curve correlating OD_{600} readings with CFU/mL values was used to estimate bacterial concentrations.

4.2.6 Nisin Preparation

A commercial nisin preparation (Nisaplin®, Danisco, Grindsted, Denmark), containing ≥1000 IU/mg of nisin and approximately 50% sodium chloride, was used in this study. A stock solution was prepared by dissolving 40,000 µg/mL of nisin in 0.02 M hydrochloric acid (HCl), and the pH was adjusted to 6.0. The solution was then sterilized using a 0.2 µm pore-size syringe filter and ready to be used.

4.2.7 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using a 96-well microtiter plate following a two-fold serial dilution. A standardised bacterial inoculum (6–7 $\log_{10}$ CFU/mL) was prepared in Brain Heart Infusion (BHI) broth. For each well, 100 µL of inoculated BHI broth was mixed with 100 µL of lemongrass or citral nanoemulsion. Two-fold serial dilutions were then prepared by transferring 100 µL from one well to the next across the plate, and 100 µL was discarded from the final well to ensure a uniform final volume of 100 µL per well. Lemongrass and citral nanoemulsions (initial concentration: 2.00%) were diluted in Brain Heart Infusion (BHI) broth containing the bacterial inoculum. Column 12 contained the highest concentration of the antimicrobial agents (1.00%) and column 3 contained the lowest concentration (0.02%). Column 1 served as the negative control (BHI only, without inoculum or antimicrobial agent), while column 2 acted as the positive control (inoculum without antimicrobial agent). After 24 h of incubation at 30°C, bacterial growth was assessed by measuring optical density at 590 nm using a microplate reader (Varioskan™ LUX, Thermo Fisher Scientific, UK). All assays were performed in triplicate. The MIC was defined as the lowest concentration of the test compound that resulted in no visible bacterial growth, corresponding to an OD590 change of less than 0.05 compared to the negative control. MIC values were expressed as percentage concentration (% v/v) because the test samples were nanoemulsion formulations rather than purified compounds. All assays were performed in triplicate.

4.2.8 Planktonic Inhibition Assay

The antimicrobial efficacy of nisin, lemongrass and citral nanoemulsions was assessed based on their ability to inactivate *B. cereus*. Briefly, 0.1 mL of the working culture prepared as mentioned in section 4.2.5 was mixed with 0.1 mL of either nisin, lemongrass or citral nanoemulsion in a 1.5 mL Eppendorf tube. The mixtures were incubated at 30°C for 1 h, after which they were centrifuged at 12 000 × *g* for 5 min at 4°C (Sigma® 6–16, John Morris Scientific Ltd., New Zealand). The resulting pellets were washed with 0.85% sterile saline, vortexed for 30 seconds (Scilogex, Germany), and re-centrifuged at 5 000 × *g* for 5 min. The final pellets were resuspended in fresh sterile saline. Serial dilutions of the treated bacterial suspensions were prepared and plated onto Tryptic Soy Agar (TSA) (Difco™, Becton, Dickinson and Company, USA), followed by incubation at 30°C for 24 h. A negative control was included using sterile saline in place of nanoemulsions. Colony-forming units per milliliter (CFU/mL) were calculated using the standard CFU formula. CFU/mL = (Number of colonies x dilution factor) / volume of culture plate. The detection limit of the plating method was 1 $\log_{10}$ CFU/mL.

4.2.9 Biofilm control assay

To assess the ability of the test agents to disrupt established biofilms, a biofilm control assay was conducted. Briefly, 0.1 mL of the working bacterial culture (prepared as described in Section 4.2.5) was inoculated into wells of a sterile 96-well microtiter plate and incubated at 30°C for 24 h to allow biofilm formation. After incubation, the supernatant was carefully removed, and each well was washed three times with sterile distilled water to remove non-adherent cells. The plate was then inverted and air-dried at room temperature (21 ± 2°C) for 30 min. Subsequently, various concentrations of the antimicrobial agents were added to the wells containing pre-formed biofilms and incubated at 30°C for 1 h. After treatment, the antimicrobial solutions were discarded, and the wells were again

washed three times with sterile distilled water and air-dried at room temperature for another 30 min.

The remaining biofilm in each well was dislodged using a sterile cotton swab, which was then immersed into 0.1 mL of 0.85% sterile saline to resuspend the cells. Serial dilutions of these suspensions were plated on Tryptic Soy Agar (TSA) (Difco™, Becton, Dickinson and Company, USA) and incubated at 30°C for 24 h. A negative control was included by replacing the nanoemulsions with sterile saline. The number of viable cells was quantified and expressed as colony-forming units per square centimeter (CFU/cm^2), calculated using the standard CFU formula; CFU/cm^2=(Number of colonies×Dilution factor)/(Volume plated (mL)×Area sam pled (cm^2)). The detection limit of this method was 2.50 $\log_{10}$ CFU/cm^2.

4.2.10 Biofilm Prevention Assay

The biofilm prevention assay was performed to evaluate whether pre-coating surfaces with the test agents could inhibit the initial formation of *B. cereus* biofilms. Briefly, 0.1 mL of varying concentrations of nisin, lemongrass nanoemulsion, or citral nanoemulsion was added to wells of a sterile 96-well microtiter plate and incubated at 30°C for 1 h to allow surface adsorption. Following incubation, the wells were gently rinsed three times with sterile distilled water to remove any unbound compounds, then air-dried at room temperature (21 ± 2°C) for 30 min. Subsequently, 0.1 mL of the working bacterial culture was added to each pre-coated well and incubated at 30°C for 24 h to allow biofilm development. After incubation, the supernatants were removed, and the wells were washed three times with sterile distilled water and air-dried again for 30 min.

To recover the attached cells, the biofilms were dislodged using a sterile cotton swab and resuspended in 0.1 mL of 0.85% sterile saline. Serial dilutions were prepared and plated on Tryptic Soy Agar (TSA) (Difco™, Becton, Dickinson and Company, USA), followed by incubation at 30°C for 24 h. A negative control was included by substituting the antimicrobial agents with sterile saline. Viable cell counts were expressed as colony-forming units per square centimeter (CFU/cm^2),

calculated using the standard CFU formula (refer to section 4.2.9). The detection limit of the assay was 2.50 $\log_{10}$ CFU/cm^2.

4.2.11 Statistical analysis

All experiments were performed in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) (IBM SPSS version 29) and, where necessary, the Tukey test ($\alpha = 0.05$) or t-test ($\alpha = 0.05$). All results were expressed as mean ± standard deviation.

4.3 Results

4.3.1 Determination of Minimum Inhibitory Concentration (MIC)

Table 4.1 presents the minimum inhibitory concentrations (MICs) of lemongrass nanoemulsion, citral nanoemulsion, and nisin against *B. cereus* isolates ATCC 14579, P4, and M2. The MICs for both lemongrass and citral nanoemulsions were consistent at 0.125% (v/v) across all three isolates, suggesting strong and uniform antimicrobial potential. Nisin showed an MIC of 2 500 µg/mL for all isolates. While the MIC values indicate effective inhibition for each treatment, direct comparison across agents is limited due to differences in concentration units and formulation types. To enable more consistent comparisons, subsequent assays were conducted using standardized multiples of MIC (1 to 5MIC). 1MIC–5MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v), 5MIC = 2.000% (v/v)).

Table 4.1. Minimum inhibitory concentrations (MICs) of lemongrass nanoemulsion, citral nanoemulsion, and nisin against *B. cereus* isolates (P4, M2, and ATCC 14579).

Antimicrobial agent	*B. cereus* isolates		
	ATCC 14579	P4	M2
Lemongrass nanoemulsion	0.125% (v/v)	0.125% (v/v)	0.125% (v/v)
Citral nanoemulsion	0.125% (v/v)	0.125% (v/v)	0.125% (v/v)
Nisin	2 500 µg/mL	2 500 µg/mL	2 500 µg/mL

4.3.2 Planktonic inhibition assay

A heatmap of Log_{10} CFU/mL values illustrates the antimicrobial effects of lemongrass nanoemulsion, citral nanoemulsion, and nisin against planktonic *B. cereus* isolates (P4, M2, and ATCC 14579) (Figure 4.1). All three treatments showed a dose-dependent reduction in CFU/mL across isolates. For isolate P4, log reduction of ≤ 5.87 Log_{10} CFU/mL was achieved by 3 MIC for all treatments, with citral and nisin reaching < 1 Log_{10} CFU/mL as early as 3 MIC. Isolate M2 showed moderate sensitivity, with lemongrass and nisin achieving < 7.18 Log_{10} CFU/mL reduction by 3MIC, while citral required up to 4 MIC for a similar effect. In contrast, ATCC 14579 was more resistant, with only nisin reducing counts to $<$ 1.00 Log_{10} CFU/mL by 3MIC, whereas lemongrass and citral showed partial reduction, maintaining values of > 2 Log_{10} CFU/mL at 5MIC. These results suggest that citral and lemongrass are highly effective against isolate P4 and M2, while nisin demonstrates the broadest spectrum of activity, including partial efficacy against the more resistant ATCC 14579 isolate.

Isolates	Treatment	Control	1MIC	2MIC	3MIC	4MIC	5MIC
P4	LG	6.87	3.00	3.44	1.00	< 1.00	< 1.00
	Citral	6.87	3.23	2.54	< 1.00	< 1.00	< 1.00
	Nisin	6.87	3.00	2.15	< 1.00	< 1.00	< 1.00
M2	LG	7.18	3.47	3.47	< 1.00	< 1.00	< 1.00
	Citral	7.18	5.15	4.30	3.33	< 1.00	< 1.00
	Nisin	7.18	3.66	3.26	< 1.00	< 1.00	< 1.00
ATCC 14579	LG	6.4	5.14	3.19	3.2	2.33	2.06
	Citral	6.4	3.77	2.23	2.84	2.67	2.43
	Nisin	6.4	3.48	3.30	< 1.00	< 1.00	< 1.00

Figure 4.1. Heatmap of the Log_{10} CFU/mL count showing the antimicrobial effects of lemongrass nanoemulsion (LG), citral nanoemulsion, and nisin against planktonic *B. cereus* isolates (P4, M2 and ATCC 14579) at increasing concentrations (1MIC to 5MIC) compared to control.

4.3.3 Biofilm control

Figure 4.2 shows a heatmap of Log_{10} CFU/cm^2 values representing the antibiofilm activity of lemongrass, citral, and nisin against *B. cereus* isolates (P4, M2, and ATCC 14579). A clear dose-dependent reduction was observed for lemongrass and citral in both isolates P4 and M2, with CFU/cm^2 counts dropping to 2.49 Log_{10} CFU/cm^2 from 2 MIC onwards, indicating strong biofilm disruption. In contrast, nisin showed a more gradual effect, requiring 5MIC to reduce isolate P4 biofilm counts < 3.00 Log_{10} CFU/cm^2. For isolate ATCC 14579, none of the treatments achieved < 2.88 Log_{10} CFU/cm^2 suggesting higher resistance. Overall, lemongrass and citral nanoemulsions were more effective in disrupting biofilm formation in isolate P4 and M2 compared to ATCC 14579.

Strain	Treatment	Control	1MIC	2MIC	3MIC	4MIC	5MIC
	LG	6.59	3.79	< 2.50	< 2.50	< 2.50	< 2.50
	Citral	6.59	3.72	< 2.50	< 2.50	< 2.50	< 2.50
P4	Nisin	6.59	4.94	4.64	4.95	3.34	2.96
	LG	6.73	4.79	3.96	< 2.50	< 2.50	< 2.50
	Citral	6.73	5.91	4.12	3.99	< 2.50	< 2.50
M2	Nisin	6.73	5.49	5.19	5.36	5.26	4.96
	LG	6.49	5.16	5.76	3.88	4.23	2.88
ATCC	Citral	6.49	6.08	5.87	3.03	3.15	3.03
14579	Nisin	6.49	5.64	5.42	5.11	5.32	4.97

Figure 4.2. Heatmap of the Log_{10} CFU/cm^2 count showing the biofilm control of lemongrass nanoemulsion (LG), citral nanoemulsion, and nisin against *B. cereus* isolates (P4, M2 and ATCC 14579) at increasing concentrations (1MIC to 5MIC) compared to control.

4.3.4 Biofilm prevention

Figure 4.3 displays a heatmap of Log_{10} CFU/cm^2 values representing the biofilm prevention effects of lemongrass nanoemulsion (LG), citral nanoemulsion, and nisin against *B. cereus* isolates P4, M2, and ATCC 14579. Across all isolates, lemongrass showed a more pronounced reduction in biofilm formation compared to citral and nisin. In isolate P4, lemongrass reduced CFU/cm^2 from 5.04 Log_{10} to 3.59 Log_{10} between 2MIC and 5MIC, while citral and nisin maintained higher counts, with minimal reduction below 5.00 Log_{10} CFU/cm^2. Similarly, isolate M2 responded better to lemongrass, reaching 3.49 Log_{10} CFU/cm^2 at 5MIC, whereas citral and nisin showed only mild reductions.

For isolate ATCC 14579, all treatments showed modest effects, with lemongrass and citral reducing counts to ~3.6–3.7 Log_{10} CFU/cm^2 at 5MIC, while nisin remained largely ineffective, with CFU/cm^2 above 5.5 Log_{10} even at the highest concentration. These findings suggest that lemongrass nanoemulsion is the most effective agent in preventing biofilm formation, particularly for the more susceptible isolates P4 and M2, while nisin was the least effective across all strains.

Strain	Treatment	Control	1MIC	2MIC	3MIC	4MIC	5MIC
P4	LG	6.88	4.59	5.04	3.73	4.96	3.59
	Citral	6.88	6.36	5.38	5.38	5.25	3.88
	Nisin	6.88	6.59	6.06	6.37	5.64	5.65
M2	LG	7.01	5.79	5.64	4.30	3.64	3.49
	Citral	7.01	6.02	5.36	5.03	5.55	4.50
	Nisin	7.01	6.69	6.06	5.94	5.74	5.57
ATCC 14579	LG	7.11	4.99	5.04	5.29	4.10	3.76
	Citral	7.11	4.99	4.84	5.58	4.74	3.62
	Nisin	7.11	5.64	5.85	6.17	5.62	5.56

Figure 4.3. Heatmap of the Log_{10} CFU/cm^2 count showing the biofilm prevention of lemongrass nanoemulsion (LG), citral nanoemulsion, and nisin against *B. cereus* isolates (P4, M2 and ATCC 14579) at increasing concentrations (1MIC to 5MIC) compared to the untreated control.

4.4 Discussion

This study evaluated the antimicrobial and antibiofilm efficacy of lemongrass and citral nanoemulsions, benchmarked against the commercially approved peptide nisin, across three *B. cereus* isolates. The findings revealed distinct activity patterns between planktonic and biofilm states, reflecting the different mechanisms of activity of the two antimicrobials and microbial isolate variation.

The uniform MIC values (0.125% (v/v)) observed for both lemongrass and citral nanoemulsions across all *B. cereus* isolates underscore their consistent antimicrobial efficacy, likely attributed to the stability of the nanoemulsion and the high purity of citral. Citral, the primary bioactive component of lemongrass, is a monoterpene with well-documented broad-spectrum antimicrobial activity. Mechanistic studies by Gandhi et al. (2019) and Kusuma et al. (2024) have shown that monoterpenes such as citral exert their bactericidal effects by disrupting the integrity of the phospholipid bilayer in bacterial cell membranes and interfering with critical enzymatic systems, ultimately leading to membrane collapse and cell death. In contrast, nisin which is a cationic bacteriocin peptide, acts through a more

specific mechanism by binding with high affinity to lipid II, thereby facilitating pore formation and inhibiting cell wall biosynthesis (Shi et al., 2024). The distinct chemical structures and mechanisms of action between plant-derived monoterpenes and peptide-based antimicrobials likely account for their differing efficacy profiles across planktonic and biofilm-associated *B. cereus* populations.

While MIC results suggested comparable inhibitory thresholds, CFU-based assays revealed isolate-specific differences, particularly under biofilm conditions. In planktonic assays, isolate P4 demonstrated the highest susceptibility, with all treatments achieving ≤ 5.87 Log_{10} CFU/mL by 3MIC. Conversely, isolate ATCC 14579 showed greater resilience, where nisin reduced CFU counts to < 6.4 Log_{10} at 3MIC, while lemongrass and citral remained above 2.0 Log_{10} CFU/mL even at 5MIC. These variations may be attributed to differences in cell wall structure, efflux pump expression, or spore-forming capacity across isolates (Singh et al., 2017).

Biofilm assays revealed a more complex picture. Despite nisin's potent planktonic activity, its effectiveness markedly declined in biofilm settings, especially against isolate ATCC 14579. This finding is consistent with previous reports indicating that nisin exhibits limited antibiofilm activity against pre-formed biofilms. Nisin was included as a comparator in this study because it is a widely used food-grade bacteriocin (E234) with well-established activity against Gram-positive bacteria and is commonly applied in food preservation systems (De Arauz et al., 2009). However, nisin is widely reported to exhibit limited antibiofilm activity against pre-formed (mature) biofilms, largely due to the protective extracellular polymeric substance (EPS) matrix and the presence of slow-growing or persister subpopulations within biofilm structures (Angelopoulou et al., 2020). Importantly, antibiofilm effects of nisin are often observed under prolonged exposure durations (typically 24–48 h) and/or when applied in combination with other approaches (e.g., membrane-disrupting compounds, enzymes, chelating agents, or physical disruption strategies) to enhance penetration and activity (Ghapanvari et al., 2022).

In the present study, the exposure duration was only 1 h, which may have been insufficient for nisin to exert meaningful disruption or killing within established

biofilms. Therefore, any comparatively lower antibiofilm performance of nisin in this study should be interpreted in light of its known limitations under short exposure conditions, rather than as evidence of poor intrinsic antimicrobial efficacy. The inclusion of nisin nevertheless provides a useful benchmark and highlights the potential of lemongrass formulations to exert more rapid effects on biofilm-associated cells.

This lowered efficacy may result from limited peptide diffusion through the EPS matrix, reduced expression of lipid II in metabolically dormant cells, and potential protease-mediated degradation which is a common feature of mature biofilms (Jancic & Gorgieva, 2021). In contrast, lemongrass and citral nanoemulsions demonstrated stronger activity in both the biofilm control assay (treatment of pre-formed biofilms) and the biofilm prevention assay using a conditioning-film approach. In particular, surface pre-conditioning with lemongrass nanoemulsion reduced CFU recovery following biofilm challenge in isolates P4 and M2, suggesting that conditioning the surface may reduce subsequent bacterial attachment and biofilm accumulation, potentially through disruption of adhesion-related processes or early matrix establishment.

Interestingly, this study found that biofilm control (treatment of established biofilms) sometimes resulted in greater CFU reduction than biofilm prevention (surface pre-coating before biofilm initiation). This may be due to longer and more direct exposure of mature biofilms to antimicrobials in control assays, allowing sustained interaction with bacterial cells over 24 h. In contrast, prevention assays typically involve brief contact of the substrate surface with the antimicrobial agent (1 h), before bacterial introduction, where agents may degrade, become diluted, or fail to remain bioactive throughout the adhesion and early matrix formation. A recent study confirmed that certain antimicrobials show higher efficacy against mature biofilms than in prevention settings, possibly due to insufficient agent retention on pre-treated surfaces and early quorum sensing events proceeding without interference (Su et al., 2022). These findings underscore the importance of optimizing formulation stability and delivery strategies to maintain antimicrobial activity during early biofilm development.

Despite a general dose-dependent reduction in CFU counts, fluctuations were observed at higher antimicrobial concentrations. Such variability reflects the metabolic and structural heterogeneity within biofilms, where gradients of oxygen, nutrients, and signalling molecules create microenvironments with variable antimicrobial susceptibility. These observations align with prior work suggesting that subpopulations within biofilms exhibit differential responses due to distinct phenotypic states, including persister cells and localized antimicrobial tolerance (Crabbe et al., 2019).

Given its GRAS status and widespread food applications, nisin served as a relevant benchmark in this study (Cedillo Olivos et al., 2024). While nisin's superior planktonic activity reaffirmed its utility against actively dividing *B. cereus*, the nanoemulsions outperformed it in biofilm control and prevention. These findings support the growing interest in plant-derived nanoformulations as promising, multifunctional alternatives to peptide-based antimicrobials, particularly in biofilm-prone applications such as food contact surfaces and medical devices.

4.5 Conclusion

This study demonstrated that lemongrass and citral nanoemulsions possess strong and consistent antimicrobial activity against *B. cereus* isolates, with MIC values of 0.125% (v/v) across all strains. Both nanoemulsions effectively reduced planktonic bacterial counts and disrupted biofilms, particularly in the more susceptible isolates P4 and M2.

Taken together, this study highlights the need for context-specific antimicrobial strategies that account for microbial phenotype and the delivery environment. While nisin remains effective in planktonic contexts, lemongrass and citral nanoemulsions show enhanced promise for biofilm-targeted interventions. Future studies should explore time-kill dynamics, surface-coating performance, and nanoemulsion stability across varying environmental conditions to support translational application in food safety and pharmaceutical systems.

4.6 Copyright information

Parts of this study is intended to be submitted to a journal for publication and the Online Statement of Contribution form is attached in Appendix III.

4.7 References

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4.8 Supplementary material

Table 4.2. Antimicrobial activity of lemongrass nanoemulsion (LG), citral nanoemulsion, and nisin against planktonic *B. cereus* isolates (P4, M2, and ATCC 14579).

Isolates	**P4**					**M2**					**ATCC 14579**				
	1 MIC	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**	**1 MIC**	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**	**1 MIC**	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**
Control	6.87 ± 0.39					7.18 ± 0.11					6.40 ± 0.12				
LG	3.00 ± 0.00	3.44 ± 0.05	1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	3.47 ± 0.67	3.47 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	5.14 ± 0.15	3.19 ± 0.65	3.20 ± 0.17	2.33 ± 0.58	2.06 ± 0.21
Citral	3.23 ± 0.34	2.54 ± 0.09	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	5.15 ± 0.43	4.30 ± 0.43	3.33 ± 0.20	<1.00 ± 0.00	<1.00 ± 0.00	3.77 ± 0.10	2.23 ± 0.40	2.84 ± 0.15	2.67 ± 0.58	2.43 ± 0.23
Nisin	3.00 ± 0.00	2.15 ± 0.21	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	3.66 ± 0.32	3.26 ± 0.24	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	3.48 ± 0.00	3.30 ± 0.42	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00

Table 4.3. Biofilm control activity of lemongrass nanoemulsion (LG), citral nanoemulsion, and nisin against planktonic *B. cereus* isolates (P4, M2, and ATCC 14579).

Isolates	**P4**					**M2**					**ATCC 14579**				
	1 MIC	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**	**1 MIC**	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**	**1 MIC**	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**
Control	6.59 ± 0.17					6.73 ± 0.34					6.49 ± 0.00				
LG	3.79 ± 0.43	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	4.79 ± 0.42	3.96 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	5.16 ± 0.41	5.76 ± 0.25	3.88 ± 0.55	4.23 ± 0.06	2.88 ± 0.12
Citral	3.72 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	5.91 ± 0.60	4.12 ± 0.21	3.99 ± 0.70	<2.50 ± 0.00	<2.50 ± 0.00	6.08 ± 0.16	5.87 ± 0.26	3.03 ± 0.08	3.15 ± 0.21	3.03 ± 0.34
Nisin	4.94 ± 0.63	4.64 ± 0.21	4.95 ± 0.45	3.34 ± 0.21	2.96 ± 0.67	5.49 ± 0.00	5.19 ± 0.00	5.36 ± 0.11	5.26 ± 0.10	4.96 ± 0.01	5.64 ± 0.21	5.42 ± 0.54	5.11 ± 0.17	5.32 ± 0.18	4.97 ± 0.04

Table 4.4. Biofilm prevention activity of lemongrass nanoemulsion (LG), citral nanoemulsion, and nisin against planktonic *B. cereus* isolates (P4, M2, and ATCC 14579).

Isolates	**P4**					**M2**					**ATCC 14579**				
	1 MIC	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**	**1 MIC**	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**	**1 MIC**	**2 MIC**	**3 MIC**	**4 MIC**	**5 MIC**
Control	6.88 ± 0.74					7.01 ± 0.33					7.11 ± 0.54				
LG	4.59 ± 0.17	5.04 ± 0.24	3.73 ± 0.34	4.96 ± 0.00	3.59 ± 0.17	5.79 ± 0.00	5.64 ± 0.21	4.30 ± 0.47	3.64 ± 0.21	3.49 ± 0.00	4.99 ± 0.71	5.04 ± 0.26	5.29 ± 0.14	4.10 ± 0.81	3.76 ± 0.36
Citral	6.36 ± 0.12	5.38 ± 0.27	5.38 ± 0.47	5.25 ± 1.57	3.88 ± 0.12	6.02 ± 0.09	5.36 ± 0.12	5.03 ± 0.09	5.55 ± 0.03	4.50 ± 0.15	4.99 ± 0.71	4.84 ± 0.50	5.58 ± 0.09	4.74 ± 1.10	3.62 ± 0.72
Nisin	6.59 ± 0.27	6.06 ± 0.10	6.37 ± 0.28	5.64 ± 0.04	5.65 ± 0.02	6.69 ± 0.34	6.06 ± 0.28	5.94 ± 0.64	5.74 ± 0.03	5.57 ± 0.03	5.64 ± 0.21	5.85 ± 0.02	6.17 ± 0.42	5.62 ± 0.07	5.56 ± 0.04

Preface

In Chapter 4, the antibacterial and antibiofilm activities of lemongrass nanoemulsion were explored, with findings indicating it demonstrated the most effective performance compared to citral and nisin. Building on these results, Chapter 5 investigates the stability of lemongrass nanoemulsion in comparison to citral standard nanoemulsion over a 6 month storage period. This chapter examines the sustained antibacterial efficacy against *B. cereus* isolates, thermal stability, and key physicochemical characteristics of the nanoemulsions, offering insights into their long-term viability as natural antimicrobial agents in food preservation.

Chapter 5. Characterization, antibacterial activity, and stability of supercritical fluid extracted lemongrass nanoemulsion on *Bacillus cereus.*

5.1 Introduction

Utilizing plant extracts as a natural food preservative has become an increasing trend as relatively cheap, readily available, environmentally friendly, natural compounds attractive to consumers and food manufacturers to enhance food safety and reduce food waste. As consumers demand healthier, less chemically processed food, plant-based preservatives present a promising alternative to synthetic chemicals. Among these, lemongrass (*C. citratus*) stands out due to its notable antimicrobial properties, primarily attributed to its high citral content, a compound recognized for its bactericidal efficacy against various fungi and foodborne pathogens including *B. cereus* (Gao et al., 2020). *B. cereus*, a spore-forming bacterium, is particularly challenging in food safety due to its ability to survive extreme environmental conditions, including heat, and its association with foodborne illnesses (Guo et al., 2021). It is known also as a significant biofilm former in dairy plants, posing a concern due to their resilience and prevalence in various milk products (Fei et al., 2019; Radmehr et al., 2020; Yang et al., 2023).

Lemongrass, beyond its antimicrobial capacity, offers other advantages when applied in food preservation. Faheem et al., (2022) highlighted that besides antimicrobial activity, lemongrass essential oil (LEO) also possesses antioxidant, antifungal, and anti-insecticidal properties, making it an excellent candidate for natural food preservative. It can be extracted using non-thermal techniques, such as supercritical fluid extraction (SFE), which preserve bioactive compounds more effectively than traditional thermal methods (Moreira et al., 2019). Nejia et al., (2013) found that compared to hydrodistillation, SFE produces 34% more essential oil and keeps more natural scents and compounds of Cupressus sempervirens. SFE of extraction *Pimenta Racemosa* performs better than steam distillation as it yields higher phenolic content in less time and offers greater flexibility for extracting diverse bioactive compounds (McGaw et al., 2016). With its high yield, enhanced selectivity, and stability, SFE is now a preferred green extraction method in pharmaceuticals, food, and cosmetics (Zhang et al., 2018). This preservation approach aligns well with the food industry's shift toward minimizing thermal processing to maintain the quality, nutritional profile, and functionality of natural preservatives (Herzyk et al., 2024). The incorporation of such compounds in

nanoemulsion form is increasingly recognized for enhancing stability, bioavailability, and controlled release, which may further extend shelf life and stability under various storage conditions (Barradas & de Holanda e Silva, 2020).

Nanoemulsions offer an advanced delivery system for essential oils like lemongrass, providing better stability against environmental factors such as light, oxygen, and temperature fluctuations (Liu et al., 2019). The nanoemulsion approach not only helps improve the longevity of active compounds but also strengthen their antimicrobial potential by facilitating more effective interactions with microbial cell walls (Mushtaq et al., 2023). The studies by da Silva Gundel et al., (2018) and Gago et al., (2019) demonstrated that lemongrass nanoemulsions significantly enhance the stability and antimicrobial activity of lemongrass oil, effectively targeting pathogens such as *P. aeruginosa*, *S. aureus*, and *E. coli*. However, these studies did not focus on the stability or efficacy of lemongrass nanoemulsions specifically against *B. cereus*, leaving a critical gap in the understanding of their potential application for combating this resilient foodborne pathogen.

This study aims to investigate the physicochemical characterization, storage stability, and antimicrobial efficacy of supercritical fluid-extracted lemongrass nanoemulsions and commercial sample of citral on *B. cereus*, with the goal of advancing food preservation methods that are both safe and effective. Ultimately, this research seeks to contribute to the development of sustainable natural food preservatives, providing a foundation for future studies on nanoemulsions of plant-based extracts as viable alternatives to synthetic additives in food preservation.

5.2 Materials and methods

5.2.1 Sample

Sample used in this chapter were prepared as described in Chapter 4, Section 4.2.1 (Sample).

5.2.2 Extract preparation

Lemongrass extracts used in this chapter were prepared as described in Chapter 3, Section 3.2.2 (Extract preparation).

5.2.3 Supercritical fluid extraction

Supercritical fluid extraction procedures and operating parameters were conducted as described in Chapter 3, Section 3.2.3 (Supercritical fluid extraction) with the modification that only the 85 bar pressure condition was used in this chapter.

5.2.4 Yield of lemongrass

Yield calculation and reporting were performed as described in Chapter 3, Section 3.2.4 (Yield of lemongrass extract). however, in this chapter, yield data are presented only for lemongrass PA.

5.2.5 Identification and quantification of active compounds in lemongrass extract using Gas chromatography mass spectrometry (GCMS)

LEO extract was dissolved in ethanol High Performance Liquid Chromatography (HPLC) grade (R & M Marketing, Essex, UK) to yield 5 mg/mL an aliquot of the extract was injected into a QP2010 Ultra gas chromatograph-mass spectrometer (Shimadzu Corporation, Kyoto, Japan) with a BP5MS column (30 m × 0.25 mm × 0.25 μm) for compound separation. Helium was used as the carrier gas at a flow rate of 3 mL/min. The oven injector temperature was 250°C; source temp 200°C. The oven temperature was 50°C, ramped up to 300°C at 15°C/min to and held for 10 min. The peaks were analysed by comparing their retention times and mass fragments patterns with standard spectra available in the Shimadzu GCMS NIST/ Wiley library.

5.2.6 Primary emulsion formation

A sodium alginate aqueous solution was made by dissolving 2.0% sodium alginate (w/w) (Ajax Finechem, Auckland, New Zealand) in miliQ water at 70°C with continuous overnight stirring until it was completely dissolved. Then, it was left to

cool at 21°C. The primary emulsion was formed by mixing the sodium alginate aqueous solution with 2.0% of lemongrass extract or citral and Tween 80 with a laboratory blender (T-25 digital Ultraturrax IKA, Staufen, Germany Ultraturrax) at 13 500 rpm for 2 to 3 min with 30 second intervals. The volume of Tween 80 used was 1:3 ratio of extract and Tween 80 following methodology proposed by (Gago et al., 2019).

5.2.7 Nanoemulsion formation

The primary emulsions were passed through the microfluidization process (M-110P, Microfluidics, USA) at 150 MPa for 3 cycles to obtain the nanoemulsions. The external coil of the microfluidizer was immersed in ice so that the nanoemulsions were cooled down at the outlet of the microfluidization unit and the temperature was kept at 10°C. Nanoemulsions were kept in capped plastic tubes and stored at 4°C room in the absence of light.

5.2.8 Nanoemulsions characterization

5.2.8.1 Droplet size, polydispersity and ζ-potential

The average droplet size of the nanoemulsions was determined by dynamic-light-scattering (DLS), using a Zetasizer Nano-ZS laser diffractometer (ATA Scientific, New South Wales, Australia), working at 633 nm and equipped with a backscatter detector (173°), which is used to specifically measure submicron particles. Polydispersity index (PDI), which represents the distribution of particle size, was also recorded from the instrument during the DLS measurement. PDI values near 1 indicate a heterogeneous or multimodal distribution of droplet sizes, whereas those near 0 give an idea of monomodal distribution (Gago et al., 2019). The electrophoretic mobility of oil droplets in nanoemulsions, also known as ζ-potential (mV) was determined by phase-analysis light scattering (PALS) using an automated capillary electrophoresis device (ATA Scientific, New South Wales, Australia), using a 633 nm laser at 25°C. This determines the surface electrical charge of the droplets dispersed in the continuous phase. Samples were prior diluted in Mili-Q water using a dilution ratio of 1: 9 sample to water. Measurements

of the parameters (droplet size polydispersity and ζ-potential) were performed over the following storage times: 0, 2, 4 and 6 months.

5.2.8.2 Antimicrobial activity.

The antimicrobial activity of lemongrass and citral nanoemulsions were evaluated using inactivation of *B. cereus*. Three isolates of *B. cereus* were used in this experiment: one reference isolate, (*B. cereus* ATCC 14579), one isolated from potato (*B. cereus* P4) and one isolated from milk (*B. cereus* M2). All isolates were provided by the Food Microbiology laboratory of Massey University. Briefly, *B. cereus* was cultured in Brain Heart Infusion (BHI) broth (BactoTM, Becton, Dickinson and Company, USA) at 30°C for overnight. Once the bacterial numbers reached 6 $\log_{10}$ CFU/mL, 0.1 mL of the bacterial culture was transferred to a 1.5 mL Eppendorf tube with 0.1 mL of lemongrass or citral nanoemulsions. To confirm that the culture reached 6 $\log_{10}$ CFU/mL, optical density (OD) measurement was performed using spectrophotometry (VarioskanTM Lux, Thermo Fisher Scientific, Massachussetts, USA) at 600 nm. A correlation curve between OD600 and CFU/mL was established, allowing the estimation of bacterial concentration based on absorbance readings. The Eppendorf tube was incubated at 30°C for 1 h, then centrifuged (Sigma® 6–16, John Morris Scientific Ltd., New Zealand) at 12 000 × *g* for 5 min at 4°C, the cell pellets after centrifugation were washed with sterile saline (0.85 %) via vortex (30 s) (Scilogex, Germany) and centrifugation (5 000 × *g* for 5 min). The concentrated cells were collected by removing saline and dissolving the final pellet in fresh sterile saline. Serial dilutions of the bacterial suspension were prepared and spread on Tryptic Soy Agar (TSA) (DifcoTM, Becton, Dickinson and Company, USA). The plates were incubated at 30°C for 24 h. A control was performed with the same method by replacing the nanoemulsions with sterile saline water. The colonies on the plates were counted and CFU/mL were calculated using the formula of CFU/mL; CFU/ml = (Number of colonies x dilution factor) / volume of culture plate.

The detection limit of the colonies on the plate is 1 Log_{10} CFU/mL. The antimicrobial activity determinations were performed just after preparation of the nanoemulsions and at each of the storage times (0, 2, 4 and 6 months).

5.2.8.3 Thermal stability

The stability of lemongrass and citral nanoemulsions were tested at different temperature and pHs according to the method described by Ramli et al., (2020), with slight modifications. For the thermal stability, nanoemulsions were exposed to the following temperatures: 30°C, 60°C and 90°C for 15 min each. A heat block (Techne® Dri-Block® OB-3, Watson Victor LTD, New Zealand) was pre-set at different temperatures (30°C, 60°C and 90°C) prior to each test. A thermometer was used to verify that the desired temperature was reached before treating the samples. After incubation, the samples were left at room temperature (21 ± 2°C) to cool completely before testing for their antimicrobial properties by using MICs analysis. Untreated extract with pH 6 at room temperature (21 ± 2°C) was used as a control.

5.2.9 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibition concentration (MIC) was performed in a 96-well microtiter plate using two-fold serial dilutions. Lemongrass or citral nanoemulsions with a concentration of 2.00% (v/v) were mixed and two-fold dilutions were prepared in Brain Heart Infusion (BHI) broth containing the inoculum. Column 12 was filled with the highest concentration of Lemongrass or citral nanoemulsions (1.00% (v/v)), while column 3 was filled with the lowest concentration of Lemongrass or citral nanoemulsions (0.02% (v/v)). Column 1 served as the negative control (no inoculum and antimicrobial agent) and column 2 served as the positive control (inoculum and antimicrobial agent). The MIC was determined as the lowest concentration of antimicrobial agent that inhibits microbial growth, and the absorbance was measured using a microtiter plate reader (Varioskan ^{TM} LUX, UK) with reading <0.05 change in optical density $(OD)_{590}$ after incubating the sample for 24 hours at 30°C. MIC values were expressed as percentage concentration (% v/v) because the test samples were nanoemulsion formulations rather than purified compounds. All assays were performed in triplicate.

5.2.10 Statistical analysis

All experiments were performed in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) (IBM SPSS version 29) and, where necessary, the Tukey test ($\alpha = 0.05$) or t-test ($\alpha = 0.05$). All results were expressed as mean ± standard deviation.

5.3 Results

5.3.1 Yield of lemongrass supercritical fluid extraction

Table 5.1 shows the yield collected from raw lemongrass at different pressure levels during supercritical fluid carbon dioxide extraction. The pressures are listed in bars and the yields are given as a percentage.

Table 5.1. Yield collected of lemongrass at different pressures level.

Pressure (bar)	Peha ayam (PA)		
	Sample (g)	**Extract collected (g)**	**Yield collected (mg/g)**
85	172.80	0.13	0.75
100	185.31	0.54	2.91
200	184.45	0.79	4.28
300	185.22	1.51	8.15

The results indicate a clear positive relationship between the applied pressure and the yield collected, highlighting the influence of pressure on the efficiency of the process. At the lowest pressure of 85 bar, the yield collected is minimal, at only 0.075% (mg/g). As the pressure increased to 100 bar, the yield more than tripled to 0.291% (mg/g). Further increases in pressure to 200 bar and 300 bar resulted in progressively higher yields of 0.428% (mg/g) and 0.815% (mg/g), respectively.

Table 5.2. Composition of bioactive components in citral and lemongrass extracted at 85 bar.

No.	Name of compound	Standard		Supercritical fluid extraction	
		Citral		Lemongrass	
		Retention time	Relative peak area (%)	Retention time	Relative peak area (%)
1	Neral	22.179	44.08	22.285	25.25
2	Geraniol	nd	nd	22.859	4.18
3	Geranial	23.561	45.98	23.740	46.44
4	Caryophyllene	nd	nd	30.497	2.11
5	Cis-.alpha.-Bergamotene	nd	nd	31.147	1.00
6	Geranial diethylacetal	nd	nd	31.262	0.75
7	Germacrene D	nd	nd	33.160	1.10
8	Bulnesene <alpha>	nd	nd	34.221	0.79
9	Muurolene <gamma>	nd	nd	34.557	0.81
10	Endo-1-bourbonanol	nd	nd	37.095	2.29
11	Junipercamphor	nd	nd	38.864	7.75
12	Viridiflorol	nd	nd	40.228	0.83
13	Intermedeol	nd	nd	40.444	1.42
14	Eudesm-7(11)-en-4-ol	nd	nd	41.871	1.29

*nd: not detected

5.3.2 Composition of active compounds by Gas Chromatography Mass Spectrometry (GCMS) analysis

The results of the Gas Chromatography Mass Spectrometry (GCMS) analysis of SFE are presented in Table 2. Comparing chemical profile of lemongrass to its standard citral extract. Peak area percentages (Area, %) were obtained from chromatogram peak integration and represent the relative abundance of each detected compound, calculated as the peak area of a compound divided by the total peak area of all identified compounds ×100. Therefore, Area (%) provides a semi-quantitative estimate of extract composition rather than absolute concentration. The main bioactive components in lemongrass oil are neral and geranial, which together make up citral, a key compound valued for its fragrance and antimicrobial properties. Accordingly, total citral content in the lemongrass extract was estimated as the combined Area (%) of neral and geranial.

In the citral standard, neral and geranial were the primary components, at 44.08% and 45.98% respectively. In the SFE lemongrass extract, while neral and geranial were still dominant (25.25% and 46.44%, respectively), additional compounds were present, demonstrating a broader chemical profile. These included geraniol (4.18%), caryophyllene (2.11%), and junipercamphor (7.75%), along with several other minor terpenes and sesquiterpenes, such as cis-α-bergamotene, germacrene D, and viridiflorol.

5.3.3 Antimicrobial activity

Table 5.3 shows the antimicrobial activity of lemongrass (LG) and citral (C) nanoemulsions at various concentrations (0.125% (v/v), 0.250% (v/v), 0.500% (v/v), and 2.000% (v/v)) against three *B. cereus* isolates (ATCC 14579, P4, and M2) over a six-month storage period at 4°C. Across all isolates, higher concentrations (0.500% (v/v) and 2.000% (v/v)) of both LG and C nanoemulsions consistently achieved the highest log reductions, indicating their ability to suppress bacterial growth. For instance, at 2 months, the 2.000% (v/v) LG concentration showed a 4.86 Log_{10} CFU/mL reduction for *B. cereus* ATCC 14579 and effectively suppressed isolate P4 and M2 to <1.00 Log_{10} CFU/mL. The effectiveness of the nanoemulsions decreased with storage time, particularly at lower concentrations. For example, for P4 with 0.125% (v/v) LG, the log reduction at 2 months was 3.66 Log_{10} CFU/mL, but this dropped by 4 months. This suggests degradation or dissipation of active components over time, reducing antimicrobial efficacy.

Isolate-specific differences are also apparent, with ATCC 14579 showing the highest resistance to the treatments compared to isolate P4 and M2. Even at 2.00% (v/v) LG, the growth of *B. cereus* ATCC 14579 remained at 2.06 Log_{10} CFU/mL on Day 0, compared to <1.00 log_{10} CFU/mL for isolate P4 and M2. Despite this resistance, *B. cereus* ATCC 14579 demonstrated relative stability in its response to the nanoemulsions over the six-month storage period, as no significant difference ($p > 0.05$) was observed in 2.00% (v/v) LG throughout the storage time. In contrast, significant differences ($p < 0.001$) were observed in *B. cereus* P4 and *B. cereus* M2.

Table 5.3. Growth of *B. cereus* isolates (log CFU/ml), after exposure (1 h) to nanoemulsions supplemented with different concentrations of lemongrass (LG), citral (C), at day 0, and after 2, 4 and 6 months of storage at 4°C.

***B. cereus* isolates**	**Concentration of nanoemulsions**	**Growth of *B. cereus* (log CFU/mL)**			
		Day 0	2 months	4 months	6 months
ATCC 14579	Control	6.50 ± 0.71^{gB}	7.26 ± 0.25^{dC}	5.83 ± 0.60^{eA}	6.00 ± 0.00^{eAB}
	0.125% LG	5.14 ± 0.15^{fB}	3.60 ± 0.43^{cA}	4.75 ± 0.21^{dB}	5.24 ± 0.34^{dB}
	0.250% LG	3.19 ± 0.65^{deA}	2.63 ± 0.35^{bcA}	3.15 ± 0.21^{bA}	4.15 ± 0.21^{cB}
	0.500% LG	3.20 ± 0.17^{cdB}	3.25 ± 0.03^{aBC}	2.36 ± 0.39^{aA}	3.50 ± 0.28^{bcC}
	2.000% LG	2.06 ± 0.21^{aA}	2.40 ± 0.46^{aA}	2.35 ± 0.49^{aA}	2.50 ± 0.71^{aA}
	0.125% C	3.77 ± 0.10^{eB}	4.30 ± 0.26^{bcA}	4.97 ± 0.06^{dC}	4.97 ± 0.06^{dC}
	0.250% C	2.23 ± 0.40^{abA}	3.23 ± 0.20^{aB}	4.00 ± 0.00^{cC}	4.00 ± 0.00^{cC}
	0.500% C	2.84 ± 0.15^{bcdB}	2.24 ± 0.34^{abA}	3.15 ± 1.62^{bB}	3.00 ± 0.00^{abB}
	2.000% C	2.43 ± 0.23^{abA}	2.80 ± 0.14^{abB}	3.15 ± 0.21^{bC}	3.00 ± 0.00^{abBC}
P4	Control	7.10 ± 0.17^{dB}	7.30 ± 0.00^{dB}	6.35 ± 0.49^{cA}	6.39 ± 0.36^{cA}
	0.125% LG	3.47 ± 0.67^{bA}	4.54 ± 0.09^{cB}	6.07 ± 0.40^{cC}	6.07 ± 0.68^{bcC}
	0.250% LG	3.47 ± 0.00^{bA}	3.60 ± 0.43^{abA}	5.30 ± 0.43^{bB}	5.24 ± 0.34^{bB}
	0.500% LG	$<1.00 \pm 0.00^{aA}$	$<1.00 \pm 0.00^{aaA}$	4.92 ± 0.11^{abB}	5.24 ± 0.34^{bB}
	2.000% LG	$<1.00 \pm 0.00^{aA}$	$<1.00 \pm 0.00^{acA}$	4.49 ± 0.03^{aC}	4.00 ± 0.00^{aB}
	0.125% C	4.30 ± 0.43^{cA}	4.24 ± 0.34^{acA}	6.79 ± 0.61^{cC}	5.65 ± 0.91^{bcB}

	0.250% C	3.33 ± 0.20 ^{bA}	3.30 ± 0.26 ^{aA}	6.25 ± 0.15 ^{cC}	5.31 ± 0.58 ^{bcB}
	0.500% C	<1.00 ± 0.00 ^{aA}	<1.00 ± 0.00 ^{aA}	4.86 ± 0.27 ^{abC}	4.15 ± 0.21 ^{aB}
	2.000% C	<1.00 ± 0.00 ^{aA}	<1.00 ± 0.00 ^{aA}	4.52 ± 0.16 ^{abC}	3.69 ± 0.53 ^{aB}
M2	Control	6.59 ± 0.25 ^{eAB}	7.03 ± 0.05 ^{be}	6.67 ± 0.58 ^{dAB}	6.26 ± 0.24 ^{fA}
	0.125% LG	3.00 ± 0.00 ^{cA}	4.15 ± 0.21 ^{dB}	6.46 ± 0.27 ^{cdC}	6.46 ± 0.15 ^{fC}
	0.250% LG	3.44 ± 0.05 ^{dB}	2.74 ± 0.37 ^{bA}	6.25 ± 0.22 ^{cdC}	5.98 ± 0.03 ^{eC}
	0.500% LG	1.00 ± 0.00 ^{aA}	<1.00 ± 0.00 ^{aA}	4.82 ± 0.31 ^{abB}	4.63 ± 0.21^{cB}
	2.000% LG	<1.00 ± 0.00 ^{aA}	<1.00 ± 0.00 ^{aA}	4.18 ± 0.24 ^{aC}	2.50 ± 0.28 ^{bB}
	0.125% C	3.23 ± 0.34 ^{cdA}	4.26 ± 0.24 ^{dB}	7.13 ± 0.38 ^{dD}	5.50 ± 0.71 ^{deC}
	0.250% C	2.54 ± 0.09 ^{bA}	3.70 ± 0.12 ^{cB}	6.39 ± 0.35 ^{cdD}	5.48 ± 0.43 ^{deC}
	0.500% C	<1.00 ± 0.00 ^{aA}	<1.00 ± 0.00 ^{aA}	5.10± 0.17 ^{bcB}	5.15 ± 0.21 ^{cdB}
	2.000% C	<1.00 ± 0.00 ^{aA}	<1.00 ± 0.00 ^{aA}	4.37 ± 0.19 ^{aC}	1.39 ± 0.12 ^{aB}

LG: lemongrass; C: citral.

The values followed by the same lowercase, in the same column and by the same uppercase in the same row are not significantly different by Tukey test, at P <0.05

5.3.4 Droplet size, polydispersity index and zeta potential

The physicochemical stability of lemongrass and citral nanoemulsions was assessed over six months of storage at 4°C (Table 5.4), focusing on parameters such as droplet size, polydispersity index (PDI), and zeta potential.

Table 5.4. Physicochemical stability parameters of lemongrass and citral nanoemulsions at 2.000% concentration over 6 months of storage at 4°C.

Duration	**0 Days**		**2 Months**		**4 Months**		**6 Months**	
Nanoemulsion	LG	C	LG	C	LG	C	LG	C
Droplet size (nm)	86.32 ± 0.66^{A}	32.86 ± 1.37^{A}	346.03 ± 21.98^{B}	161.60 ± 43.69^{AB}	516.63 ± 20.24^{C}	281.65 ± 81.53^{BC}	494.47 ± 8.88^{C}	350.27 ± 85.93^{C}
Polydispersity index	0.50 ± 0.00^{A}	0.85 ± 0.04^{B}	0.89 ± 0.14^{A}	0.54 ± 0.18^{A}	0.85 ± 0.07^{A}	0.33 ± 0.03^{A}	0.81 ± 0.27^{A}	0.89 ± 0.13^{B}
Zeta potential (mV)	-44.01 ± 1.69^{C}	-31.08 ± 0.45^{A}	-42.26 ± 0.86^{C}	-36.83 ± 3.25^{AB}	-38.94 ± 1.18^{B}	-38.35 ± 0.96^{B}	-33.63 ± 1.45^{A}	-32.88 ± 2.57^{AB}

LG: lemongrass; C: citral.

The values followed by the same uppercase letter in the same row are not significantly different by Tukey test, at $P < 0.05$

Nanoemulsion stability is often evaluated through key physical characteristics such as droplet size, polydispersity index (PDI), and zeta potential. These parameters are critical in determining the stability, homogeneity, and functionality of nanoemulsions in various applications. Droplet size indicates the average diameter of the dispersed phase in the emulsion and affects stability, with smaller droplet sizes typically correlating with greater resistance to coalescence (McClements, 2012). The polydispersity index (PDI) reflects the size distribution of droplets within the emulsion, with values closer to 0 indicating a more uniform size distribution, essential for physical and chemical stability (Danaei et al., 2018). Zeta potential measures the surface charge of droplets and serves as an indicator of electrostatic repulsion between particles; higher absolute values signify greater colloidal stability by preventing aggregation (Honary & Zahir, 2013).

The data (Table 5.4) demonstrates significant changes ($p < 0.001$) in the physical stability of lemongrass nanoemmulsion (LG) over time, specifically in droplet size and zeta potential measurements. Similarly, for citral nanoemulsions (C), significant changes ($p < 0.001$) are observed in droplet size and the polydispersity index (PDI). Initially, at Day

0, citral nanoemulsions showed smaller droplet sizes (32.86 ± 1.37 nm) compared to LG nanoemulsions (86.32 ± 0.66 nm). However, over time, both formulations showed an increase in droplet size, with citral nanoemulsions reaching 350.27 ± 85.93 nm and LG nanoemulsions increasing to 494.47 ± 8.88 nm by 6 months. This growth in droplet size suggests coalescence or aggregation of the droplets, which could compromise the stability of the nanoemulsions. Notably, LG nanoemulsions consistently showed larger droplet sizes than C nanoemulsions at all time points, indicating potential differences in their structural stability under similar storage conditions.

Figure 5.1 illustrates the relationship between droplet size and the antimicrobial activity of lemongrass nanoemulsion against *B. cereus* isolate P4. Initially, the droplet size is small (~100 nm) but increases steadily over time, reaching approximately 500 nm by 6 months. The antimicrobial activity, represented by the microbial count (log CFU/mL), remains low (<1.0 $\log_{10}$ CFU/mL) during the first 2 months. However, at 4 months, there is a sharp increase in the microbial count (~4.0 $\log_{10}$ CFU/mL), indicating a weakening of antimicrobial activity. A similar trend is observed at 6 months, confirming a decline in antimicrobial effectiveness over time. This chart suggests a possible correlation between droplet size and antimicrobial activity, with reduced effectiveness occurring as the droplet size reaches its maximum.

To further visualise this association independent of time, droplet size was plotted directly against *B. cereus* plate counts (log CFU/mL) for *B. cereus* isolate P4 treated with 2.0% (v/v) lemongrass nanoemulsion (Figure 5.2). The scatter plot demonstrates an overall positive relationship between droplet growth and bacterial survival, supporting the conclusion that increased droplet size contributes to diminished antimicrobial efficacy during storage.

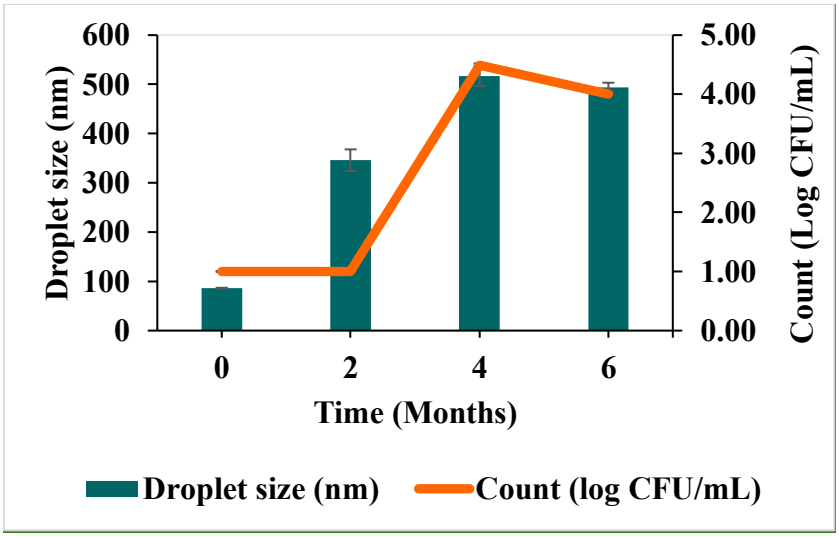

Figure 5.1. Correlation between droplet size and antimicrobial activity of lemongrass nanoemulsion (2.0%(v/v)) against *B. cereus* isolate P4.

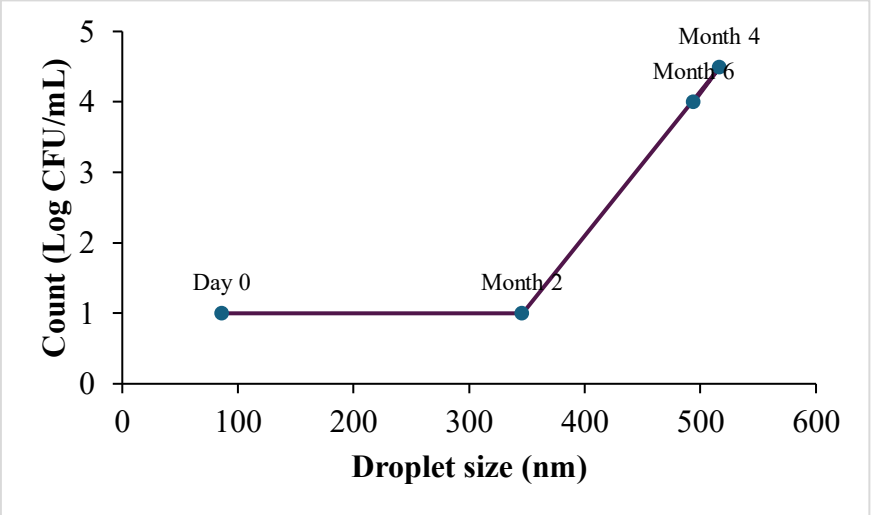

Figure 5.2. Relationship between droplet size and antibacterial efficacy of lemongrass nanoemulsion during storage.

The PDI values also reveal insights into the uniformity of the nanoemulsion formulations. For LG nanoemulsion, the PDI increased from 0.50 ± 0.00 on Day 0 to 0.81 ± 0.27 at 6 months, indicating a broader size distribution and reduced stability over time. In contrast, C nanoemulsions showed fluctuating PDI values, with an initial high value of 0.85 ± 0.04 at Day 0, decreasing to 0.33 ± 0.03 at 4 months before rising again to 0.89 ± 0.13 at 6 months. These fluctuations suggest that citral nanoemulsions experience temporary stabilization but ultimately face instability like lemongrass nanoemulsions.

The zeta potential values, which indicate the surface charge and colloidal stability, decreased over time for both LG and C nanoemulsions. Initially, LG nanoemulsions had a zeta potential of -44.01 ± 1.69 mV, which increased to -33.63 ± 1.45 mV at 6 months. Similarly, C nanoemulsions started at -31.08 ± 0.45 mV and decreased to -32.88 ± 2.57 mV by 6 months. While the negative charge remains sufficient to impart some stability, the reduction in zeta potential suggests a decline in repulsive forces, potentially contributing to aggregation and droplet growth.

5.3.5 Thermal stability

The minimum inhibitory concentration (MIC) of citral and lemongrass nanoemulsions remained constant at 0.125% (v/v) across all tested temperatures (21.0°C, 30.0°C, 60.0°C, and 90.0°C) for the three *B. cereus* isolates: ATCC 14579, P4, and M2 (Table 5.5) This consistency indicates that the formulations retained antimicrobial activity following heat exposure up to 90°C, suggesting good thermal stability under the tested conditions.

Table 5.5. Thermal stability of MIC values for lemongrass and citral nanoemulsions against all three *B. cereus* isolates.

Temperature (°C)	MIC for lemongrass nanoemulsion (% v/v)	MIC for citral nanoemulsion (% v/v)
21	0.125	0.125
30	0.125	0.125
60	0.125	0.125
90	0.125	0.125

5.4 Discussion

Supercritical Fluid Extraction (SFE) is a highly efficient method for isolating bioactive compounds from natural sources like lemongrass, utilizing the unique properties of supercritical carbon dioxide to achieve selective extraction while preserving the integrity

of sensitive bioactives. Under optimized conditions, SFE at 300 bar achieved a peak yield of 0.815% (mg/g), illustrating its capability to maximize extraction efficiency. These results are consistent with Rodrigues et al. (2018), who observed enhanced yields for eucalyptus leaves under similar pressure conditions when combined with co-solvents. However, as Pokrovskiy et al. (2019) noted, the relationship between pressure and yield is not universally linear; certain matrices, such as black coffee oil and cannabis, exhibit reduced yields at elevated pressures due to plant-specific matrix composition and saturation effects.

While high pressures offer substantial yields, 85 bar was identified as the optimal extraction pressure for lemongrass due to its practical and functional advantages. Extracts obtained at this moderate pressure retained a liquid state, avoiding the waxy, semi-solid phase that can complicate solubility at higher pressures. Furthermore, from the preliminary study, lemongrass extracts obtained at 85 bar exhibited superior antimicrobial activity against Gram-positive and Gram-negative bacteria (Table 5.6 in supplementary material) These findings align with Calva-Cruz et al. (2021), who demonstrated that optimized SFE conditions for *Lippia graveolens* produced antimicrobial compounds effective against multidrug-resistant pathogens, *Enterococcus faecalis* and *S. aureus* isolates even under conditions that were less than those required for maximum yield.

Nanoemulsions formulated with lemongrass and citral in the present study consistently demonstrated strong antimicrobial potential, largely attributed to their small initial droplet sizes, often below 200 nm, which enhance stability and microbial interaction. Both lemongrass and citral nanoemulsions exhibited comparable antimicrobial activity, suggesting that citral, the major active compound in lemongrass oil, is primarily responsible for the observed efficacy. Moreover, findings from our preliminary study (Table 5.7 in supplementary material) revealed that lemongrass nanoemulsions exhibited significantly higher antimicrobial activity compared to LEO, achieving comparable effects at five times lower concentrations. For instance, citral and lemongrass nanoemulsions reported in this study have particle sizes ranging from 32 to 86 nm, which gradually increased over time due to coalescence and aggregation during storage (Figure 5.1). Citronella oil nanoemulsions exhibited optimized particle sizes around 79 nm, with minimal changes over 28 days under low-temperature storage, underscoring the

importance of controlled environments for stability (Somala et al., 2022). Notably, smaller droplet sizes, particularly those below 100 nm, are associated with enhanced antimicrobial efficacy due to improved interactions with microbial membranes (Sedaghat Doost et al., 2020).

Zeta potential, a measure of electrostatic stability, initially exceeded -30 mV in most nanoemulsions, indicating sufficient colloidal stability. However, over six months, lemongrass nanoemulsions showed a decline in zeta potential from -44 mV to -33 mV, reflecting reduced electrostatic repulsion and a heightened risk of droplet aggregation. These trends are consistent with findings by Carvalho et al., (2018), who emphasized the critical role of maintaining a zeta potential above -30 mV to prevent destabilization. Stability-enhancing agents, such as polymer encapsulation or synergistic surfactants, have been shown to mitigate these declines, maintaining stability under various storage conditions.

The antimicrobial activity of lemongrass nanoemulsions was particularly prominent during the first four months of storage, achieving significant log reductions against *B. cereus* ATCC 14579 and complete suppression of isolate P4 and M2 isolates at higher concentrations (2.00% (v/v)). This efficacy aligns with studies by Garcia et al., (2022) and do Carmo Silva et al. (2020) which attribute such performance to enhanced solubility, bioavailability, and microbial membrane interactions facilitated by nanoemulsion formulation. However, in the present study, degradation of antimicrobial activity was observed that started at four months and was associated with droplet coalescence and declining zeta potential. These challenges underscore the need for advanced stabilization strategies, such as polymer encapsulation (Maurya et al., 2021) or controlled-release systems (Jamali et al., 2021), to extend functional efficacy. Studies by Gago et al. (2019) highlighted the critical role of optimal storage conditions in maintaining nanoemulsion stability. Specifically, the highest concentration of lemongrass nanoemulsion (2.50%(v/v)) retained its antimicrobial efficacy against *E. coli* for up to six months when stored at 1°C, compared to significantly reduced stability at 25°C.

In the present study, the antimicrobial effects of lemongrass oil and citral were more pronounced against Gram-positive bacteria compared with Gram-negative organisms,

which is consistent with previous findings that essential oils often exhibit reduced activity against Gram-negative bacteria due to their more complex cell envelope. For instance, Alsakhawy et al. (2024) reported that free lemongrass oil and lemongrass-oil-loaded nanoparticles produced markedly stronger effects in Gram-positive bacteria than Gram-negative strains, attributing this to structural differences in the bacterial cell wall that restrict essential oil penetration. Nevertheless, other studies have demonstrated that lemongrass oil can be effective against Gram-negative pathogens under certain delivery conditions. Notably, Hyun et al. (2015) observed strong inhibitory effects of LEO in the vapour phase against Gram-negative foodborne pathogens including *E. coli* O157:H7 and *S.* Typhimurium*,* highlighting that antimicrobial outcomes may depend strongly on how the essential oil is applied, as well as strain-specific susceptibility and experimental conditions. Accordingly, the weaker activity observed in the present study against Gram-negative bacteria may reflect limited penetration of citral through the Gram-negative outer membrane, together with formulation-related factors such as droplet growth during storage and matrix interactions that reduce effective contact between the antimicrobial and bacterial cells.

When comparing stability across time, lemongrass nanoemulsions demonstrated relatively prolonged stability compared to other essential oils. For example, citronella oil nanoemulsions exhibited degradation in particle size and antimicrobial activity within 28 days (Touayar et al., 2023), and thymol nanoemulsions showed a decline in efficacy after two months due to electrostatic instability (Kumari et al., 2018). In contrast, for this study, lemongrass nanoemulsion maintained stability for up to four months under optimized storage conditions, with particle sizes increasing from an initial 86.32 ± 0.66 nm to over 350 nm. Notably, both nanoemulsions lemongrass and citral displayed comparable efficacy and stability over time, underscoring their potential as robust antimicrobial agents with consistent performance during extended storage. This highlights the importance of optimal storage conditions to maintain their functionality and efficacy.

Thermal stability testing has reinforced the robustness of lemongrass and citral nanoemulsions across a broad temperature range (21°C to 90°C), making them suitable for heat-intensive applications in food manufacture. Singh et al. (2020) similarly reported that surfactant-stabilized nanoemulsions effectively resist thermal degradation,

maintaining structural integrity and efficacy under industrial processing conditions. Consistent efficacy across *B. cereus* ATCC 14579, isolate P4, and M2 underscores their broad-spectrum applicability. Research conducted by Borba et al. (2019) demonstrated that all nanoemulsions showed exceptional resistance to droplet coalescence during thermal treatments and under various storage conditions, exhibiting low Ostwald ripening rates. However, the retention of β-carotene within the nanoemulsions depended on storage temperature, the exclusion of light, and the encapsulation efficiency (%EE). Studies by da Silva Gundel et al. (2018) further emphasized the importance of thermal stability in preserving antimicrobial properties during extended use.

Despite their promising performance, isolate-specific differences in susceptibility present challenges. For instance, *B. cereus* ATCC 14579 demonstrated greater resistance compared to isolate P4 and M2, highlighting the necessity of tailoring nanoemulsion formulations to target diverse pathogens. Similar variability has been reported by do Carmo Silva et al. (2020) in the susceptibility of *L. monocytogenes* isolates, attributed to genetic and structural differences among microbial species.

The variability in efficacy of nanoemulsions against different isolates of *Bacillus cereus* highlights isolate-dependent susceptibility, influenced by genetic, structural, and physiological differences among isolates. Resistant isolates, such as ATCC 14579, demonstrate higher tolerance compared to more susceptible isolates like P4 and M2. This resistance may come from unique genetic and structural characteristics, including differences in cell wall composition, charge, and permeability, which alter their interaction with nanoemulsion components like citral or lemongrass oil (Ehling-Schulz et al., 2019). Moreover, resistant isolate like ATCC 14579 may possess more effective repair mechanisms for cell membrane damage, enabling them to maintain viability over extended treatment periods, whereas susceptible isolates experience a decline in efficacy over time.

Genomic studies further emphasize this variability, showing distinct phylogenetic groupings and differences in virulence-associated genes among isolates (Didouh et al., 2023). This genomic diversity directly impacts susceptibility to antimicrobials and other interventions, as evidenced by biofilm composition differences, such as extracellular DNA (eDNA) content, which can influence resistance to enzymatic degradation and

antimicrobial agents (Lim et al., 2021). Ultimately, the interaction between nanoemulsions and *B. cereus* isolates is a complex interplay of isolate-specific traits, emphasizing the importance of understanding variability in pathogen behavior to develop effective and consistent antimicrobial strategies.

5.5 Conclusion

This study demonstrates that supercritical fluid-extracted lemongrass and citral nanoemulsions exhibit strong antimicrobial activity and stability, particularly under optimized storage and thermal conditions. Despite slight variations in droplet size and zeta potential, both nanoemulsions proved effective for controlling *B. cereus*. Notably, the highest yield does not equate to maximum antimicrobial efficiency. An optimized extraction pressure of 85 bar at 40°C provided the lowest minimum inhibitory concentration (MIC) activity, highlighting the importance of tailored extraction conditions.

Throughout storage, higher concentrations of nanoemulsions (0.500% and 2.000%) consistently achieved the highest log reductions, effectively suppressing bacterial growth. Both nanoemulsions remained efficient for up to four months, but their stability declined thereafter, as indicated by increased droplet size, polydispersity index, reduced zeta potential, and increased viability of *B. cereus*.

Both formulations exhibited similar antimicrobial behaviour, despite isolate-dependent variations among *B. cereus*. Moreover, the nanoemulsions demonstrated thermal stability, maintaining efficacy even under high-temperature exposure, underscoring their robustness in heat-intensive applications.

To enhance the functionality and expand the applications of lemongrass and citral nanoemulsions, advanced stabilization techniques, such as polymer encapsulation or synergistic emulsifiers, are recommended. Future research should also explore their impact on sensory properties, including aroma, taste, and texture, to ensure consumer acceptance in food products. Investigating these aspects could significantly extend their shelf life and utility in industrial, clinical, and food preservation.

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5.7 Copyright information

Part of this work has been published in Food Bioscience Journal, and the Online Statement of Contribution form is attached in Appendix IV.

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5.9 Supplementary material

Table 5.6. The antibacterial activity of lemongrass extract against Gram-positive and Gram-negative bacteria.

Bacteria	Pressure	MIC (% v/v)	MBC (% v/v)
B. cereus	85	0.313	0.625
	100	> 2.500	> 2.500
S. aureus	85	0.156	0.313
	100	0.313	0.313
E. coli	85	0.625	0.625
	100	1.250	1.250
Salmonella spp.	85	0.625	0.625
	100	1.250	1.250

*MIC = Minimum Inhibition Concentration; MBC = Minimum Bactericidal concentration

Table 5.7. The minimum inhibitory concentration (MIC) of lemongrass and citral extract and nanoemulsion.

Sample	**Minimum Inhibitory Concentration (MIC) (% v/v)**
Lemongrass extract	0.630
Lemongrass nanoemulsion	0.125
Citral extract	0.160
Citral nanoemulsion	0.125

Preface

In Chapter 5, the stability of lemongrass nanoemulsion was examined, revealing that its antimicrobial activity remained effective for up to four months. The nanoemulsion also demonstrated stability under thermal treatment up to 90°C, although some changes were observed in its physicochemical characteristics over time. These findings provide important insights into the shelf-life and functional of lemongrass nanoemulsion. Building on this, Chapter 6 investigates the underlying mechanisms of its antimicrobial action through a series of kinetic assays, flow cytometry, ATP and membrane integrity assays, as well as microscopic analyses.

Chapter 6. Lemongrass Nanoemulsion Disrupts Bacterial Membranes: Mechanistic Study Against *Bacillus cereus.*

6.1 Introduction

B. cereus is a Gram-positive, spore-forming bacterium widely recognized for causing foodborne illnesses, including diarrheal and emetic syndromes. This pathogen poses a significant public health threat due to its ability to produce a range of potent toxins, such as the emetic toxin cereulide, which induces nausea and vomiting, and enterotoxins like hemolysin BL and cytotoxin K, which lead to diarrhoea (Dietrich et al., 2021). *B. cereus* is notorious for its resilience in food production, where it forms robust biofilms and spores that are highly resistant to heat, desiccation, and chemical treatments. These traits enable it to persist and contaminate a variety of food products, including processed foods and ready-to-eat meals, leading to substantial economic losses and food spoilage. (Huang et al., 2021).

B. cereus exhibits antimicrobial resistance, including resistance to β-lactams and other antibiotics, which complicates treatment strategies and increases the likelihood of foodborne outbreaks (Navaneethan & Effarizah, 2021; Sornchuer et al., 2024). Nevertheless, many *B. cereus* strains remain susceptible to aminoglycosides, clindamycin, chloramphenicol, erythromycin, and vancomycin (Cha et al., 2023). Beyond antimicrobial resistance, its ability to form spores and biofilms further enhances its survival, persistence, and contamination potential in food systems, making it a particularly challenging foodborne pathogen (Choi & Kim, 2020). Some *B. cereus* strains can proliferate at refrigeration temperature, reaching concentrations that pose a risk to human health. This makes them a potential hazard in the consumption of minimally processed chilled foods (Webb et al., 2019).

To address these challenges, there has been growing interest in natural antimicrobial agents as alternatives to synthetic preservatives and antibiotics. This change is mainly caused by concerns over antimicrobial resistance, which necessitate the search for effective, sustainable alternatives (Qadri et al., 2022). Additionally, increasing awareness of the potential health risks associated with synthetic preservatives has led to a preference for natural, safer food preservation methods. Among these, lemongrass (*Cymbopogon* spp.) has emerged as a particularly promising candidate due to its major bioactive compound, citral. Citral has been extensively studied for its strong antimicrobial properties, which include disrupting bacterial cell membranes, interfering

with metabolic processes, and effectively inhibiting microbial growth (Gao et al., 2020). Lemongrass has been shown to suppress the growth of various foodborne pathogens, including *S.* Typhimurium, *S. aureus*, and *E. coli*, with citral identified as the key antimicrobial agent driving its efficacy (Alzobaay & Kadhim, 2018). However, despite its antimicrobial potency, lemongrass essential oil (LEO) presents challenges for direct application in food systems due to its volatility and low solubility in water. These limitations reduce its stability and efficacy in complex food matrices. To overcome these issues, researchers have developed lemongrass nanoemulsions, which significantly improve the stability, bioavailability, and antimicrobial efficacy of lemongrass oil, making it a more viable option for industrial applications (Ashaq et al., 2024).

Despite these advances, there is a noticeable gap in research regarding the effects of lemongrass nanoemulsion specifically on *B. cereus*. While the antimicrobial properties of lemongrass nanoemulsion have been demonstrated against other foodborne pathogens, such as *S. aureus* and *E. coli*, its efficacy and mechanisms of action against *B. cereus* remain unexplored. This study aims to address this gap by investigating the antimicrobial activity of lemongrass nanoemulsion against *B. cereus* and elucidating its mechanisms of action. By evaluating changes in intracellular ATP levels, membrane potential, and cell membrane microstructures through advanced imaging techniques, this research seeks to provide a comprehensive understanding of how lemongrass nanoemulsion can disrupt the physiology and viability of *B. cereus*. These findings could pave the way for the development of natural, effective strategies to control *B. cereus* in food systems.

6.2 Materials and Methods

6.2.1 Sample

Raw lemongrass (*Cymbopogon citratus*) variety of Peha ayam was purchased from a farmer in Beranang, Selangor, Malaysia and the plant taxonomic identification was verified in Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) under the voucher specimen number of MFI 0217/21.

6.2.2 Nanoemulsion formation

Nanoemulsions were prepared as described in Chapter 4, Section 4.2.4.

6.2.3 Bacterial strains and culture conditions

Two isolates of *B. cereus* were used one reference isolate, (*B. cereus* ATCC 14579) and one isolated from potato (*B. cereus* P4). All isolates were provided by the Food Microbiology laboratory of Massey University. Before each experiment, stock cultures from 80°C were inoculated into brain heart infusion (BHI) broth (Bacto™, Becton, Dickinson and Company, USA) at 30°C for 18-24 h. The cultures were then streaked onto tryptone soy agar (TSA) (Difco™, Becton, Dickinson and Company, USA) and grew at 30°C for 18-24 h. Then a loopful of each isolate was inoculated into 5 mL BHI and incubated for 18-24 h at 30°C as a working culture. After the incubation, the cultures were centrifuged (Sigma® 6–16, John Morris Scientific Ltd., New Zealand) at 5 000 x *g* for 10 mins at room temperature (21 ± 2°C). The cell pellets after centrifugation were washed with 0.85 % sterile saline water and the cell pellets were diluted to a cell concentration of 6 $\log_{10}$ CFU/mL. To confirm that the culture population reached 6 $\log_{10}$ CFU/mL, optical density (OD) measurement was performed using spectrophotometry (Varioskan™ Lux, Thermo Fisher Scientific, Massachussetts, USA) at 600 nm. A correlation curve between OD600 and CFU/mL was established, allowing the estimation of bacterial concentration based on absorbance readings.

6.2.4 Growth curves and kinetic parameters

Growth of *B. cereus* isolates in Brain Heart Infusion (BHI) broth at 30 °C was assessed using a microplate-based optical density method. Briefly, *B. cereus* isolates were grown to a cell concentration of 5-6 $\log_{10}$ CFU/ mL in BHI and transferred into each well of a 96-well microtiter plate (Falcon® Corning, Arizona, USA). Lemongrass nanoemulsion was added to the cultures to obtain final concentration of 1/8MIC, 1/4MIC, 1/2MIC, and MIC, and BHI without nanoemulsion was used as a negative control. The plates were incubated at 30°C, and cell growth was monitored at 590 nm using a multimode microplate reader (Varioskan™ Lux, Thermo Fisher Scientific, Massachussetts, USA) at regular time intervals. Growth curves were generated by plotting optical density values against incubation time to describe overall growth dynamics under each treatment condition.

Specific growth rate (μ) was calculated as the slope of ln-transformed OD_{590} values versus time during the exponential growth phase, with time intervals selected based on the linear region of each growth curve. For each condition, two time points (t_1 and t_2) corresponding to the linear region of ln-transformed optical density values were selected, and μ was determined using the following equation:

$$\mu = (\ln OD_2) - (\ln OD_1) / (t_2 - t_1)$$

where OD_1 and OD_2 represent optical density measurements at times t_1 and t_2 (hours), respectively. Growth rate values were expressed as h^{-1} and reported as mean ± standard deviation from replicate experiments.

6.2.5 Total cellular ATP concentration

The method described by Shi et al. (2016) was followed with slight modification. Briefly, the *B. cereus* inoculum at cell concentration of 6 $\log_{10}$ CFU/mL was placed into a 1.5 mL Eppendorf tube for lemongrass nanoemulsion treatment. Lemongrass nanoemulsion was added to each tube to achieve final concentrations of 0 (control), 1MIC, and 2MIC, 3MIC, 4MIC, respectively. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)). Then the samples were incubated at 30°C for 1 h. To extract the ATP, cells were lysed on ice by ultrasound for 5 min (brand), and centrifuged at 5000 × *g* for 5 min. The top layer was retrieved and stored on ice to prevent ATP loss until measurement. ATP values represent total ATP recovered from cell lysates rather than exclusively intracellular ATP content. ATP was measured with an ATP assay kit (A-22066) by Molecular Probes (Eugene, OR), which is based on a luciferase–luciferin bioluminescence reaction. Luminescence was recorded as relative luminescence units (RLU), and ATP concentration (μmol) was calculated from the RLU values using an ATP standard curve generated in the same assay. After adding 50 μL of ATP assay mix to 50 μL of supernatant in white, opaque, 96-well microtiter plates Jetbiofil® Guangzhou, China), the supernatant ATP concentrations were measured with a microplate reader (Polarstar Omega, BMG Labtech, Germany) and recorded as the intracellular ATP concentration.

6.2.7 Membrane potentials

The method described by Shi et al. (2016) was followed (with slight modification) to determine the membrane potentials. Briefly, the *B. cereus* inoculum at cell concentration of 6 $\log_{10}$ CFU/mL was placed into a 1.5 mL Eppendorf tube for lemongrass nanoemulsion treatment. Next, 50 µL of cell suspensions were placed in black, opaque, 96-well microtiter plates (Nunclon™ Delta Surface, Denmark) for 1 h at 30°C, then 1 µM of the membrane potential-sensitive fluorescent probe bis-(1, 3-dibutylbarbituric acid) trimethine oxonol (DiBAC4 (3); Molecular Probes, Sigma, Louis, USA) was added and incubated for 30 min at 30°C, followed by the addition of lemongrass at five concentrations (0, 1MIC, 2MIC, 3MIC, 4MIC). 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)). Fluorescence was then measured on a fluorescence microplate reader (Varioskan™ Lux, Thermo Fisher Scientific, Massachussetts, USA) at excitation and emission wavelengths 492 and 515 nm, respectively. The excitation and emission slit widths were 3 and 5 nm, respectively. Background fluorescence resulting from the medium was determined and the results corrected as necessary.

6.2.8 Flow cytometry

Briefly, *B. cereus* inoculum at a cell concentration of 6 $\log_{10}$ CFU/mL was prepared in a 1.5 mL Eppendorf tube for lemongrass nanoemulsion treatment. The nanoemulsion was added to each tube to achieve final concentrations of 0 (control), 1MIC, 2MIC, 3MIC, and 4MIC. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)). The samples were then incubated at 30°C for 1 h. After incubation, cells were collected by centrifugation (12,000 × *g*, 5 min), washed with saline to remove excess antimicrobial compounds, and re-suspended in sterilized saline for further analysis.

Prior to flow cytometry (FC) analysis, aliquots of each sample were prepared as unstained cells and live/dead-stained cells. Cells were stained for 10 min at room temperature (21 ± 2°C) using Syto9 and propidium iodide (PI) fluorescent dyes (Live/Dead BacLight Viability kit, Molecular Probes, Eugene, OR). FC analysis was performed using a BD

Accuri™ C6 Plus Flow Cytometer (BD Biosciences, USA). The samples were excited at 488 nm, with green Syto9 fluorescence detected using the FL1 detector (FITC-A channel, 530 ± 30 nm) and red PI fluorescence measured using the FL3 detector (PerCP-A channel, > 670 nm). The sample flow rate was set to slow mode, and a total of 10,000 events were collected per sample for analysis.

The cell population was gated based on forward scatter (FSC) and side scatter (SSC) plots. To minimize background noise in the flow cytometry signals, unstained cell samples were used to set thresholds for FSC and SSC channels. "Live" cell regions were determined using log-phase cells as controls, while "dead" cell regions were defined based on ethanol-treated samples.

6.2.9 Fluorescence microscopy

B. cereus inoculum at a cell concentration of 6 $\log_{10}$ CFU/mL was prepared in a 1.5 mL Eppendorf tube for lemongrass nanoemulsion treatment. The nanoemulsion was added to each tube to achieve final concentrations of 0 (control), 1MIC, 2MIC, 3MIC, and 4MIC. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)). The samples were then incubated at 30°C for 1 hour. Sample cells were collected by centrifugation (12 000 × *g*, 5 min), washed in saline water to remove excess antimicrobial compounds, and re-suspended in sterilized saline water for Syto9/PI staining with (Live/Dead BacLight Viability kit, Molecular Probes, Eugene, OR). A 5 µL volume of cell culture was mounted onto microscopy slides and left for15 minutes until it was dry. Then 5 µL of mixed Syto9/PI staining was put on the dried culture and incubated at room temperature (21 ± 2°C) for 10 min. Ethanol was used as a positive control. The slides were examined using an epifluorescent microscope (Nikon eclipse Ni, USA). U-RFL-T mercury lamp was used as the excitation source, and the fluorescence signals were collected by two separate emission filters for Syto9 (FITC, 465–49 nm) and PI (TRITC, 510–550 nm). Images were visualized and analyzed using NIS-elements D software and Image J software.

6.2.10 Transmission electron microscopy (TEM)

Cells were incubated with 4MIC of lemongrass nanoemulsion in a 1.5 mL Eppendorf tube at 30°C for 1 h. Control cells were incubated without lemongrass nanoemulsion. After incubation cells were harvested by centrifugation at 12 000 x *g* for 5 min and washed thrice with sterilized saline water afterwards. Cell pellets were obtained from each control or treated cell suspension and fixed with Karnovsky fixative. The cells were post-fixed with 1% osmium tetroxide for 1 h at room temperature (21 ± 2°C). This was followed by dehydration in a graded acetone series (50%, 75%, 95% and 100%) for 1 h. The final dehydration was carried out in absolute ethanol for 1 h. The cleaning was done with discarding the 100% acetone, then 50:50 resin (without catalyst) and acetone mixture were added and incubated overnight on a rotator with the lids closed. Infiltration was carried out on a rotator in two steps: a mixture of propylene oxide and araldite (1:1) overnight and two changes in pure Araldite (Selly's, New Zealand) for 2 h at room temperature (21 ± 2°C). Cells were allowed to polymerize in an oven at 60°C for 48 h. Finally, 40–60-nm-thick sections were collected on 300 mesh copper grids, stained with lead citrate, and were viewed under the transmission electron microscope (FEI, TECNAI G2 Spirit BioTwin, Netherlands).

6.2.11 Statistical analysis

All experiments were performed in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) and, where necessary, the Tukey test ($\alpha = 0.05$) or t-test ($\alpha = 0.05$). All results were expressed as mean ± standard deviation.

6.3 Results

6.3.1 Effect of lemongrass nanoemulsion on *B. cereus* growth

Lemongrass nanoemulsion strongly inhibited the growth of *B. cereus* ATCC 14579 and P4 in a concentration-dependent manner, as shown in Figures 6.1 and 6.2. At the MIC (0.125%), ln(OD_{590}) values remained close to baseline throughout the 24 h incubation period, indicating complete suppression of exponential growth.

Consequently, growth curves at this concentration showed no detectable increase in biomass over time. At sub-inhibitory concentrations (1/2 MIC and below), Exponential growth was maintained but occurred at progressively reduced rates with increasing nanoemulsion concentration. This was reflected by reduced slopes in the ln(OD_{590}) versus time plots, indicating concentration-dependent reductions in specific growth rate (μ) (Tables 6.1 and 6.2). Although biomass accumulation was initially suppressed at higher sub-MIC levels, ln(OD) values increased at later time points, indicating partial recovery of growth.

For *B. cereus* P4 (Figure 6.2), exponential growth was also observed under control conditions; however, the onset of exponential growth occurred later and progressed more gradually compared with *B. cereus* ATCC 14579. Overall, untreated control cultures exhibited rapid exponential growth with the steepest ln(OD_{590}) slopes, consistent with uninhibited growth under the experimental conditions.

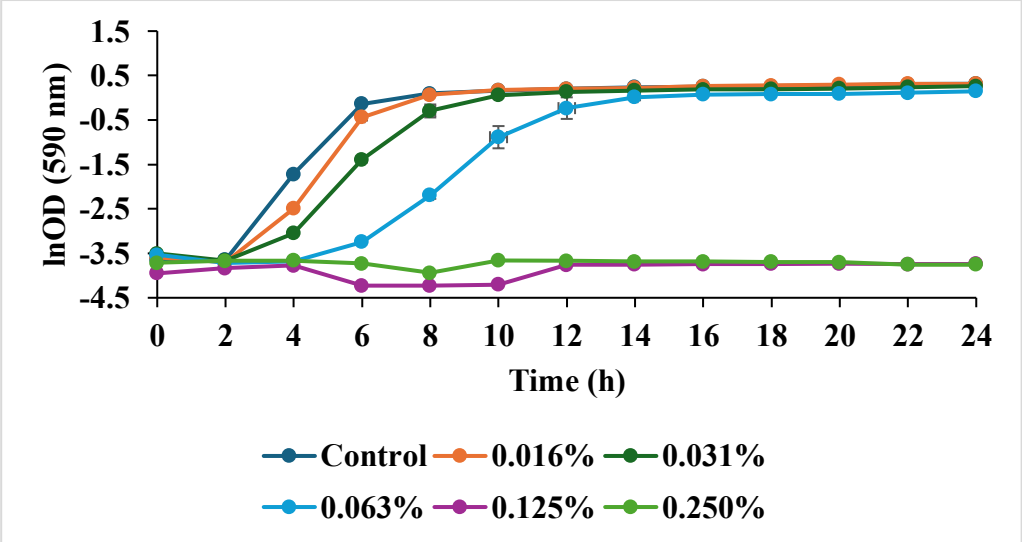


Figure 6.1. Kinetic growth curves of *B. cereus* ATCC 14579 in BHI broth at 30 °C with lemongrass nanoemulsion (0.016–0.125% v/v), expressed as ln(OD_{590}) over 24 h.

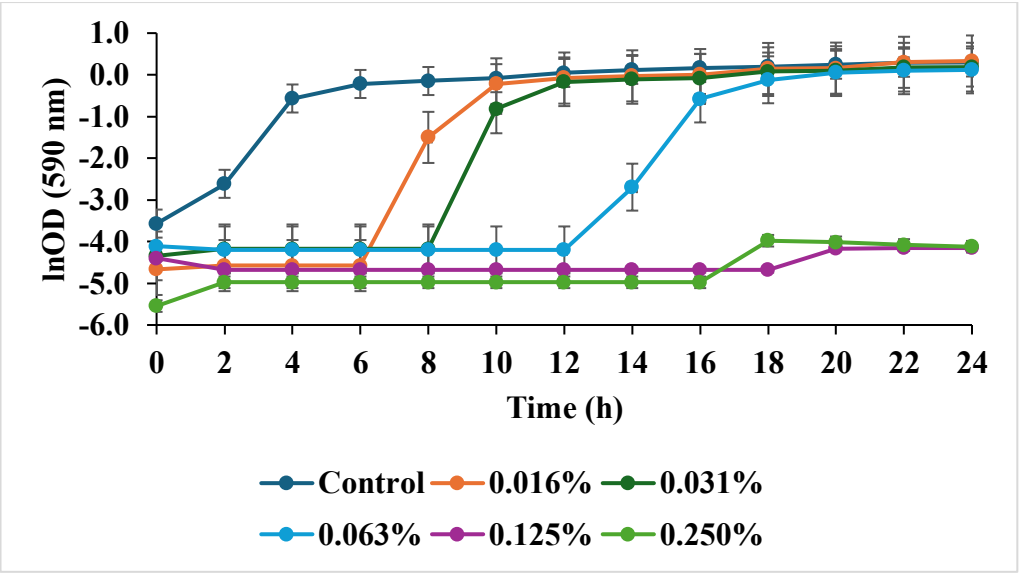


Figure 6.2. Kinetic growth curves of *B. cereus* ATCC 14579 in BHI broth at 30 °C with lemongrass nanoemulsion (0.016–0.125% v/v), expressed as ln(OD_{590}) over 24 h.

Table 6.1. Growth rate (μ) of *B. cereus* ATCC 14579 cells grown in BHI with different concentration of lemongrass nanoemulsion.

Lemongrass nanoemulsion (% v/v)	Time interval used (h)	μ (h^{-1})
Control	4–8	0.455
0.016	4–8	0.638
0.031	6–10	0.363
0.063	8–12	0.489
0.125	–	*Not detected
0.250	–	*Not detected

Specific growth rate (μ) was calculated as the slope of ln(OD_{590}) versus time during the exponential growth phase. *Not detected indicates that no exponential growth phase was observed.

Table 6.2. Growth rate (μ) of *B. cereus* P4 cells grown in BHI with different concentration of lemongrass nanoemulsion.

Lemongrass nanoemulsion (% v/v)	Time interval used (h)	μ (h^{-1})
Control	4–8	0.105
0.016	8–12	0.356
0.031	10–14	0.177
0.063	14–18	0.644
0.125	–	*Not detected
0.250	–	*Not detected

Specific growth rate (μ) was calculated as the slope of $\ln(OD_{590})$ versus time during the exponential growth phase. *Not detected indicates that no exponential growth phase was observed.

6.3.2 Effect of lemongrass nanoemulsion on ATP production of *B. cereus*

The graphs (Figures 6.3 and 6.4) demonstrate that lemongrass nanoemulsion exhibits a concentration-dependent effect on cell-associated ATP levels of *B. cereus*. ATP concentrations were calculated from relative luminescence units (RLU) using a standard curve; therefore, RLU and ATP values are not independent measures. Across concentrations, ATP exhibited a non-linear response, decreasing from the control to 1–2MIC and subsequently increasing at 3–4MIC. At 1MIC and 2MIC, ATP levels were significantly reduced, consistent with suppression of cellular metabolic activity. However, a partial increase in ATP signal was observed at higher concentrations (3–4MIC). It is important to note that ATP was measured after cell lysis, and therefore the results reflect total ATP recovered from lysed bacterial cells at the time of sampling, rather than intracellular ATP dynamics or extracellular ATP release. The bacteria-only control (no extract) provided the baseline ATP signal; however, an extract-only (nanoemulsion without bacteria) control was not included. Therefore, potential assay interference or background luminescence contributed by the nanoemulsion matrix cannot be excluded, particularly at higher concentrations where non-linear ATP responses were observed.

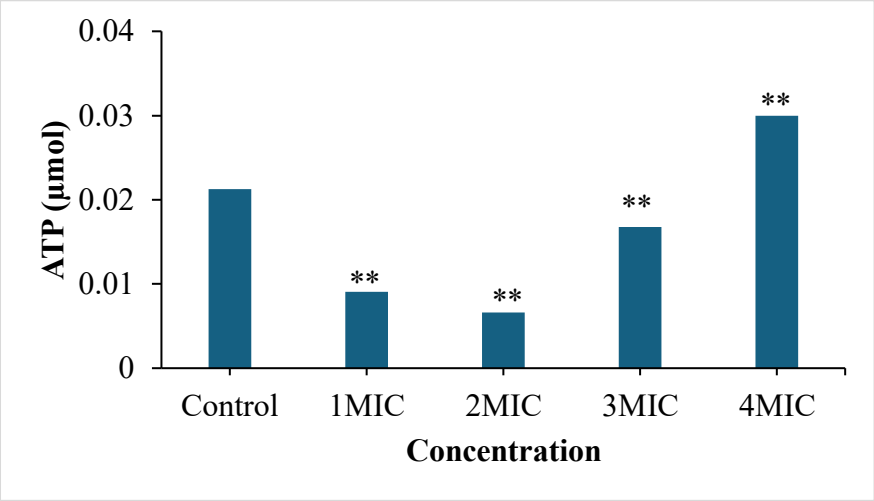


Figure 6.3. Effect of lemongrass nanoemulsion on ATP production of *B. cereus* ATCC 14579 following 1 h treatment. Each bar represents the mean ± SD of three independent experiments, ** indicates $p < 0.001$ versus the control group. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).

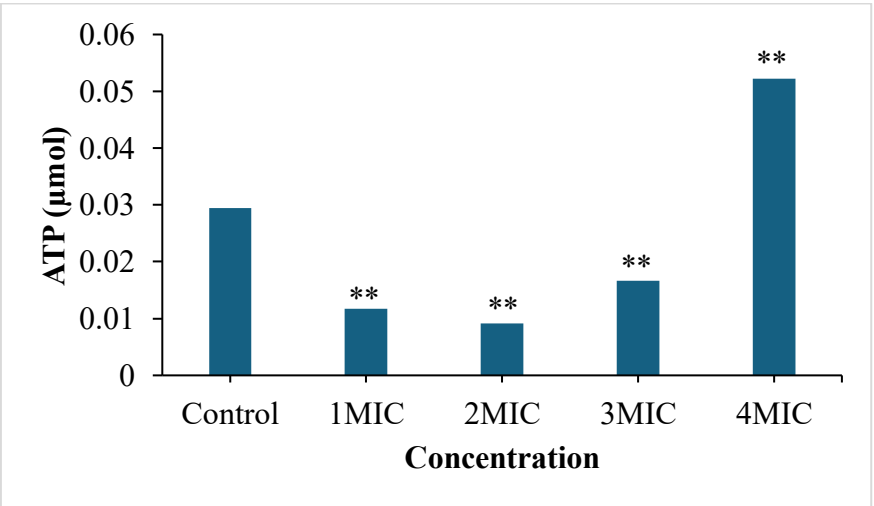


Figure 6.4. Effect of lemongrass nanoemulsion on ATP production of *B. cereus* P4. Each bar represents the mean ± SD of three independent experiments, ** indicates $p < 0.001$ versus the control group. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).

6.3.3 Effect of lemongrass nanoemulsion on membrane potential of *B. cereus*

Figure 6.5 demonstrates a concentration-dependent increase in fluorescence intensity for both *B. cereus* ATCC 14579 and P4, upon exposure to lemongrass nanoemulsion. In the control group, both isolates exhibited low fluorescence intensity, indicating minimal baseline fluorescence, At 1MIC and 2MIC, fluorescence intensity increased for both strains, suggesting enhanced membrane permeability or uptake of the fluorescent marker, with ATCC 14579 consistently showing slightly higher values than P4. This trend continued at 3MIC and 4MIC, with the highest fluorescence intensity observed at 4MIC for both isolates. Overall, the results highlight a concentration-dependent effect of lemongrass nanoemulsion on fluorescence intensity, with ATCC 14579 exhibiting marginally higher responses compared to P4.

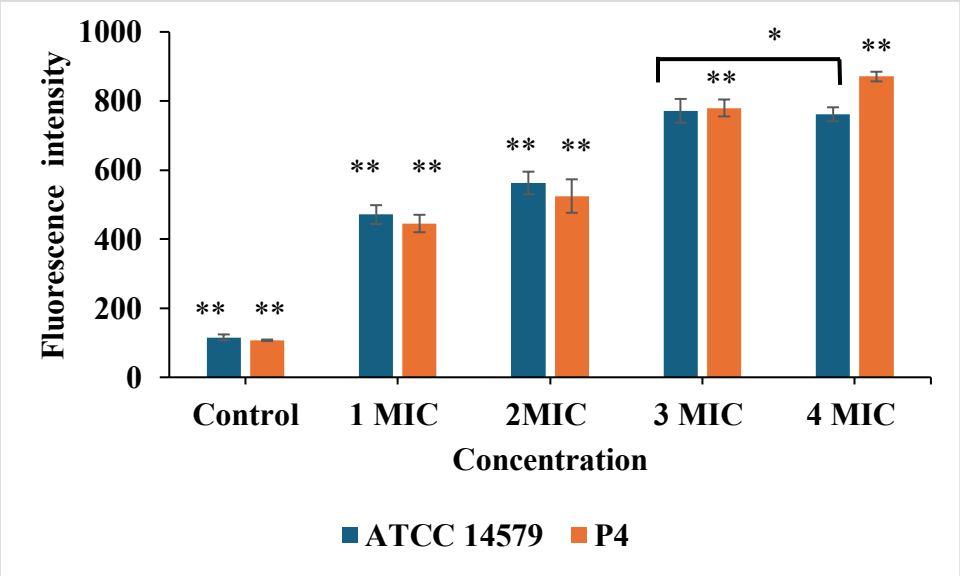
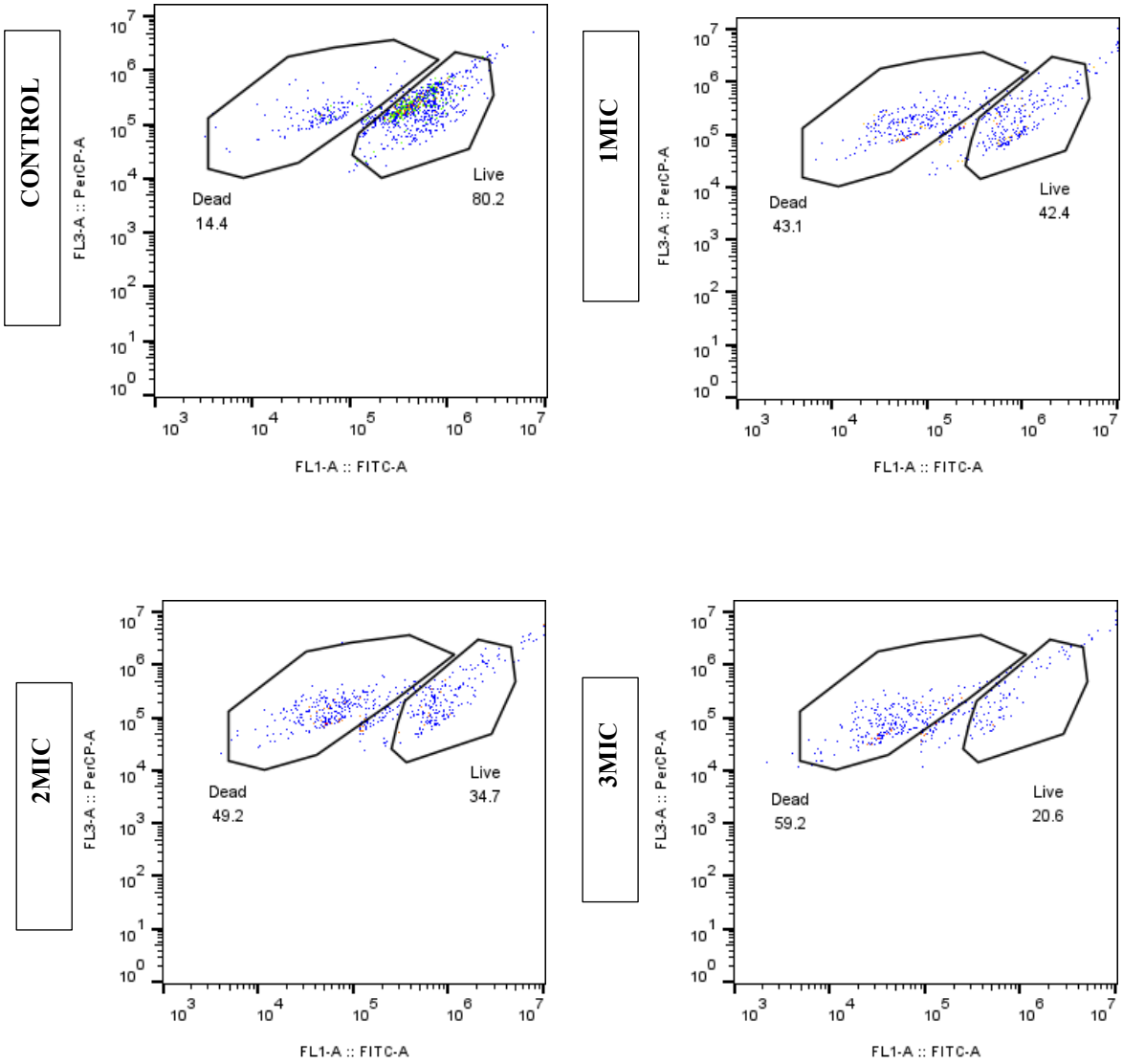


Figure 6.5. Effect of lemongrass nanoemulsion on membrane potential of *B. cereus* ATCC 14579 and *B. cereus* P4. Each bar represents the mean ± SD of three independent experiments, * and ** indicate $p < 0.001$ versus the control group. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).

6.3.4 Flow cytometric assessment of the antimicrobial activity of lemongrass nanoemulsion on *B. cereus*

Flow cytometry analysis (Figure 6.6 and 6.7) reveals the effect of lemongrass nanoemulsion on *B. cereus* ATCC 14579 and *B. cereus* isolate P4 viabilities using LIVE/DEAD BacLight staining. The dot plots show a clear shift from green fluorescence (FL1, indicating viable cells) to red fluorescence (FL3, indicating dead cells) as the concentration increased from 1MIC to 4MIC. The positive control (ethanol) showed a predominantly red population, confirming the validity of the assay.



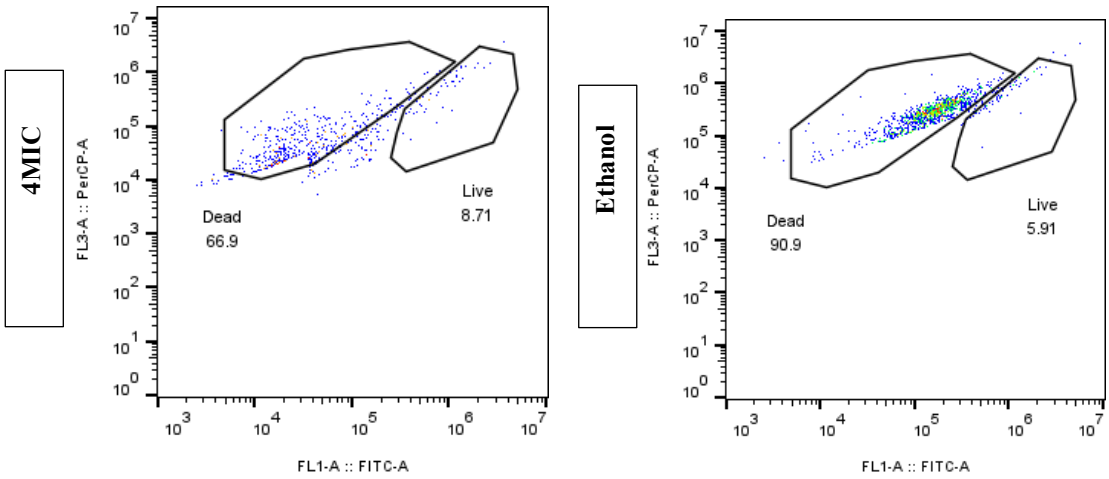
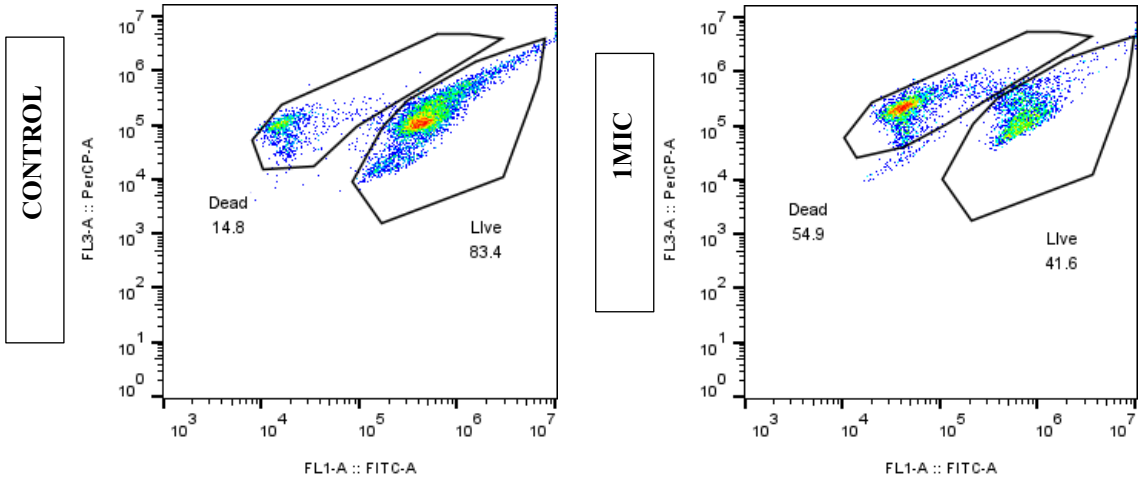


Figure 6.6. Flow cytometry (FC) dot plots of FL1, FITC-A (green fluorescence) vs. FL3, PerCP-A (red fluorescence) of *B. cereus* ATCC 14579 control cells, 1 hr treatment with 1MIC, 2MIC, 3MIC, 4MIC lemongrass nanoemulsion and with positive control, ethanol. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).



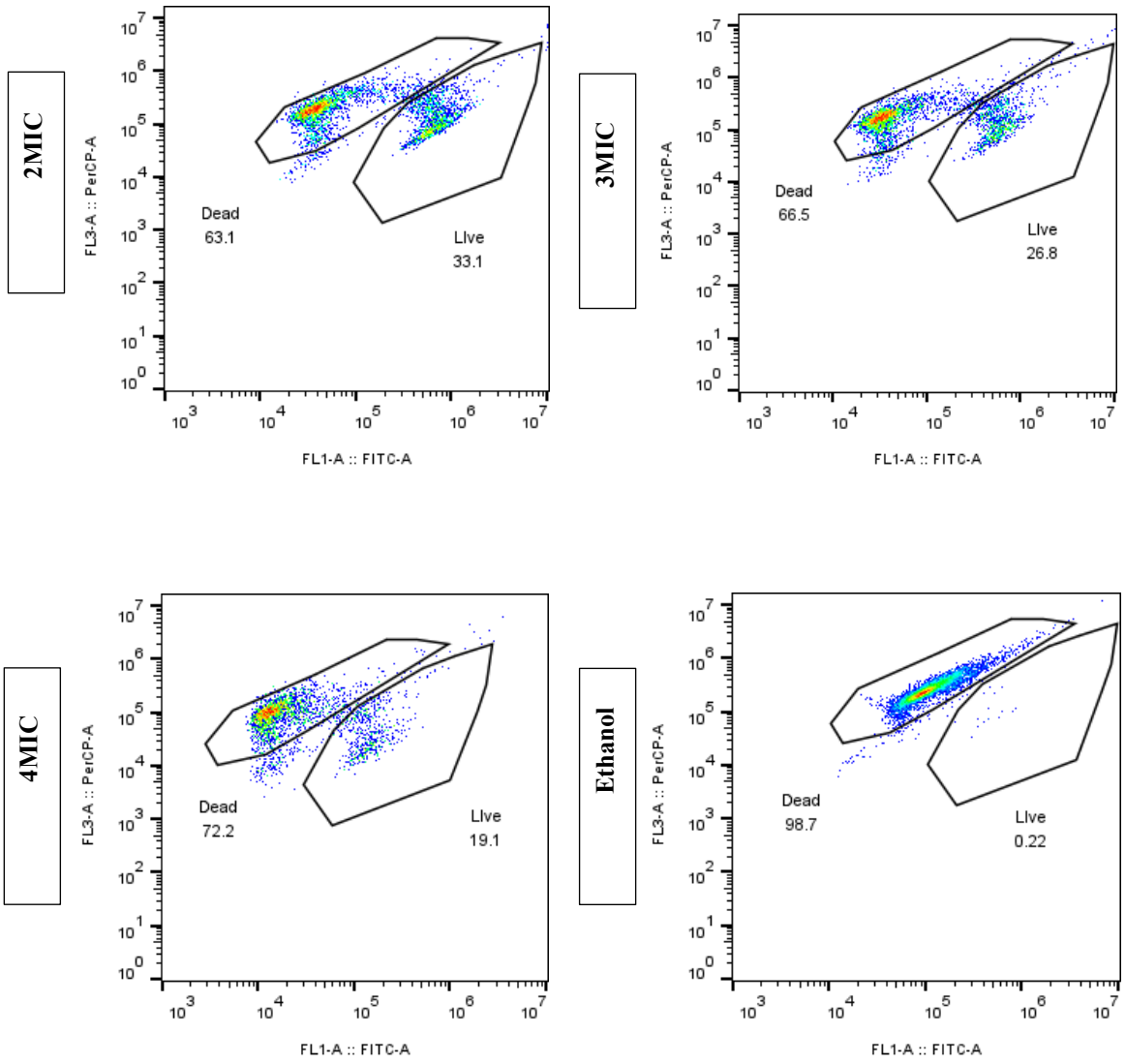
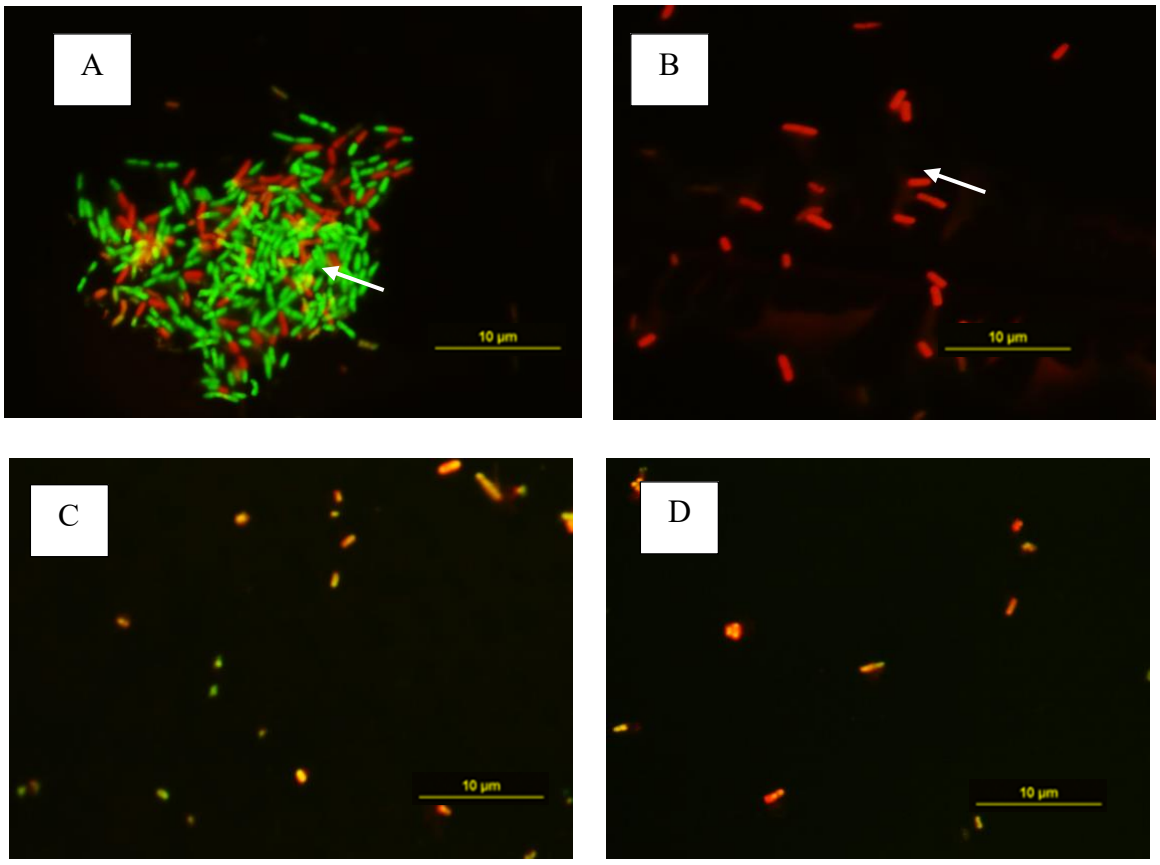


Figure 6.7. Flow cytometry (FC) dot plots of FL1, FITC-A (green fluorescence) vs. FL3, PerCP-A (red fluorescence) of *B. cereus* isolate P4 control cells, 1 hr treatment with 1MIC, 2MIC, 3MIC, 4MIC lemongrass nanoemulsion and with positive control, ethanol. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).

6.3.5 Fluorescent microscopic verification of live/dead stainings on *B. cereus* cells after treated with lemongrass nanoemulsion

The LIVE/DEAD BacLight staining images (Figures 6.8 and 6.9) illustrate the concentration-dependent bactericidal effects of lemongrass nanoemulsion on *B. cereus* ATCC 14579 and P4 isolates. In the untreated control (A), both *B. cereus* (ATCC 14579 and P4) exhibit predominantly green fluorescence, indicating a high proportion of live cells with intact membranes. The ethanol-treated positive control (B) shows mostly red fluorescence, reflecting extensive cell death and membrane disruption. With 1MIC (C) and 2MIC of lemongrass nanoemulsion (D) a noticeable reduction in green fluorescence was observed, with an increasing presence of red fluorescence, indicating partial bacterial mortality. At 3MIC (E) and 4MIC (F), red fluorescence dominates, signifying widespread bacterial lysis and loss of viability. Both isolates exhibited similar trends, with a progressive shift from green to red fluorescence as the nanoemulsion concentration increased, confirming the dose-dependent bactericidal activity of lemongrass nanoemulsion on *B. cereus*.



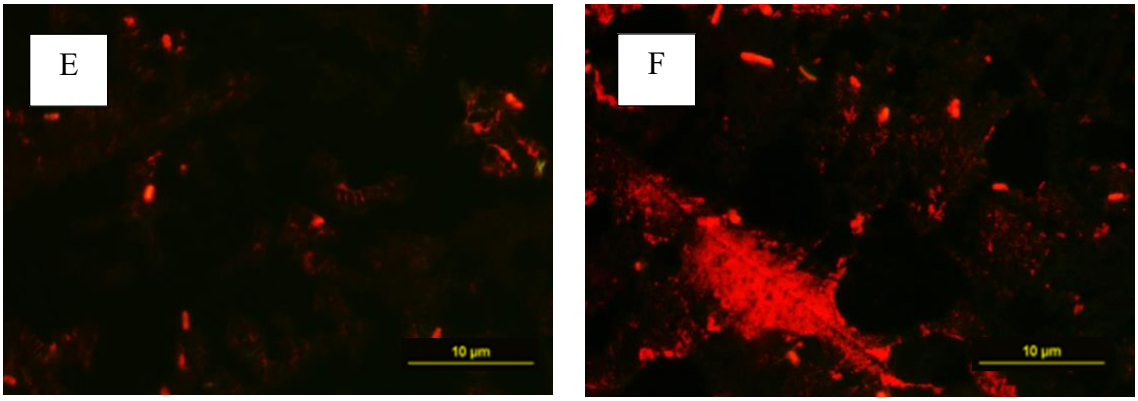
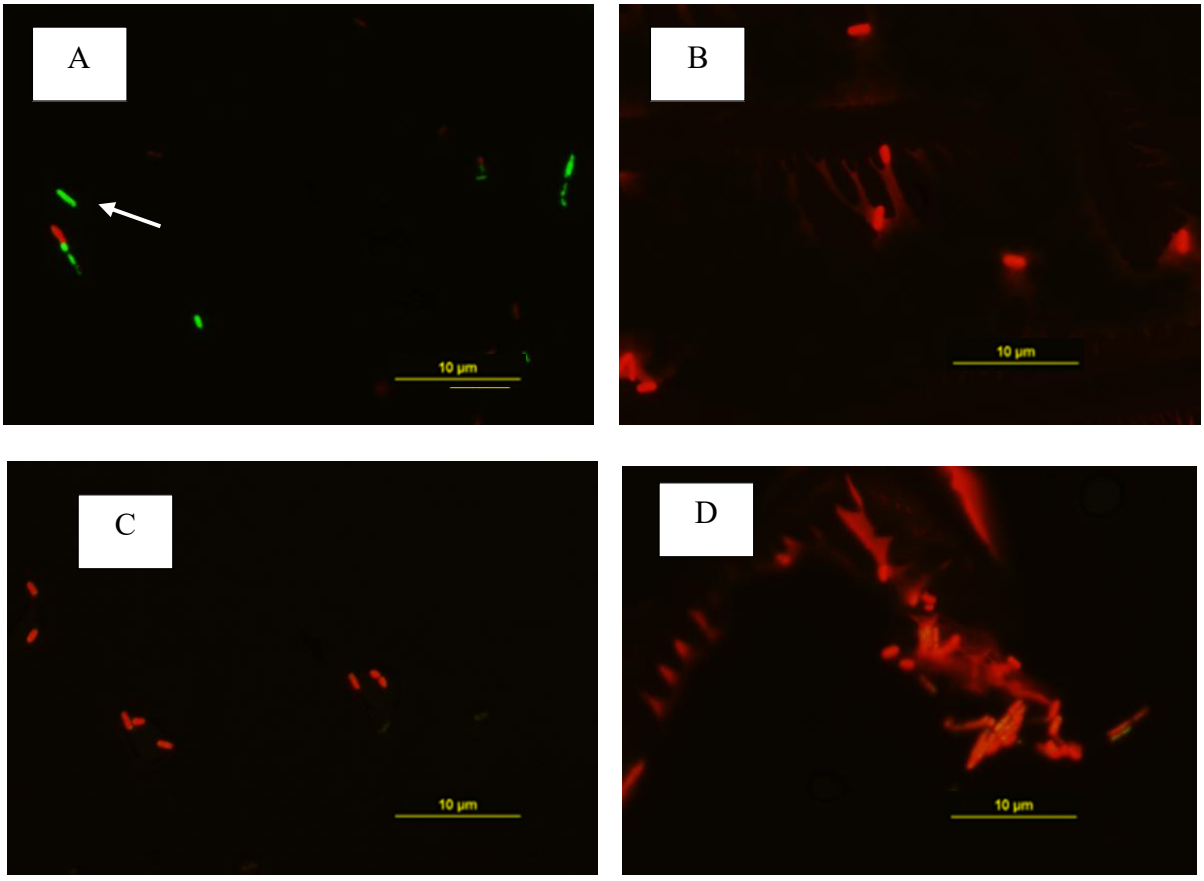


Figure 6.8. LIVE/DEAD BacLight staining of *B. cereus* ATCC 14579 untreated (A) Treatment with ethanol as a positive control; (B) Treatment with 1MIC of lemongrass nanoemulsion; (C) Treatment with 2MIC of lemongrass nanoemulsion; (D) Treatment with 3MIC of lemongrass nanoemulsion (E) and treatment with 4MIC of lemongrass nanoemulsion. White arrows indicate the live (green) or dead (red) cell. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).



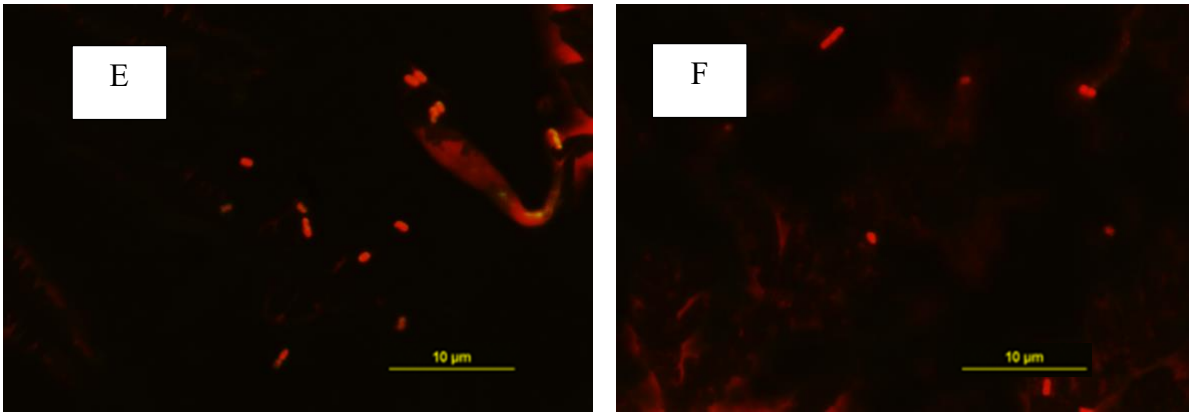


Figure 6.9. LIVE/DEAD BacLight staining of *B. cereus* isolate P4 untreated (A) Treatment with ethanol as a positive control; (B) Treatment with 1MIC of lemongrass nanoemulsion; (C) Treatment with 2MIC of lemongrass nanoemulsion; (D) Treatment with 3MIC of lemongrass nanoemulsion (E) and treatment with 4MIC of lemongrass nanoemulsion. White arrows indicate the live (green) or dead (red) cell. 1MIC–4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).

6.3.6 The effect of lemongrass naoemulsion on cell morphology and intracellular component of *B. cereus*

Transmission electron microscopy (Figure 6.10) reveals the ultrastructural damage inflicted by lemongrass nanoemulsion on *B. cereus* ATCC 14579 and isolate P4. Untreated cells (A, B) exhibit intact membranes and well-preserved cellular structures. In contrast, cells treated with 4MIC lemongrass nanoemulsion (C, D) show severe morphological alterations, including loss of uniformity in the cell wall boundary, Irregular defined cell membrane and disruption of cytoplasmic integrity.

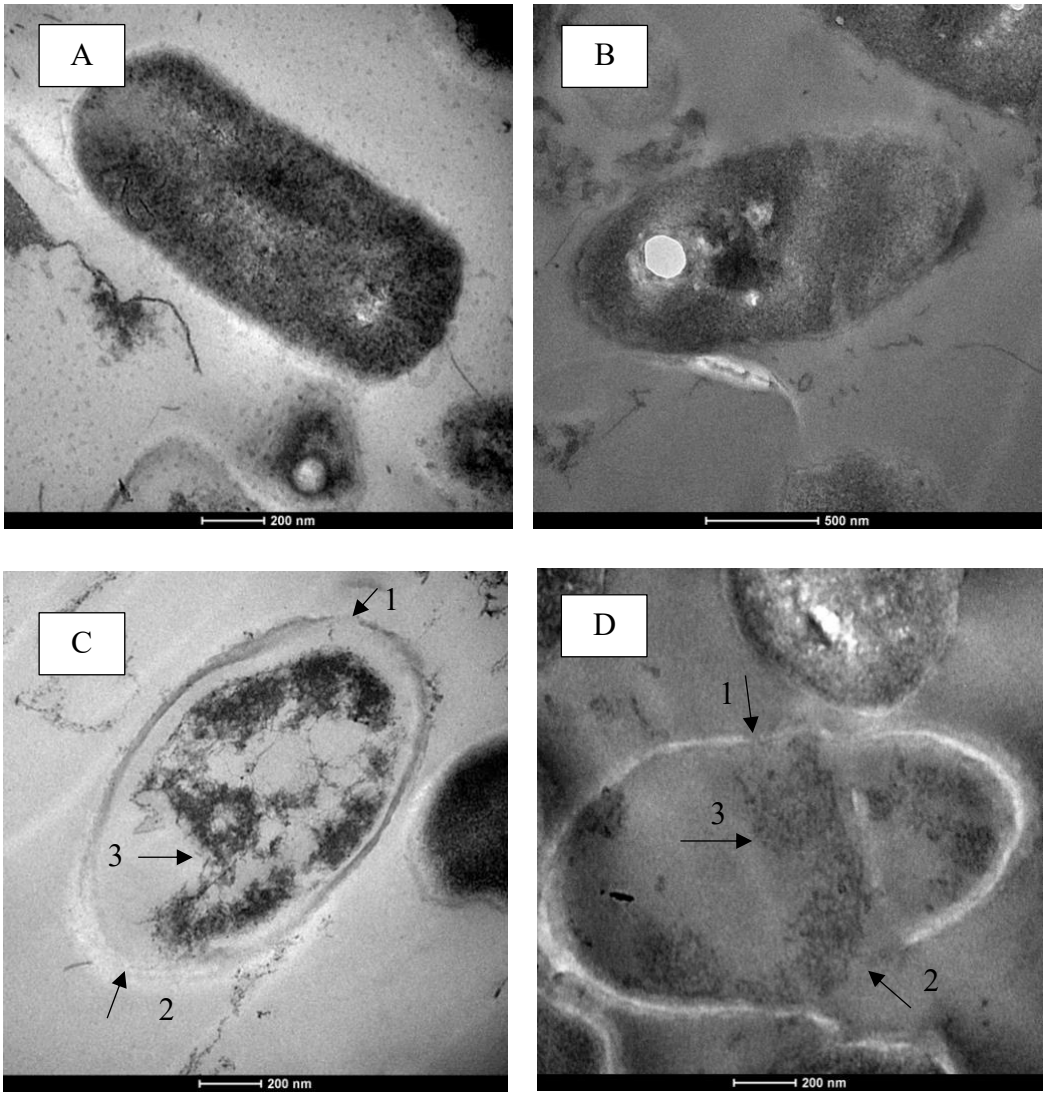


Figure 6.10. The transmission electron photography of *B. cereus* P4 (A, C) and *B. cereus* ATCC 14579 (B, D). The treatments are (A, B) untreated; (C, D) treated with 4MIC lemongrass nanoemulsion. Arrows indicate (1) Loss of uniformity in the cell wall boundary; (2) Irregular defined cell membrane; (3) Disruption of cytoplasmic integrity. 4MIC represent concentration levels derived from a two-fold (doubling) serial dilution series based on the MIC value. (1MIC = 0.125% (v/v), 2MIC = 0.250% (v/v), 3MIC = 0.500% (v/v), 4MIC = 1.000% (v/v)).

6.4 Discussion

The growth kinetics of *B. cereus* ATCC 14579 and the isolate P4 demonstrated a clear concentration-dependent suppression of bacterial growth in the presence of lemongrass nanoemulsion (Figures 6.1 and 6.2; Tables 6.1 and 6.2). At the MIC (0.125% v/v) and

above, exponential growth was not detected for either isolate over the 24 h incubation period, with ln(OD_{590}) values remaining close to baseline, indicating a strong bacteriostatic effect under the conditions of this assay.

At sub-inhibitory concentrations (1/2 MIC (0.063% v/v), 1/4 MIC (0.031% v/v), and 1/8 MIC (0.016% v/v)), exponential growth was retained but occurred later and over progressively shorter time intervals as nanoemulsion concentration increased. This was reflected by delayed increases in ln(OD_{590}) values and by the concentration-specific exponential windows used to estimate the specific growth rate (μ). Consequently, μ values varied across concentrations and strains and were interpreted relative to each curve's exponential phase rather than at a fixed time point. Apparent increases in μ at certain sub-MIC concentrations reflect steeper slopes over brief, delayed exponential phases rather than enhanced overall growth, as biomass accumulation remained delayed and reduced compared with untreated controls. This contrasts with the fixed-time growth rate approach reported by Zaheer et al. (2021), which was appropriate in their study because exponential growth occurred over a common time window across all treatments.

Comparative analysis revealed strain-dependent differences in sensitivity to growth suppression, with the isolate P4 exhibiting slower baseline growth and more pronounced delays in exponential growth onset than ATCC 14579. This highlights variability in susceptibility among *B. cereus* strains and underscores the importance of including both reference strains and field isolates in antimicrobial assessments.

Overall, the combined analysis of growth curves and growth rate estimates indicates that lemongrass nanoemulsion suppresses *B. cereus* growth primarily by limiting the ability to enter and sustain exponential growth. These findings are consistent with previous reports describing the antibacterial activity of lemongrass essential oil and its nanoemulsion formulations, which have been linked to disruptions of membrane integrity and cellular metabolism (Burt, 2004; Calo et al., 2015; Noorbakhsh et al., 2025).

A deeper understanding of bacterial viability and metabolic activity can be achieved through adenosine triphosphate (ATP) quantification, which serves as an indicator of overall cellular integrity and metabolic collapse rather than a standalone metabolic pathway. It plays an essential role in biosynthesis, transport, motility, and its presence

depends on intact membranes and functional cellular systems (Vestergaard et al., 2022). As depicted in Figures 6.3 and 6.4, ATP levels decreased at 1MIC and 2MIC, correlating with a reduction in bacterial viability and metabolic activity. This decline aligns with previous reports indicating that essential oil-based antimicrobials disrupt bacterial membranes, leading to ATP leakage and energy depletion, ultimately resulting in cell death (Espina et al., 2011).

Interestingly, at higher concentrations (3MIC and 4MIC), ATP levels increased sharply, suggesting a biphasic response, a reaction characterized by two distinct phases, often involving an initial inhibitory effect followed by a secondary compensatory effect. This pattern is commonly observed in biological systems, including microbial stress responses and cellular metabolism (Calabrese, 2013). This pattern contrasts with the findings of Shi et al. (2016), who reported a consistent decrease in intracellular ATP following citral exposure. This variation may be attributed to several factors. Notably, Shi et al. (2016) investigated *Cronobacter sakazakii*, whereas the present study examined *B. cereus*, a spore-forming Gram-positive bacterium with distinct membrane composition, stress response pathways, and energy regulation mechanisms.

The increase in ATP signal at higher concentrations may be explained by a combination of biological responses and assay-related factors. A surviving subpopulation of bacteria may activate stress response pathways, including altered energy metabolism or efflux systems, in an attempt to counteract antimicrobial stress (Gill & Holley, 2006; Zack et al., 2024). Alternatively, extensive membrane disruption at elevated nanoemulsion concentrations may lead to leakage of intracellular ATP into the extracellular environment, which can subsequently be detected by the bioluminescence assay (Shi et al., 2016).

Importantly, as ATP was quantified after cell lysis using a luciferase–luciferin assay, the measured signal reflects total cell-associated ATP rather than intracellular ATP dynamics alone. Thus, ATP levels should be interpreted as an indicator of overall cellular integrity rather than a specific metabolic pathway. As the control condition consisted of bacteria only (without extract), the absence of an extract-only control represents a limitation when interpreting ATP elevations at higher concentrations. Accordingly, the increase in ATP signal observed at 3–4MIC should be interpreted cautiously and does

not necessarily indicate enhanced bacterial viability or the presence of a fundamentally different antimicrobial mechanism.

Understanding the resting membrane potential is crucial as it reflects the electrochemical gradient maintained by bacterial cells, which is essential for ATP production, nutrient transport, and cellular homeostasis (Benarroch & Asally, 2020). Disrupting this potential can impair vital cellular functions, leading to bacterial death, making it a key target for antimicrobial agents like lemongrass nanoemulsion (Begum et al., 2024). Figure 6.5 illustrates the effect of lemongrass nanoemulsion on the membrane potential of *B. cereus* ATCC 14579 and isolate P4, as indicated by fluorescence intensity. The increase in fluorescence intensity with increasing nanoemulsion concentrations suggests a progressive depolarization of the bacterial membrane. This pattern is consistent with the mode of action of antimicrobial agents that disrupt membrane integrity, leading to ion leakage, loss of membrane potential, and eventual cell death (Molina-Hernandez et al., 2021).

At lower concentrations (1MIC and 2MIC), there was a noticeable increase in fluorescence intensity compared to the control cells, indicating the initial stages of membrane depolarization. As the concentration increases (3MIC and 4MIC), the fluorescence intensity increased further, suggesting extensive membrane damage and loss of potential. This aligns with previous studies reporting that essential oils and nanoemulsions act primarily by disrupting the lipid bilayer, leading to increased membrane permeability (Maurya et al., 2021).

The flow cytometry (Figure 6.6 and 6.7) analysis demonstrated that *B. cereus* ATCC 14579 and P4 exhibited a strong dose-dependent response, with a progressive decline in live cell percentages correlating with increasing lemongrass nanoemulsion concentrations. One key challenge in analyzing the flow cytometry data was the presence of autofluorescent particles, which affected the interpretation of the Syto 9 and PI plots. Autofluorescent particles were smaller in size for *B. cereus* P4, allowing effective exclusion using forward scatter (FSC)-based gating. However, in *B. cereus* ATCC 14579, autofluorescent particles overlapped with bacterial populations, necessitating a more refined not gating strategy to remove them from the final analysis. Such autofluorescence-related challenges have been reported in microbial flow cytometry

studies and must be carefully controlled to prevent misinterpretation of viability assays (Iyengar et al., 2024; Pilkington, 2024).

Fluorescent microscopic imaging (Figures 6.8 and 6.9) using the LIVE/DEAD BacLight staining technique provided direct visual evidence of the antibacterial effect of lemongrass nanoemulsion on *B. cereus* ATCC 14579 and isolate P4. The green fluorescence observed in the untreated control samples indicated intact, viable bacterial cells with preserved membrane integrity, while increasing concentrations of lemongrass nanoemulsion resulted in a progressive shift toward red fluorescence, indicative of membrane damage and cell death.

The images reveal a dose-dependent reduction in viable cells, with the transition from intact bacterial clusters in the control to scattered, compromised cells at higher nanoemulsion concentrations. At sub-lethal concentrations (1MIC), a mixed population of live (green) and dead (red) cells was visible, suggesting partial membrane permeability and early-stage bacterial stress. As the concentration increased (2–4MIC), a predominance of red-fluorescent cells was observed, indicating extensive membrane disruption leading to bacterial death. This aligns with findings from previous studies demonstrating that citral, the major bioactive compound in lemongrass, disrupts bacterial lipid bilayers, increasing permeability and causing leakage of intracellular components (Gutierrez-Pacheco et al., 2023; Mukarram et al., 2021).

The transmission electron microscopy (TEM) images provide ultrastructural evidence of the antibacterial effect of lemongrass nanoemulsion on *B. cereus* ATCC 14579 and isolate P4. The untreated bacterial cells (Figure 6.10a and 6.10b) exhibited an intact cell membrane and cytoplasmic organization, maintaining their characteristic rod shape with dense intracellular contents. In contrast, cells treated with 4MIC lemongrass nanoemulsion (Figure 6.10C and 6.10D) displayed pronounced ultrastructural alterations, including deformation of the cell envelope, irregular and less distinct membrane boundaries, and regions of reduced cytoplasmic electron density, as indicated by the arrows in the images. These observations indicate substantial compromise of cellular ultrastructure and envelope integrity following treatment.

While TEM does not allow direct determination of specific antimicrobial mechanisms, the observed morphological changes are consistent with membrane-associated damage commonly reported for essential oil–based antimicrobials. Similar envelope deformation and loss of structural integrity in *B. cereus* cells have been reported following exposure to thyme essential oil using environmental scanning electron microscopy (Kang et al., 2018).

6.5 Conclusion

Overall, lemongrass nanoemulsion exhibits potent antibacterial activity against *B. cereus* ATCC 14579 and isolate P4, demonstrating concentration-dependent inhibition with isolate-specific variations. The combined evidence from membrane potential analysis, fluorescence microscopy, and transmission electron microscopy indicates substantial compromise of cell envelope integrity and cellular ultrastructure following treatment. While these observations are consistent with a membrane-associated mode of antimicrobial action, they do not permit direct confirmation of cell lysis. These findings highlight lemongrass nanoemulsion as a promising natural antimicrobial for food safety, pharmaceuticals, and biocontrol, underscoring the need for further research into its molecular mechanisms and synergistic applications.

6.6 Acknowledgement

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6.7 Copyright information

Parts of this study is intended to be submitted to a journal for publication and the Online Statement of Contribution form is attached in Appendix V.

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Chapter 7. Final discussion and Future work

7.1 Final discussion

This study investigated the antimicrobial potential of lemongrass (*Cymbopogon citratus*) nanoemulsion against *Bacillus cereus*, a spore-forming and biofilm-producing foodborne pathogen. The research began by using supercritical fluid extraction (SFE), identified from the literature as the most effective method for extracting citral (Ashaq et al., 2024). Citral is an active compound responsible for antimicrobial activity (Gutierrez-Pacheco et al., 2023). The antimicrobial efficacy of these extracts was compared with the primary emulsions to evaluate their effectiveness against *B. cereus* in different food matrices, specifically rice milk, soy milk, and milk protein concentrate (MPC) (Chapter 3). To improve efficacy, Chapter 4 explored the application of nanoemulsion technology. Nanoemulsions of Lemongrass extract, and commercial citral were tested and compared to nisin as nisin is a well described commercial antimicrobial. The antimicrobial efficacy of these formulations was assessed against *B. cereus* as planktonic cells and biofilm cells and explored the potential to prevent biofilm formation, as biofilms are a source of contamination in the food industry. In Chapter 5, the nanoemulsions were characterized to determine droplet size, polydispersity index, and conductivity. Stability testing was also conducted to evaluate resistance to heat and shelf-life. Finally, the mechanism of action of the lemongrass nanoemulsion was elucidated to understand how it exerts its antimicrobial effect against *B. cereus* cells (Chapter 6). This project was designed to address the following research questions:

7.1.1 How effective is supercritical fluid-extracted lemongrass oil in inhibiting *B. cereus* in different food matrices, and does emulsification improve its antimicrobial performance?

This study provided compelling evidence supporting the efficacy of SFE extraction and the functional advantage of emulsification. The lemongrass extract derived from the Peha Ayam (PA) variety at 85 bar exhibited the highest citral content (90.06%) and demonstrated the most potent antimicrobial activity, with the lowest MIC (0.313% (v/v)) and MBC (0.625% (v/v)) values across all isolates tested. When formulated as an emulsion, the antimicrobial efficacy of both lemongrass and citral improved significantly ($p<0.05$), achieving MIC values of 0.125% (v/v) which is five times lower than the crude extract, highlighting the role of emulsification in enhancing bioavailability and

membrane interaction. Application studies in soy milk, rice milk, and milk protein concentrate (MPC) matrices further demonstrated that both emulsions (lemongrass and citral) produced concentration-dependent inhibition of *B. cereus*. Among these, soy milk supported the most effective bacterial reduction, whereas rice milk exhibited comparatively reduced efficacy, potentially due to its higher carbohydrate content offering protective buffering to *B. cereus*. In contrast, variations in MPC concentrations (5%, 10%, and 15%) did not significantly ($p>0.05$) influence the antimicrobial outcomes, suggesting that protein level was not a critical factor. These findings collectively indicate that optimized SFE, particularly at lower pressures preserving citral integrity, coupled with emulsion formulation, offers a robust natural antimicrobial strategy against *B. cereus* in various food systems.

7.1.2 How effective are lemongrass and citral nanoemulsions in inhibiting both planktonic and biofilm *B. cereus* compared to nisin across different isolates?

Both lemongrass and citral nanoemulsions exhibited consistent and potent antimicrobial activity against planktonic *B. cereus*, with minimum inhibitory concentrations (MICs) of 0.125% (v/v) across all tested isolates (P4, M2, and ATCC 14579). In contrast, nisin required a higher MIC of 2 500 µg/mL, equivalent to 0.25% (w/v), to achieve comparable inhibition. This highlights the greater apparent potency of the lemongrass and citral-based nanoemulsions under the tested conditions. When applied in dose-dependent CFU-based assays, both lemongrass and citral nanoemulsions achieved planktonic reductions in isolates P4 and M2, reaching ≥5.8 $\log_{10}$ CFU/mL reduction at 3–4MIC. However, isolate ATCC 14579 exhibited greater tolerance, with lemongrass and citral treatments resulting in ≤2.0 $\log_{10}$ CFU/mL reduction even at 5MIC. In contrast, nisin achieved >4.5 $\log_{10}$ CFU/mL reduction at 3MIC for ATCC 14579, underscoring isolate-specific differences in susceptibility and suggesting that nisin maintained superior activity in this strain under planktonic conditions.

In biofilm control assays, lemongrass nanoemulsion consistently outperformed citral and nisin, reducing viable biofilm-associated cells in isolates P4 and M2 by >4.0 $\log_{10}$ CFU/cm^2 at concentrations as low as 2MIC. These log reductions were significantly ($p<0.05$) higher than those observed for nisin, which showed ≤1.5 $\log_{10}$ CFU/cm^2 reduction under similar conditions. The enhanced biofilm efficacy of lemongrass may be

attributed to the nanoemulsion's ability to disrupt matrix integrity and interfere with adhesion-related pathways. Nisin showed limited efficacy against biofilms, particularly for ATCC 14579, likely due to restricted diffusion, lower lipid II expression in sessile cells, and potential peptide degradation (Musiejuk & Kafarski, 2023), In biofilm prevention assays, which assessed antimicrobial pre-coating of surfaces before bacterial inoculation, lemongrass again showed the greatest efficacy, especially for the more susceptible isolates. Biofilm control was generally more effective than prevention, likely due to longer antimicrobial exposure and uninterrupted early quorum sensing in the latter. Overall, lemongrass nanoemulsion exhibited the most consistent and robust performance across all conditions, supporting its potential as a natural, plant-based alternative to synthetic or peptide antimicrobials for managing both planktonic and biofilm-associated *B. cereus* contamination in food safety contexts.

7.1.3 What are the key physicochemical properties of lemongrass nanoemulsion, and how do they contribute to its stability and potential antimicrobial effectiveness?

The key physicochemical properties of lemongrass nanoemulsion including droplet size, polydispersity index (PDI), conductivity, and stability played, a pivotal role in supporting both its shelf-life and antimicrobial performance. The optimized formulation using sodium alginate (0.2%) and Tween 80 (0.5%) as stabilizers, coupled with high-pressure homogenization at 150 MPa, resulted in a stable oil-in-water system with droplet sizes consistently below 100 nm and PDI values under 0.3. These characteristics indicate a highly monodisperse and uniform emulsion, which enhances physical stability and prevents coalescence or phase separation (Ho et al., 2022). Conductivity measurements confirmed the integrity of the continuous aqueous phase, indicating good dispersion and system uniformity (Preeti et al., 2023). Heat stability assays revealed no significant ($p>0.05$) changes in antimicrobial efficacy, as the minimum inhibitory concentration (MIC) values remained stable even after exposure to temperatures of up to 90°C for 15 min. Similarly, shelf-life assessments demonstrated that the nanoemulsion maintained its antimicrobial effectiveness over time, achieving consistent log reductions in *B. cereus* colony counts for up to four months under dark storage at 4°C.

These findings underscore the importance of the nanoemulsion's physicochemical attributes particularly its small droplet size and narrow polydispersity index which increase surface area contact with bacterial membranes and enable uniform, controlled release of bioactive compounds. These nano-scale characteristics enhance the diffusion and membrane penetration of citral, the primary antimicrobial agent in lemongrass oil, thereby contributing to more rapid and potent bacterial inhibition. Collectively, the physicochemical stability and structural integrity of the lemongrass nanoemulsion are fundamental to its sustained antimicrobial performance and support its potential application as a natural preservative in food safety systems.

7.1.4 What are the observed patterns of antimicrobial activity of lemongrass nanoemulsions against planktonic *B. cereus* cells, and what might these suggest about potential modes of action?

The antimicrobial activity patterns observed for lemongrass nanoemulsions against planktonic *B. cereus* cells revealed both consistency and concentration-dependence, providing insights into the potential modes of action. Across two isolates (P4 and ATCC 14579), the lemongrass nanoemulsion exhibited uniform MIC values of 0.125% (v/v), indicating a broad-spectrum baseline efficacy. Planktonic inhibition assays, CFU based, revealed a clear dose-dependent pattern. For isolate P4, treatment with 3 MIC resulted in a mean reduction of 5.87 $\log_{10}$ CFU/mL, while increasing the concentration to 4MIC produced a slightly higher reduction of 6.12 $\log_{10}$ CFU/mL, suggesting saturation of the bactericidal effect beyond 3MIC. In contrast, isolate ATCC 14579 showed greater tolerance as 3MIC yielded a 3.85 $\log_{10}$ CFU/mL reduction, which improved to 5.02 $\log_{10}$ at 4MIC, indicating a stronger concentration‑response effect in this more resilient isolate.

A rapid decline in CFU counts was observed early after adding the lemongrass extract nanoemulsion, implying membrane-disruptive action consistent with the physicochemical nature of citral and the nanoemulsion. The nanoemulsion's small droplet size and enhanced dispersibility may have promoted fast absorption and interaction with bacterial cell membranes (Tanuku et al., 2024), facilitating cytoplasmic leakage and disruption of essential cellular processes. These findings align with known mechanisms of citral, which include compromising membrane integrity, interfering with the proton motive force, and potentially altering intracellular pH and enzyme function (Dai et al.,

2025; Gutierrez-Pacheco et al., 2023). The rapid, concentration-dependent inhibition, combined with uniform efficacy across diverse isolates, supports the hypothesis that lemongrass nanoemulsions exert their antimicrobial effect through a multi-targeted membrane-disruption mechanism enhanced by nanoencapsulation. This mechanism is particularly valuable in the food industry where rapid microbial control is essential.

7.2 Limitations

Despite the promising findings generated from this study, several limitations must be acknowledged to contextualize the scope of the results and consider directions for future research.

This study was conducted during the COVID-19 pandemic, which impacted the research timeline. As part of the 2021 cohort, laboratory work was delayed by approximately one year due to international border closures and restricted campus access in New Zealand. These unforeseen circumstances limited the immediate commencement of experimental work and postponed access to essential equipment. Notably, the microfluidizer required for producing nanoemulsions was not initially available within the host laboratory. As a result, the early-phase application study was conducted using raw SFE lemongrass extract and its emulsion, prior to full nanoemulsion formulation. While this provided a valuable baseline for comparison between crude and emulsified extracts, fully characterized nanoemulsions were only possible later in the study once access to the microfluidizer was secured. These logistical constraints inevitably shaped the sequencing of the experiments and should be considered when interpreting the scope and progression of findings.

7.3 Future Research Direction

The findings of this study highlight the antimicrobial potential of lemongrass nanoemulsion against *B. cereus* in model systems. As this is the first study to examine the use of supercritical fluid-extracted lemongrass oil as a nanoemulsion for controlling *B. cereus*, it represents a novel approach to control this foodborne pathogen. However, several key areas warrant further exploration to support translation into commercial applications and deepen scientific understanding.

1. Validation in real food systems

While this study demonstrated efficacy in simplified food matrices (soy milk, rice milk, and milk protein concentrate), future studies should evaluate the performance of lemongrass nanoemulsion in more complex and commercially relevant food systems. This includes ready-to-eat meals, processed dairy products, high-fat matrices (e.g., cheese, cream-based sauces), and high-protein foods such as meat or plant-based foods. The influence of variables such as pH, temperature, food structure, and storage duration on antimicrobial persistence and release kinetics must be investigated. Real-time testing under industrial storage and processing conditions (e.g., pasteurization, freezing, packaging environments) will also provide useful information for industrial application.

2. Improved Formulation Stability

Although the current nanoemulsion demonstrated strong thermal and shelf stability, its long-term robustness under diverse food processing conditions may be further enhanced. Advanced delivery systems such as biopolymer encapsulation, layer-by-layer nanocoating, and microencapsulation techniques could be explored to improve physical and oxidative stability while modulating controlled release of bioactive compounds. Incorporating edible coatings or emulsifiers with targeted release mechanisms may also extend antimicrobial activity during food storage and distribution.

3. Broader Antimicrobial Spectrum Testing

This study focused specifically on *B. cereus* as a model spore-forming and biofilm-producing pathogen. To increase the scope of lemongrass nanoemulsions, future research should investigate antimicrobial performance against a wider range of foodborne pathogens, including *L. monocytogenes*, *E. coli* O157:H7, *Salmonella spp.*, and *S. aureus*. Additionally, given the resilience of biofilms in food processing environments, mixed-species biofilm models should be incorporated to evaluate how lemongrass nanoemulsions perform under complex microbial interactions.

4. Incorporation of Molecular Profiling Tools

To elucidate the mechanisms underlying the antimicrobial and anti-biofilm actions of lemongrass nanoemulsions, future studies should integrate transcriptomic, proteomic, or metabolomic approaches. These methods would allow in-depth profiling of microbial

responses at the cellular and molecular levels, revealing critical stress pathways, membrane disruption processes, or adaptive resistance mechanisms in *B. cereus* and other pathogens. Such insights would also help in the rational design of combination therapies and formulation adjustments to delay resistance development.

5. Sensory Evaluation and Regulatory Assessment

For commercial adoption, it is critical to evaluate the sensory acceptability of lemongrass nanoemulsion in various food matrices. Comprehensive sensory profiling that covers aroma, taste, texture, and aftertaste should be conducted using trained panels and consumer testing. These evaluations will determine organoleptic compatibility and acceptance thresholds.

6. Toxicity and Safety Evaluation

Although citral and lemongrass are classified as GRAS at low concentrations, the nanoformulation may alter bioavailability and pharmacokinetics, potentially influencing toxicity profiles. Future studies should include detailed in vitro and in vivo toxicity assessments, including cytotoxicity to intestinal and hepatic cell lines, genotoxicity, and acute/sub-chronic animal models. Particular attention should be given to potential impacts on gut microbiota and cumulative exposure in vulnerable populations (e.g., children, pregnant women). Ensuring the safety of lemongrass nanoemulsion in both food and human health contexts is critical for responsible commercialization.

7.4 Final conclusion

This thesis explored the development and application of lemongrass nanoemulsions as natural antimicrobial agents against *B. cereus*, addressing the global need for safer, plant-based food preservatives. Using an integrated approach encompassing SFE, nanoformulation, food matrix application, and mechanistic studies, it demonstrated that citral-rich extracts, especially those derived under optimized pressure and temperature conditions, showed potent antimicrobial activity. This efficacy was enhanced through nano-sizing, enabling stable performance under heat and storage conditions and promoting rapid bacterial inhibition through membrane disruption.

Conceptually, this work contributes to food microbiology by underscoring the importance of formulation science, food-context sensitivity, and mechanism-driven design in developing sustainable antimicrobial solutions. By aligning green extraction methods with nano-delivery technologies, the study presents a viable alternative to synthetic preservatives. Future research should now focus on sensory evaluation, toxicological safety, regulatory compliance, and scalability to support commercial translation across food, pharmaceutical, and biocontrol industries.

7.5 References

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Appendices

Appendix I Online Statement of Contribution (Chapter 2)

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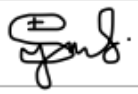
Student's signature:		Main supervisor's signature:	Steve Flint Digitally signed by Steve Flint Date: 2025.07.09 12:00:22 +12'00'

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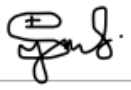
Student's signature:		Main supervisor's signature:	Steve Flint Digitally signed by Steve Flint Date: 2025.07.09 12:00:55 +12'00'

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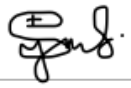
Student's signature:		Main supervisor's signature:	Steve Flint Digitally signed by Steve Flint Date: 2025.07.09 12:01:22 +12'00'

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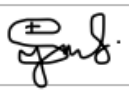
Student's signature:		Main supervisor's signature:	Steve Flint Digitally signed by Steve Flint Date: 2025.07.09 12:01:42 +12'00'

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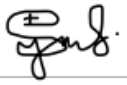
Student's signature:		Main supervisor's signature:	Steve Flint Digitally signed by Steve Flint Date: 2025.07.09 12:02:02 +12'00'

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Appendix VI First page of published paper 1

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Characterization, antibacterial activity, and stability of supercritical fluid extracted lemongrass nanoemulsion on *Bacillus cereus*

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ABSTRACT

Natural food preservation is a sustainable approach to extend shelf life, combat foodborne pathogens and enhance food safety. *Bacillus cereus*, a resilient contaminant, poses challenges due to its spore-forming ability and association with foodborne illnesses. This study investigates the characterization, antimicrobial activity, and stability of lemongrass (*Cymbopogon citratus*) nanoemulsions, extracted using supercritical fluid extraction (SFE), and their efficacy against *B. cereus* isolates (ATCC 14579, P4, and M2). Lemongrass oil was extracted at 85, 100, 200, and 300 bar, with the highest yield (0.815 %) obtained at 300 bar. Nanoemulsions were formulated with lemongrass extract and commercial citral, characterized for droplet size, polydispersity index (PDI), conductivity, and zeta potential, and assessed for antimicrobial activity. Lemongrass nanoemulsions initially had droplet sizes of 86.32 ± 0.66 nm, but increased over six months due to coalescence, with PDI values rising from 0.50 ± 0.00 to 0.81 ± 0.27, indicating reduced stability. Although zeta potential declined from −44.01 ± 1.69 mV to −33.63 ± 1.45 mV, it remained within the stable range (>±30 mV), maintaining sufficient electrostatic repulsion to prevent rapid aggregation. At 2.0 % concentration, nanoemulsions effectively suppressed *B. cereus* isolates (<1.00 CFU/mL), though efficacy declined after four months with increasing droplet size. Lemongrass nanoemulsions exhibited comparable antibacterial activity and stability trends to citral, suggesting that whole lemongrass extract retains its bioactivity as effectively as its major compound. Improved stabilization strategies, such as polymer encapsulation, could enhance shelf life, expanding applications in food preservation.

1. Introduction

Utilizing plant extracts as a natural food preservative has become an increasing trend as relatively cheap, readily available, environmentally friendly, natural compounds attractive to consumers and food manufacturers to enhance food safety and reduce food waste. As consumers demand healthier, less chemically processed food, plant-based preservatives present a promising alternative to synthetic chemicals. Among these, lemongrass (*Cymbopogon citratus*) stands out due to its notable antimicrobial properties, primarily attributed to its high citral content, a compound recognized for its bactericidal efficacy against various foodborne pathogens and fungi including *Bacillus cereus* (Gao et al., 2020). *B. cereus*, a spore-forming bacterium, is particularly challenging in food safety due to its ability to survive extreme environmental conditions, including heat, and its association with foodborne illnesses (Guo et al., 2021). It is known also as a significant biofilm former in dairy plants, posing a concern due to their resilience and prevalence in various milk products (Fei et al., 2019; Radmehr et al., 2020; Yang et al., 2023).

Lemongrass, beyond its antimicrobial capacity, offers other advantages when applied in food preservation. Faheem et al., (2022) highlighted that besides antimicrobial activity, lemongrass essential oil also possesses antioxidant, antifungal, and anti-insecticidal properties, making it an excellent candidate for natural food preservative. It can be extracted using non-thermal techniques, such as supercritical fluid extraction, which preserve bioactive compounds more effectively than traditional thermal methods (Moreira et al., 2019). Nejia et al., (2013) found that compared to hydrodistillation, supercritical fluid extraction produces 34 % more essential oil and keeps more natural scents and compounds of *Cupressus sempervirens*. Supercritical fluid extraction of extraction *Pimenta Racemosa* performs better than steam distillation as it yields higher phenolic content in less time and offers greater flexibility for extracting diverse bioactive compounds (McGaw et al., 2016). With its high yield, enhanced selectivity, and stability, supercritical fluid