ABSTRACT

Title of thesis: THREE *Clostridium* SPECIES WITH HEALTH IMPARTING PROPERTIES: *In vitro* SCREENING FOR PROBIOTIC POTENTIAL

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This research aimed to unlock the probiotic potential of the genus *Clostridium*, which is often overshadowed by the predominant focus on pathogenic species. The study specifically targeted three promising *Clostridium* species: *C. disporicum*, *C. celatum*, and *C. vincentii*, which have shown potential in mitigating diet-induced obesity. Despite the challenges presented by the anaerobic growth requirements of *Clostridium* bacteria, the study capitalized on their capacity to sporulate. This characteristic provides an avenue to use them as probiotics, with resilient and dormant spores capable of surviving food processing and harsh stomach conditions. The resilience of these spores was examined by exposing them to oxygen, heat, gastrointestinal juices, and bile salts. The spores survived oxygen exposure, exhibited resilience to both bile salts and gastric acids, and demonstrated a survival temperature of 70°C. When exposed to suitable germination conditions *in vitro*, the spores successfully germinated. The study assessed the colonization potential of the bacteria by evaluating their adhesion ability, and all bacteria were found to have the adhesion ability. Furthermore, a safety assessment was conducted by examining hemolytic activity and antibiotic susceptibility to selected antibiotics. The bacteria were found to be susceptible to the antibiotics and did not exhibit hemolytic activity. Bile salt hydrolase (BSH) activity and antibacterial activities were also assessed, and none of the bacteria exhibited BSH activity or antibacterial activity. Antioxidant tests revealed that *C. vincentii* had the highest

antioxidant properties. Assessment of anti-inflammatory properties showed that *C. celatum* downregulated the gene expression of cytokine inflammation markers IL-6, IL-1, and iNOS while upregulating TGF-β expression. In summary all 3 bacterial species showed good probiotic potential from the *in vitro* tests. Particularly the formation of resistant spores that later germinated to vegetative cells that produced molecular patterns with antioxidant and anti-inflammatory properties. This necessitates further studies on their probiotic properties.

THREE *CLOSTRIDIUM* SPECIES WITH HEALTH IMPARTING PROPERTIES: *IN VITRO* SCREENING FOR PROBIOTIC POTENTIAL

by

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1.0 INTRODUCTION

1.1 Gut Microbiota

The human body hosts various bacteria, viruses, and other unicellular eukaryotes. These microbes inhabit nearly all surfaces of the body, with a significant concentration found in the gut. The gut microbiome, a community of microorganisms residing in the gastrointestinal tract begins developing immediately after birth and evolves as an individual ages. These microorganisms directly or indirectly affect human health, and there is a core microbial community present in every individual (Pushpanathan et al., 2019).

This gut ecosystem consists of strict anaerobes, facultative anaerobes, and aerobes. Profiling of the 16S rRNA gene sequence has identified bacteria belonging to various phyla, including *Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria*, *Verrucomicrobia*. Among these, the predominant ones are *Bacteroidetes* and *Firmicutes* (Pushpanathan et al., 2019). The composition and diversity of microorganisms present in the gut microbiota is influenced by several host factors such as diet, age, lifestyle and environmental conditions, with diet playing the most significant role (Vijay et al., 2022). The gut microbiota feeds on undigested food residues, mucus secreted in the gut wall lining and dead cells to maintain high population levels (Liu et al., 2021).

The gut microbiota synthesizes different metabolic products (metabolites) that positively or negatively affect the hosts health. The metabolites include short-chain fatty acids, vitamins, and various health-beneficial products like analgesics and antioxidants. Some microbes have potential to produce harmful products such as neurotoxins, immunotoxins, and carcinogens (Liu et al., 2021). These bacterial metabolites can enter the bloodstream through the gut and subsequently regulate the expression of specific genes that affect the host immune and metabolic processes.

Maintaining balance in the gut microbiota is crucial, as an imbalance of these microorganisms can lead to metabolic disorders.

1.2 Probiotics

Probiotics are non-pathogenic bacteria that when ingested, confer health benefits to an individual (FAO/WHO, 2006). They offer various health benefits, such as balancing the gut microbiota, inhibiting the growth of pathogenic bacteria, strengthening the guts barrier function, alleviating gastrointestinal infections, and improving body immunity (García-Ruiz et al., 2014). In recent years, the use of probiotics to beneficially modulate the microbiome and combat infections threatening human and animal health has been on the rise. They are sometimes used as therapeutics, thereby reducing burdens associated with dissemination of antibiotic resistance resulting from overuse and misuse of antibiotics (Stavropoulou & Bezirtzoglou, 2020). On going research includes exploring probiotic applications in areas such as metabolic syndromes (obesity, cardiovascular disease, and diabetes), anticancer activities, and psychotropic effects via the gut-brain axis (Zaylaa et al., 2018).

Numerous studies have focused on the search for new bacteria and strains that provide specific health benefits. Since these microorganisms colonize the human gut, their evaluation in terms of health promotion is crucial (Pradhan et al., 2020). Most probiotics consumed are typically derived from humans because strains from human sources adapt easily to conditions in the human gastrointestinal tract, facilitating successful gut colonization (Argyri et al., 2013). Various genera, including *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, and diverse yeast strains, have been investigated for their probiotic potential. FAO/WHO (2002) established specific criteria that a bacterial strain must meet to be designated as a probiotic. *In vitro* analyses play a crucial

role in assessing and selecting strains with enhanced probiotic potential, thereby contributing to a better understanding of the strain and some of the mechanisms underlying its beneficial effects (Casarotti et al., 2017).

1.3 Genus Clostridium

1.3.1 Classification

Clostridium are gram-positive, rod-shaped, spore-forming anaerobic bacteria found in diverse environments, including water, soils, the intestinal tract, and other biotopes. *Clostridium* belongs to the phylum *Firmicutes*, which includes gram-positive bacteria with semi-rigid cell walls (Stojanov et al., 2020). Moving down the taxonomic tree, it is identified in the class *Clostridia*, containing the order *Clostridiales*, within which the family *Clostridiaceae* includes the genus *Clostridium* (Uzal et al., 2016).

Earlier, the classification of *Clostridium* was solely based on physiological and morphological characteristics. However, studies over time have shown increasing heterogeneity among them (Guo et al., 2020). This genus has been extensively restructured, and many species have been moved to other genera. Species such as *C. botulinum*, *C. chauvoei*, *C. haemolyticum*, *C. novyi*, *C. perfringens*, *C. septicum*, and *C. tetani* have been shown to belong to *Clostridium* because they have a common ancestry with *C. butyricum*, which is known to be a true *Clostridium*. Species that are termed as true *Clostridium* fall under cluster I and are referred to as *Clostridium sensu stricto*. Other species that do not fall into this genus are reclassified into other genera (Uzal et al., 2016).

More studies by Lawson et al. (2016) reclassified *C. difficile* and *C. mangenotii* to a new genus *Clostridioides* and family *Peptostreptococcaceae* respectively. Other reclassification examples

include *C. aminovalericum*, *C. jejunse*, and *C. xylanovorans* being moved to a new genus *Anaerocolumna*, and family *Lachnospiraceae*, *C. lituseburense*, *C. bartlettii*, *C. glycolicum*, and *C. mayombei* to the genus *Romboutsia* and family *Peptostreptococcaceae* (Seong et al., 2018). The reclassification of different genera and species due to their internal structure and phylogenetic knowledge will necessitate future revisions within *Clostridium* cluster I.

1.3.2 Distribution and Colonization of Clostridium Species in Gut

The most predominant organisms in the gut microbiota are classified into three strict anaerobes, which include, *Clostridium* cluster IV, and *Clostridium* cluster XIVa. *Clostridium* are recognized for their ability to form endospores, which potentially enables them to withstand the harsh conditions in the gastrointestinal tract and the external environment (Yuji et al., 2012). Members of the *Clostridium* are among the first bacteria to colonize the gut, and their presence is detectable in feces within the first week of birth (Guo et al., 2020). Most *Clostridium* species tend to establish a commensal relationship within the gastrointestinal tract, but some species such as *Clostridium difficile*, *Clostridium perfringens*, and *Clostridium tetani* have the capacity to produce toxins, making them pathogenic (Robert et al., 2019).

I.4. Preliminary Studies- Beneficial Clostridium

In a study conducted in outbred Sprague-Dawley rats, a correlation was observed between the abundance of the genus *Clostridium* in the gut microbiota and development of obesity in response to a high fat diet. Rats that remained lean despite the high fat diet exhibited a higher proportion of *Clostridium* cluster I. Specifically, *Clostridium* accounted for 9% of the total gut bacteria composition. Rats that easily developed obesity (obese-prone) had only 0- 0.03% *Clostridium* (Obanda et al., 2021).

In a separate study conducted in C57BL6J mice aimed at identifying dietary interventions with the potential to prevent diet induced obesity, six vegetables were screened. *Urtica dioica*, (stinging nettle) showed the ability to protect against diet induced obesity. Supplementing a high fat diet with *Urtica dioica* mitigated fat accumulation, prevented insulin resistance, and increased the representation of genus *Clostridium*. Metagenomic sequencing of the gut contents using 16S rRNA gene analysis revealed that at the species level, *Clostridium vincentii* and *Clostridium disporicum* were found to be abundant (Fan et al., 2021).

This research therefore focused on exploring the probiotic potential of the genus *Clostridium*, particularly the three promising species *C. disporicum*, *C. celatum*, and *C. vincentii*. These are the species that were highly represented in obese-resistant rats and mice fed with the vegetable *Urtica dioica*. The study capitalized on their ability to form resilient spores that have potential to survive oxygen exposure, the upper gut harsh conditions and germinate upon reaching the lower gut. The research also explored the safety and potential health impacts of these bacteria. This was accomplished through *in vitro* characterization and *in silico* pathway exploration.

1.5. Justification

The health benefits of *Clostridium* have not been well established mainly because they are strict anaerobes and a lot of research on this genus focuses on the pathogenic species that cause disease. Exploring the probiotic potential of the genus *Clostridium*, is an area that is overshadowed by extensive knowledge and a greater research focus on pathogenic *Clostridium* species. The focus was on three *Clostridium* species: *C. disporicum*, *C. celatum*, and *C. vincentii*. Their strict anaerobic nature poses a challenge for incorporating them into food, necessitating exploration of spores. It is necessary to explore the resilience of the spores and ability to germinate after exposure

to environmental conditions, specifically oxygen and harsh upper gut acids. It is likely that if the spores survive and reach the lower gut, they can germinate and produce proteins and/or metabolites that influence the host's health and phenotype. The spores can be added to foods during processing or taken as a supplement. By focusing on the probiotic potential of these specific *Clostridium* species, this research addressed a critical knowledge gap: practical applications of these bacteria as probiotics for protection against disease.

1.6 Objectives

Objective 1: Stimulate the sporulation of *Clostridium* sp. and determine the resilience of the spores to environmental and gastrointestinal conditions.

Sporulation was induced by exposing cultures of *C. vincentii*, *C. celatum*, and *C. disporicum* to caffeine, as shown by (Labbe et al., 1981). The resilience of the spores was determined by exposing them to oxygen and heat. To simulate gastrointestinal conditions, spores were exposed to gastric acids and bile salts. Subsequently, the viability of the resilient spores was examined by exposing them to suitable germination conditions and observing their ability to germinate.

Objective 2: Determine the colonization potential, safety and health impacts of *Clostridium* through *in vitro* characterization

Spores were cultured into vegetative cells under anaerobic conditions. From these cultures, bacterial cell-free supernatant (CFS) was isolated and used to assess antibacterial activity, bile salt hydrolase activity, antioxidant activity, hemolytic activity, anti-inflammation effects and screened for ability to activate Toll-like Receptors (TLRs) in intestinal cells.

Objective 3: Conduct an *in-silico* search of virulence factor genes and antimicrobial resistance genes

Leveraging existing data from scientific repositories, a comprehensive analysis of the genetic information pertaining to *C. vincentii, C. celatum,* and *C. disporicum* was conducted. The focus was on ascertaining the absence of pathogenic potential and antibiotic resistance in the selected species.

2.0 LITERATURE REVIEW

2.1 Gut Microbiota

The microbiota residing in the gastrointestinal (GI) tract is composed of bacteria, archaea, viruses, and eukaryotic microorganisms. This community has co-evolved with host organisms, establishing a complex and mutually beneficial relationship (Haque & Haque, 2017). The abundance of microorganisms in the gut is estimated to be ten times greater than the number of human cells. Moreover, the genomic content of the microbial community exceeds that of the human genome by more than 150 times (Adak and Khan, 2018).

Initial gut colonization in humans occurs in the early stages of newborn life. Maternal microbiota plays a significant role in this initial microbial seeding. Traditionally, microbiota development is associated with birth, but emerging research challenges this notion by detecting microorganisms in prenatal tissues such as the placenta (Kjersti et al., 2014). Maintaining a balance between the host and gut microbiota is crucial for human health. Any alteration or disturbance of this equilibrium is associated with numerous human diseases, including hypertension, diabetes, inflammatory bowel disease, obesity, and cardiovascular disease (Julius et al., 2017).

The composition of the gut microbiota changes from early life to become relatively stable in adults and differs for each individual. Various factors influence the microbial constitution in mammals, beginning with seeding upon birth. Among these factors is host genetics. For instance, genetically obese mice exhibit a different proportion of major bacterial groups in their intestines compared to genetically lean mice (Sekirov et al., 2010). Another significant factor is the use of antibiotics, which profoundly impacts the microbiota and is associated with antibiotic resistance. Extensive

research demonstrates alterations in the gut microbiota following an antibiotic regime (Fishbein et al., 2023)

2.1.2 Composition of the Gut Microbiota

The gut microbiota is a complex system that coexists with its host, constituting approximately one kilogram of the host's body weight and functioning akin to an organ that impacts the host's health through endocrine-like, immunological, and metabolic functions (Geng et al., 2022). Initially, understanding the microbiota posed challenges due to the difficulty in culturing several species of bacteria that comprise gut bacteria. In the 1980s, a breakthrough occurred with the use of 16S rRNA gene sequencing, enabling the identification and classification of bacteria without relying on traditional culturing techniques (Olsen et al., 1986). More recently metagenomic sequencing allows identification of these gut microbes at species level and their function.

The gut microbiota contains bacteria, viruses, and yeasts. Metagenomic sequencing of fecal samples from healthy individuals has identified the predominant members of the gut microbiota as *Bacteriodetes* and *Firmicutes*. Other taxa detected through these sequencing efforts include *Actinobacteria, Proteobacteria,* and *Verrucomicrobia*. Recent studies have also revealed colonization of the gut by methanogenic archaea and multiple phages (Geng et al., 2022). A healthy gut microbiota is characterized by high taxonomic diversity, microbial gene richness, and a stable core microbiota. However, the composition of the gut microbiota varies significantly among individuals, influenced by factors such as age and environmental conditions (Hou et al., 2022) and health of the host and medications taken including antibiotics. Moreover, within the gastrointestinal tract, the microbiota varies across different anatomical regions. For example, *Proteobacteria* specifically family *Enterobacteriaceae* are prevalent in the small intestine but not

in the colon, whereas in the colon, *Bacteriodetes* specifically families *Bacteroidaceae*, *Prevotellaceae*, and *Rikenellaceae* are abundant (Hou et al., 2022).

2.1.3 *Clostridium* sensu stricto

Clostridium is composed of gram-positive, strict and facultative anaerobes exhibiting various phenotypes, including thermophilic, acidophilic, and psychrophilic characteristics. The 16S rRNA gene, one of the most conserved genes for taxonomy, classifies *Clostridium* as a highly heterogeneous group (Rossi-Tamisier et al., 2015).

The classification of *Clostridium* has undergone multiple revisions, resulting in the reassignment of many organisms previously classified as *Clostridium* to new genera. Early taxonomy, based on phenotypic characteristics, attributed all gram-positive, anaerobic, spore-forming bacilli to the genus *Clostridium*. However, with the advent of DNA sequencing and phylogenetic analysis, more insights into the taxonomy of this genus have emerged (Alou et al., 2018b). Current consensus recognizes *Clostridium* cluster I, termed *Clostridium sensu stricto*, as the true *Clostridium* genus (Cruz-Morales et al., 2019). The branching pattern of the 16S rRNA gene tree suggests that only a specific subset of known *Clostridium* species, forming a distinct clade (Cluster I), should be considered genuine representatives of the genus *Clostridium* (Clostridium sensu stricto) (Li et al., 2023b).

Currently, *Clostridium sensu stricto* encompasses approximately 118 species with validly published and correct names. Notable members include significant human and animal pathogens such as *C. botulinum* and *C. tetani*, as well as industrially relevant bacteria like *C. acetobutylicum* and *C. saccharobutylicum* (Li et al., 2023b). A substantial proportion of *Clostridium sensu stricto* species were initially isolated from host-associated habitats such as feces and infections in humans

and animals. Recent metagenomic studies have identified *Clostridium sensu stricto* as a pivotal and, in some cases, dominant member of the endosphere community. While the proposal by Collins et al. (1994) to confine the genus *Clostridium* to cluster I represents a crucial step in clarifying its taxonomy, it is noteworthy that cluster I also includes certain members of other genera, including *Anaerobacter*, *Eubacterium*, and *Sarcina* (Li et al., 2023b).

2.1.4 Role of Gut Microbiota in Health

The gut microbiota establishes a mutually beneficial relationship with the gut mucosa, exerting metabolic, immunological, and gut-protective functions in a healthy individual. Acting as a distinct organ system, these bacteria are essential for maintaining the physiological functions of adult tissues and organs. They achieve this by synthesizing various vitamins, aiding in food digestion, producing lactic acid, enhancing intestinal motility, inhibiting the growth of pathogenic microbes, and stimulating the immune system (Wang et al., 2022). Below, we delve into the detailed functions of the gut microbiota in maintaining health.

Antimicrobial Protection

Numerous gut bacteria actively produce antimicrobial compounds and compete for nutrients and attachment sites along the gut lining, thereby preventing colonization by pathogens. They employ antagonistic strategies such as competitive substrate removal, accumulation of D-amino acids, lowering of oxidation-reduction potential, and co-aggregation. Additionally, these bacteria produce metabolic by-products that can inhibit the growth of surrounding bacteria (Garcia-Gutierrez et al., 2018).

Experiments have shown non-pathogenic bacteria competing for attachment sites on intestinal epithelial cells, effectively preventing the adhesion and infiltration of pathogenic enteroinvasive

bacteria (Guarner et al., 2003). In laboratory studies, the collective action of the gut microbiota is evident as they compete for nutrients in their environment, leveraging their numerical advantage to outcompete harmful bacteria and establish dominance. Furthermore, many gastrointestinal bacteria possess the ability to produce bacteriocins, antimicrobial peptides that inhibit the growth of competing microbes (Garcia-Gutierrez et al., 2018).

Immunomodulation

The intestinal epithelium serves as the primary interface facilitating interactions between the immune system and the external environment. It maintains a delicate balance between mounting rapid defensive immune responses against invading microbial pathogens and tolerating non harmful or beneficial commensal microbes (Liébana-García et al., 2021). Ongoing interactions between the intestinal epithelium, microbiota, and its metabolites significantly influence the development of the host's immune system (Bull et al., 2014). The gut microbiota plays a pivotal role in modulating gut immunity in coordination with both innate and adaptive immune systems. Key components and cell types involved include gut-associated lymphoid tissues (GALT), effector and regulatory T cells, IgA-producing B (plasma) cells, Group 3 innate lymphoid cells, as well as resident macrophages and dendritic cells in the lamina propria (Jandhyala, 2015).

Commensal microorganisms also play a crucial role in the maturation of the immune system and helping distinguish between pathogenic and commensal bacteria. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) found on epithelial cell membranes, enable the immune system to recognize and respond to these microorganisms and their secretions (metabolites or proteins) (Al-Rashidi, 2022). Indigenous microflora retained by the epithelium typically do not

activate TLRs, while pathogenic bacteria, possessing virulence factors, can breach the epithelial barrier and later activate TLRs on dendritic cells and macrophages (Al-Rashidi, 2022).

The gut microbiota also triggers regulatory responses, crucial for suppressing inflammatory reactions to ingested food and other orally administered antigens, a process known as oral tolerance (Sudo et al., 1997). Foxp3+ regulatory T (Treg) cells play a central role in this process, maintaining peripheral and mucosal homeostasis throughout the host's life. Disruptions in Treg cell homeostasis can lead to loss of oral tolerance and abnormal effector responses in the gut (Josefowicz et al., 2012). While Foxp3+ Treg cells can originate in the thymus, the gastrointestinal tract is a site for inducing Treg cells in response to oral antigens (Sun et al., 2007). Optimal tolerance to commensal and environmental antigens requires the combined influence of thymus-dependent and gastrointestinal-induced Treg cells (Josefowicz et al., 2012). Mucosal dendritic cells, such as CD103+ CD11b+ DCs, play a critical role in this process by producing regulatory factors like TGF-β and retinoic acid (RA), which promote Treg cell induction (Sun et al., 2007). Probiotics recognized for conferring health benefits, may exert their effects in part through the induction or expansion of Treg cells. This regulatory effect has been implicated in managing inflammatory diseases and atopic eczema in neonates and infants, potentially involving modulation of mucosal dendritic cells towards a pro-regulatory function (Karimi et al., 2009).

Tissue-specific elements such as vitamin A and MUC2 (a mucus glycoprotein synthesized by intestinal goblet cells) contribute to the regulatory specialization of mucosal dendritic cells (Shan et al., 2013). This pathway is crucial for maintaining mucosal homeostasis, as a portion of induced Treg cells in colonic tissue exhibits specificity for antigens derived from the commensal microbiota (Lathrop et al., 2011).

Studies indicate that the gut microbiota regulates the expansion of specific lymphocyte subsets, including T helper 17 (TH17) cells, a CD4+ cell lineage important for defense and implicated in autoimmune diseases due to their production of pro-inflammatory cytokines. Antibiotic treatment or germ-free conditions have been shown to reduce Th17 cell populations in the gut. Conversely, certain bacteria related to Clostridia, particularly segmented filamentous bacteria, promote Th17 cell differentiation by stimulating the expression of serum amyloid A protein (SAA) and inducing IL-6 and IL-23 release from CD11-expressing lamina propria dendritic cells (Al-Rashidi, 2022).

2.1.5 Toll-Like Receptors (TLRs) Pathway

TLRs are part of the pattern recognition receptors (PRRs) that detect and respond to pathogen-associated molecular patterns (PAMPs) from pathogens like bacteria and viruses, as well as damage-associated molecular patterns (DAMPs) released from damaged cells. TLRs play a crucial role in the innate immune system, the first line of defense, by sensing and responding to these PAMPs. Upon sensing their ligands, TLRs initiate a variety of downstream signaling cascades such as inflammasome signaling pathways, type I interferon, and nuclear factor kappa B (NF-κB), which lead to the production of corresponding pro-inflammatory or antiviral cytokines and chemokines (Kaur & Ali, 2022).

Currently, ten TLRs have been discovered. They are divided into two groups. First, cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10), which mainly recognize microbial membrane compounds. Second, intracellular TLRs (TLR3, TLR7, TLR8, and TLR9), which recognize microbial nucleic acids from bacteria and viruses (Kawasaki and Kawai, 2014). Each PAMP is recognized by different TLRs: lipopolysaccharides (TLR4), lipopeptides (TLR2 with

TLR6 or TLR1), double-stranded RNA (TLR3), flagellin (TLR5), single-stranded RNA (TLR7/8), and CpG motif-containing DNA (TLR9) (Kaur & Ali, 2022).

TLRs are expressed on various cells in the immune system, including natural killer cells, B cells, dendritic cells, and macrophages, as well as on the surface of epithelial and endothelial cells (Wicherska-Pawłowska et al., 2021). The receptors mediate immunity by initiating a cascade of signals in the innate immune system, leading to the production of antiviral and pro-inflammatory cytokines. Additionally, TLRs induce an antigen-specific adaptive immune response by promoting the maturation of dendritic cells, which helps in the clearance of invading pathogens (Duan et al., 2022).

After the attachment of the ligand, the signaling cascades begin with the dimerization of the intracellular Toll/Interleukin-1 receptor (TIR) domains. These TIR dimers facilitate the binding of TIR-containing adaptor proteins, including Myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), TIR-domain-containing adaptor protein/MyD88-adaptor-like (TIRAP/MAL) and TRIF-related adaptor molecule (TRAM). Depending on the adaptor protein attached, the TLR signaling pathway is divided into two categories: MyD88-dependent and TRIF-dependent. MyD88 is used by all TLRs to activate NF-κB and MAPKs, which subsequently lead to the production of pro-inflammatory cytokines. Conversely, TRIF binds exclusively to TLR3 or TLR4 and initiates an alternative signaling pathway that activates MAPKs, IRF3, and NF-κB, resulting in increased synthesis of pro-inflammatory cytokines and type I interferons (Duan et al., 2022; Wicherska-Pawłowska et al., 2021).

Negative regulation of these signaling pathways is crucial to prevent excessive secretion of pro-inflammatory cytokines, which could lead to autoimmune disorders or chronic inflammatory conditions. This regulation is achieved through targets that impair signal transduction at various stages. For instance, activation of the MyD88-dependent pathways is inhibited by ST2825, SOCS-1, and Cbl-b, while TRIF signaling is inhibited by sterile α and TIR motif-containing protein (SARM) and TRIF-associated G protein (TAG) (Wicherska-Pawłowska et al., 2021). Some potential probiotic *Lactobacillus* strains have been shown to reduce inflammation by mediating the negative regulation of the TLR pathway (Kanmani & Kim, 2020).

2.2. Probiotics

Probiotics are living microorganisms that, when administered in adequate amounts, confer health benefits on the host. To qualify as a probiotic, the specific strain's genus and species must be identified, which is crucial for linking it to health effects and facilitating accurate surveillance and epidemiological studies (FAO/WHO 2002). Non-living elements, such as dead bacteria or bacterial by-products, have been suggested to offer health benefits, but they do not meet the criteria for probiotics because they lack viability upon administration (Sanders et al., 2007). Identification of probiotic bacteria strains involves a combination of phenotypic tests followed by genetic identification using molecular techniques such as DNA/DNA hybridization or 16S rRNA sequencing (FAO/WHO 2001).

2.2.1 Desirable Properties of Probiotics

For a potential probiotic species or strain to exert its beneficial effects, it is expected to exhibit certain desirable properties. Below is a discussion of some of the properties of probiotics, which can be assessed in vitro.

Tolerance to Gastric Acid and Bile salts

Probiotics should survive in gastrointestinal conditions and reach alive in the small intestine, where they colonize and impart positive health benefits to the host. The decrease in the viability and survival is due to the presence of pepsin enzymes and low pH of gastric juice (Wendel, 2022). The probiotics should withstand harsh gastrointestinal conditions (gastric juice and bile salt) to exert positive health benefits to humans. Gastric juice and bile salt-resistance is considered one of the prime criteria in the selection of probiotics (Larsen et al., 2018).

Antibiotic Susceptibility

Antibiotic resistance is considered an undesirable trait for probiotics (Anokyewaa et al., 2021) because these strains may have the potential to transfer antibiotic-resistance genes to pathogenic bacteria in the intestinal environment, posing a risk to public health (Casarotti et al., 2017). If a bacterium is found to be resistant to antibiotics in testing, it may not be suitable for use as a probiotic.

Bile Salt Hydrolase Activity

The presence of Bile Salt Hydrolase (BSH) in strains is proposed as a key factor for screening probiotics with the potential to reduce serum cholesterol levels). BSH is an intracellular enzyme that catalyzes the hydrolysis of amide bonds between glycine or taurine and the steroid nucleus of bile salts. This reaction is followed by microbial transformation of the resulting bile acids (Horackova et al., 2020). Bacteria commonly used as probiotics, particularly those from the genera *Lactobacillus, Bifidobacterium*, and *Enterococcus*, exhibit BSH activity (Hernández-Gómez et al.,

2021). *Lactiplantibacillus plantarum*, extensively investigated as a probiotic, has displayed activity against four distinct conjugated bile acids, as evidenced by Hernández-Gómez et al. (2021), highlighting the capacity of bacteria to show BSH activity.

Antioxidant Properties

Probiotics possess the capability to directly counteract oxidants through the expression of antioxidant enzymes such as Superoxide Dismutase (SOD) These bacteria have the potential to serve as effective carriers for various antioxidant enzymes (Hoffmann et al., 2021). Notably, the probiotic *Clostridium butyricum*, known for its butyrate production, has demonstrated the ability to induce antioxidative enzymes in rats afflicted with nonalcoholic fatty liver disease, thereby mitigating hepatic oxidative stress (Kumar et al., 2007). Furthermore, studies have revealed that the culture supernatant, intact cells, and intracellular cell-free extracts of *Bifidobacterium animalis* exhibit *in vitro* scavenging of hydroxyl radicals and superoxide anions, while concurrently enhancing antioxidase activities in mice *in vivo* (Shen et al., 2010).

Antimicrobial Activity

Probiotics are currently suggested as a viable and environmentally friendly alternative to antibiotics owing to their antagonistic activities against pathogenic bacteria (Amoah et al., 2021). This antagonistic behavior is primarily attributed to their capacity to secrete bacteriocins and other compounds. Previous reports have highlighted the antimicrobial properties of *Bacillus* species against pathogenic bacteria (Amoah et al., 2021).

Hemolytic Activity

According to the European Food Safety Authority (EFSA), it is strongly recommended to evaluate hemolytic activity when considering isolated bacteria for use in food products, even if they have Generally Recognized as Safe (GRAS) or Quality Presumption of Safety (QPS) status (FAO/WHO, 2006). The absence of hemolytic activity is considered a crucial safety criterion when selecting probiotic strains (FAO/WHO, 2002). This is because bacteria lacking hemolysin are deemed non-virulent, and the absence of hemolysis ensures that opportunistic virulence is less likely to emerge among strains (Peres et al., 2014). Many bacteria studied as potential probiotics do not exhibit the ability to produce hemolysins (Yasmin et al., 2020).

α-Glucosidase Activity Inhibition Capacity

The inhibition of α-glucosidase activity is known to reduce glucose absorption from the host intestines. Consequently, this may decrease energy intake and aid in mitigating weight gain (Wei et al., 2022). Lactic acid bacteria have been demonstrated to possess the ability to inhibit α-glucosidase activity, and many of them have been utilized as probiotics (Chen et al., 2014). Despite the recognized impact, the precise mechanism through which lactic acid bacteria perform this inhibition remains unclear.

Triglyceride Lowering Capacity

In the presence of excess fat provision, inhibiting lipid over-absorption is recognized as an effective approach to alleviating obesity syndrome. The reduction in cholesterol and triglyceride absorption from the host gut contributes to decreasing their levels in circulation and tissues, thereby alleviating obesity symptoms (Haslam et al., 2005). A study by Wei et al., (2022) showed that *Lactiplantibacillus* bacteria have the ability to lower the rate of triglyceride absorption.

2.2.2 Mechanisms of Probiotic Activity

Probiotics employ diverse mechanisms of action, although the precise ways in which they exert their effects remain not fully understood. Valuable insights into these mechanisms come from documented outcomes in animal models and *in vitro* experiments. One potential avenue involves enhancing the barrier functions of the gut mucosa. Certain strains of *Lactobacillus and Bifidobacterium*, in conjunction with structural components and metabolites produced by microbes, have exhibited the capacity to activate signaling pathways in epithelial cells (Stetinova et al., 2010). Furthermore, specific probiotics such as *Lactobacillus rhamnosus* have been noted to impact the expression and/or positioning of tight junction proteins in both *in vivo* and *in vitro* models (Rose et al., 2021).

Probiotics also have the potential to modulate the functions of the immune system. Specifically, *L. acidophilus* has been identified as capable of influencing toll-like receptors (TLRs) and the proteoglycan recognition proteins in enterocytes. This modulation results in the activation of dendritic cells and a subsequent T-helper 1 response in lymphocytes (Daliri et al., 2015). The consequential stimulation of T-helper 1 cytokines may suppress T-helper 2 responses, which are associated with atopic issues (Cosmi et al., 2014). Additionally, probiotics could act by inhibiting the growth of pathogenic bacteria through the production of broad-spectrum bacteriocins (Hardy et al., 2013).

2.2.3 Probiotics in Obesity

Research emphasizes the potential of probiotics in addressing obesity, with various bacterial strains exhibiting favorable anti-obesity outcomes. These effects encompass the reduction of tissue

inflammation, endotoxemia, adiposity, body weight, leptin levels, and energy intake (Torres et al., 2015). Nevertheless, the majority of these studies have relied on rodent models, emphasizing the need for more research involving human subjects to substantiate the applicability of these probiotic strains in treating or preventing obesity. Among the probiotic species demonstrating anti-obesity effects, the most prevalent ones are *Bifidobacterium* and *Lactobacillus spp*. Additionally, *Akkermansia muciniphila* has emerged as a key gut bacterium influencing host metabolism, capable of counteracting metabolic effects induced by a high-fat diet, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance (Everard et al., 2013).

A recent study conducted on Sprague-Dawley rats unveiled a significant correlation between the prevalence of the *Clostridium* genus in the gut microbiota and susceptibility to diet-induced obesity (Obanda et al., 2021). Additionally, a decrease in the abundance of *C. butyricum* has been observed in obese individuals; however, it has been demonstrated that orally administering *C. butyricum* can alleviate obesity-related phenotypes. This probiotic bacterium, recognized for its butyrate production, has the capacity to stimulate the secretion of gastrointestinal hormones and modify the function of the parenteral organ (Liao et al., 2023).

3.0 MATERIALS AND METHODS

3.1. Testing Spore resilience

3.1.1 Growth and Sporulation of Bacteria

The three bacterial species, *C. celatum* (ATCC, Manassas, Virginia), *C. disporicum*, and *C. vincentii* (DSMZ, Göttingen, Germany), were cultured under an anaerobic hood (Anaerobe Systems, Morgan, CA) with 90% nitrogen, 5% carbon dioxide, and 5% hydrogen at 37°C for 48 hours in Reinforced Clostridium Broth (RCB). To induce sporulation, the RCB media was supplemented with caffeine, as shown by Labbe et al. (1981). Sporulation levels were assessed for 14 days using phase-contrast microscopy.

3.1.2 Purification and Quantification of Spores

Vegetative cells and endospores were separated by the procedure outlined by Yang et al. (2011). Briefly, after centrifugation at 6300×g, 10 washes with DI water, and sonication at 25 kHz, the suspension was incubated overnight in lysozyme (0.2 mg/ml) and trypsin (0.1 mg/ml) at 30°C with constant stirring to lyse and degrade the vegetative cells. Cultures were centrifuged in eight cycles at 6300×g and washed in sterile DI water until 99.9% of the cells were fully refractile. Spores were quantified using the direct plating method on *Reinforced Clostridium Agar* (RCA). The viable spore count was determined by calculating colony-forming units per milliliter (CFU/ml) after a 48-hour incubation period at 37°C. Spores were then stored in 4 degrees Celsius until use.

3.1.3 Tests for Resilience to Environmental Conditions

Tolerance to Oxygen: Spores were centrifuged (9000 rpm, 4°C, 3 min) and suspended in non-reduced PBS-cys buffer, as shown by Kadowaki et al. (2023). The cell suspension was exposed to air at room temperature for 21 days on a petri dish. Survival was ascertained by subsequent plating on Reinforced *Clostridium* Agar (Kadowaki et al., 2023).

Tolerance to Gastric Acid: Spore suspensions were centrifuged (5000 rpm, 5°C, 15 mins). The pellet was re-suspended in a 10 ml PBS buffer and incubated in gastric acid for 1, 2, and 3 hours. After each time frame, spores were evaluated for viability by plating on RCA to determine their potential to germinate. The survival percentage was calculated using the formula outlined by Nath et al. (2020).

$$\textbf{Bacterial survival rate}\ (\%) = \frac{\textbf{CFU assay}}{\textbf{CFU control}} \times \mathbf{100}$$

Tolerance to Bile Acids: This test was conducted following the method described by Nath et al. (2020). Approximately 100 µl of spores were inoculated into freshly prepared RCB medium supplemented with 0.3% bile salts for 4 hours at 37°C. Germination and growth were monitored by determining absorbance at 600 nm. After the 4 hours, the viability of spores was also assessed by spreading 100 µl of the culture onto an RCA plate, and CFUs were determined.

Survival at high temperature: The test was conducted following the protocol described by Li et al. (2021) with some modifications. The spore suspension, stored at 4°C, was adjusted to an optical density of 0.5 at 600 nm (OD600). The spores were exposed to wet heat at various temperatures

up to 100°C for 10 minutes. Following heat treatment, the spores were plated to quantify the number of colony-forming units (CFUs).

3.2 Testing Colonization, safety and health imparting potential

3.2.1 Colonization Potential

Adhesion Ability: Adhesion ability was determined through the assessment of cell hydrophobicity and the auto-aggregation index, as shown by Lee and Kim (2019). Briefly, 1 ml of xylene was mixed with 3 ml of the Intact Cell Solution (ICS) and maintained at 37°C until phase separation was observed. The bottom phase was utilized to measure the hydrophobicity by reading absorbance at 600 nm. The hydrophobicity of the cells was quantified using the following equation:

$$\textbf{Hydrophobicity}\ (\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

Where A_0 represents the initial absorbance (O.D 600) of samples measured at t = 0 h and A_t represents final absorbance

The auto-aggregation index was determined as follows:

$$\textbf{Auto} - \textbf{aggregation}\ (\%) = \left(1 - \frac{A_{24}}{A_0}\right) \times 100$$

where, A_{24} represents the OD_{600} nm measured of the upper layer at t = 24 h.

3.2.2 Determining the Safety of the Bacteria

Antibiotic Susceptibility: Using the Kirby–Bauer disk diffusion method on RCA media, bacteria were evenly spread, and antibiotic discs were placed equidistantly on the surface of freshly prepared RCA plates. The plates were incubated at 37°C for 24-48 hours before determining the diameter of the zone of inhibition. The antibiotic resistance profile of the bacteria was categorized following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016). The antibiotics utilized in this study included tetracycline (30 µg), ciprofloxacin (30 µg), and ceftriaxone (30 µg) as per Nath et al. (2020) and following the protocol established by Bauer et al. (1966).

Hemolytic Activity

Using a method described by Jeon et al. (2017), Columbia blood agar plates incorporating 5% (w/v) sheep blood were used. Germinated bacterial cells were streaked onto blood agar plates and incubated at 37°C for 24 hours. The presence of clear zones around the bacterial colonies on the plates indicated β-hemolysis, the green-hued zone surrounding the colonies indicated α-hemolysis, while no change around the colonies suggesting γ-hemolysis was checked.

3.2.3 Determining the Health Impacts of the Bacteria

3.2.3.1 Bile Salt Hydrolase Activity (BSH)

Qualitative analysis of bile salt hydrolase activity was conducted following the protocol outlined by (Hernández-Gómez et al., 2021c) with few modifications. Briefly, the *Clostridium* species were streaked on tryptic soy agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA) (Sigma-Aldrich, St. Louis, MO, USA) and 0.37 g/L CaCl2. The plates were then

incubated at 37°C for 72 hours. After incubation, the presence of deconjugated bile acid precipitation zones (opaque halos) around bacterial colonies was checked to determine bile salt hydrolase activity. Control plates without TDCA supplementation were used.

3.2.3.2 Antioxidant Activity

Antioxidant activity of *Clostridium* of both Bacteria Extracellular Vesicles and cell-free supernatant was conducted using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay as outlined by Son et al. (2017). The antioxidant activity was calculated based on the absorbance values using the following equations:

$$\textbf{DPPH radical scavanging activity}\ (\%) = \left(1 - \frac{(A_{sample} - A_{blank})}{A_{control}}\right) \times 100$$

For the isolation of BEVs, a protocol by Wei et al. (2022) was followed with a few modifications. The bacteria were grown in RCM broth until the stationary phase. Bacterial cells were then removed from the cultures by low-speed centrifugation (4000 × g for 20 min at 4°C) and filtration through 0.22 μM pore membranes. Cell-free cultures were subjected to ultracentrifugation (100,000 × g for 2 h at 4°C), and the supernatants were discarded, leaving behind the pellet. Minimal PBS was added to resuspend the pellet, which was subsequently stored at -80°C until use.

3.2.3.5 Antibacterial Activity against indicator bacteria and opportunistic pathogens

Antibacterial activity was assessed using the agar diffusion test as described by Wang et al. (2018). In summary, 100 μl of cell-free supernatant was introduced into 7-mm diameter wells made in tryptic soy agar plates pre-seeded with indicator bacteria (*Escherichia coli, Staphylococcus aureus*, *Shigella flexneri, Listeria innocua, Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*). The

plates were then incubated at either 30°C or 37°C for 48 hours, and the diameter of the inhibition zones were measured to evaluate antibacterial activity.

3.2.3.6 Anti-inflammatory properties

To assess the anti-inflammatory properties in RAW 264.7 macrophages, protocols by Khanna et al. (2020) and De Marco et a*l.* (2018) were followed with a few modifications. Inflammation was induced in the cells using 100 ng/ml Lipopolysaccharide (LPS) from *E. coli (*O111:B4*).* For treatment with cell-free supernatant (CFS), 10 µl of CFS from each bacteria was used. The negative control consisted of cells without any treatment, while the positive control involved cells treated with LPS only. The treatment groups had CFS from the bacteria. The cells were allowed to grow to confluency, after which 10 µl of the CFS was added to the treatment groups for 18 hours. Subsequently, 100 ng/ml LPS was added and left for 6 hours to induce inflammation. RNA was isolated from the cells and then synthesized into cDNA, which was used for RT-qPCR to assess the gene expression of inflammatory cytokines IL-6, IL-1, iNOS, and TGF-beta. RNA was separately extracted from each treatment group. TRIzol reagent was added to each well to lyse the cells. RNA extraction was performed following the method outlined by Abcam (Waltham, MA, USA). The quantity and quality of the RNA were measured using a Qubit 4 fluorometer (Thermo Fisher Scientific, Rockville, MD), and only samples with an RNA integrity number (RIN) above 7 were selected for cDNA synthesis. The High-Capacity cDNA Reverse Transcription Kit (Qiagen, Germantown, MD) was used for cDNA synthesis, starting with 2,000 ng of RNA. For RT-PCR, 10 ng of cDNA, a 5 µM primer mix (primer sequences are listed in Table 3.1), and PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Rockville, MD) were combined in a final volume of 10 µL. The PCR cycling conditions were as follows: 2 minutes at 50°C, 2 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and

72°C for 1 minute. The relative expression levels of target mRNAs were normalized to β-actin as the endogenous control. Data analysis was performed using the 2−ΔCT method, and fold changes in expression were compared between experimental groups.

All samples were processed in triplicate

Table 3.1: Primers Used in RT qPCR

Cytokine	Forward Primer	Reverse Primer
IL-6	GACAGCCACTCACCTCTTCA	TTCACCAGGCAAGTCTCCTC
TGF Beta	GACCGCAACAACGCCATCTA	GGCGTATCAGTGGGGGTCAG
IL-1	ACCTGCTGGTGTGTGACGTTCC	GGGTCCGACAGCACGAGGCT
iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC
B-Actin	GGCACCACACCTTCTACAATG	GGGGTGTTGAAGGTCTCAAAC

3.2.3.7. TLR Pathway Screening.

The TLR pathway screening was conducted using the QuantiNova LNA PCR Focus Panel Human Toll-Like Receptor Signaling Pathway (Qiagen, Gaithersburg, MD), which features a panel of 84 genes central to TLR-mediated signal transduction and innate immunity. Caco-2 cells were grown to confluence and allowed to differentiate. Concurrently, bacteria were cultured until reaching the plateau phase, and their cell-free supernatant was obtained by centrifuging the bacterial culture at 5000 RPM for 10 minutes. Ten microliters (1% v/v) of the bacterial cell-free supernatant were then added to the differentiated Caco-2 cells and incubated for 24 hours. Cells were collected in Trizol reagent and RNA was extracted. This RNA was then synthesized into cDNA using

QuantiTect Reverse Transcription Kit (Qiagen, Gaithersburg, MD), which was subsequently loaded onto the RT² Profiler PCR Array plate containing the 84 TLR pathway genes. The qPCR test was performed according to the manufacturer's instructions, and the results were analyzed to assess gene expression.

3.1 Screening safety of the bacteria

Whole genome sequencing data of the 3 *Clostridium* species were obtained from NCBI RefSeq and GenBank. This data was utilized to confirm the absence of virulence factor genes and antimicrobial resistance genes. For antimicrobial resistance, a protocol formulated by Abriouel et al. (2022) was followed, where the genome was checked for resistance genes using ResFinder and NCBI databases, based on nucleotide sequence similarity, with an identity of 100%. Regarding virulence factors (VFs), sequences underwent annotation through reciprocal BLAST against the Virulence Factors of Bacterial Pathogens (VFDB) database, also using nucleotide sequence similarity identity of 100%.

3.3. STATISTICAL ANALYSIS

All tests were performed in triplicate, with results presented as mean ± standard error of the mean. Statistical evaluation was carried out using one-way ANOVA, followed by the Tukey post hoc test of multiple comparisons. The data were computed using GraphPad Prism 9 (San Diego, CA, USA), with statistical significance defined as P-values less than 0.05.

4.0 RESULTS

4.1 Stimulate the sporulation of *Clostridium* sp. and determine the resilience of the spores to environmental and gastrointestinal conditions.

4.1.1 Spore Formation and Purification

C. vincentii, C. celatum, and *C. disporicum* were successfully cultured. Treatment with caffeine gave variable results among the 3 species. Both phase-bright spores and phase-dark vegetative cells were observed in the control and caffeine-treated samples. Following treatment with lysozyme and trypsin, only phase-bright spores were retained as show in figure 4.1 below.

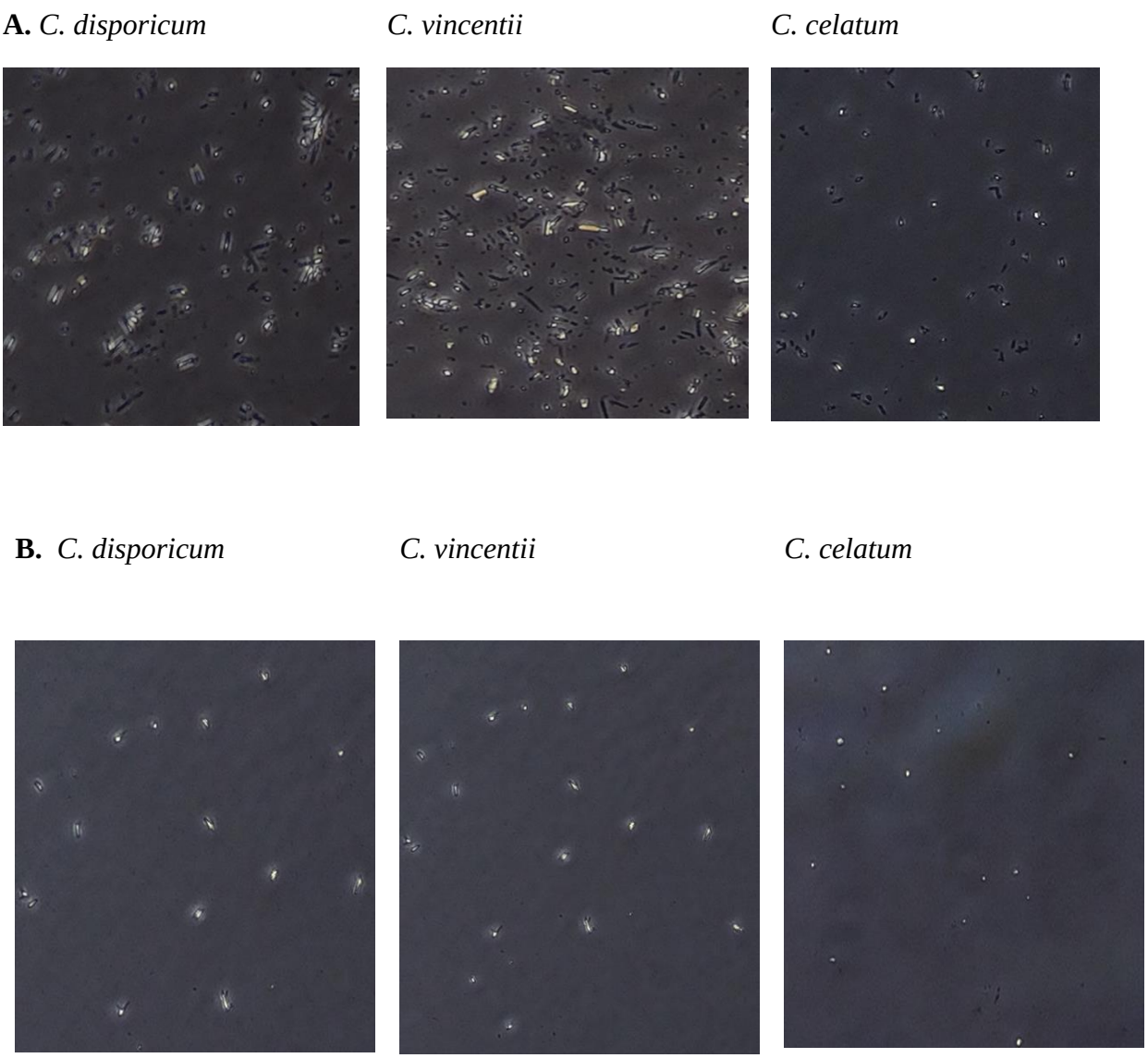


Figure 4.1: Phase contrast microscopic images of spores and vegetative cells.
Each bacteria was grown in RCM with or without caffeine for 14 days. After purifying spores using Trypsin and lysozyme, samples were observed by Phase contrast microscopy. **(A)** Phase contrast microscopic images of vegetative cells (phase dark) and spores (phase bright) post-sporulation before isolation of spores. **(B)** Phase contrast microscopic images after isolation of spores illustrating the predominance of phase bright spores over phase dark vegetative cells. Field of view was 130 µM.

4.1.2 Effect of Caffeine on Sporulation

Caffeine exhibited a significant effect (P<0.05) on the formation of spores in *C. vincentii*. In *C. disporicum*, there was no statistically significant difference. Surprisingly, in *C. celatum*, caffeine had a detrimental impact on sporulation as shown in figure 4.2.

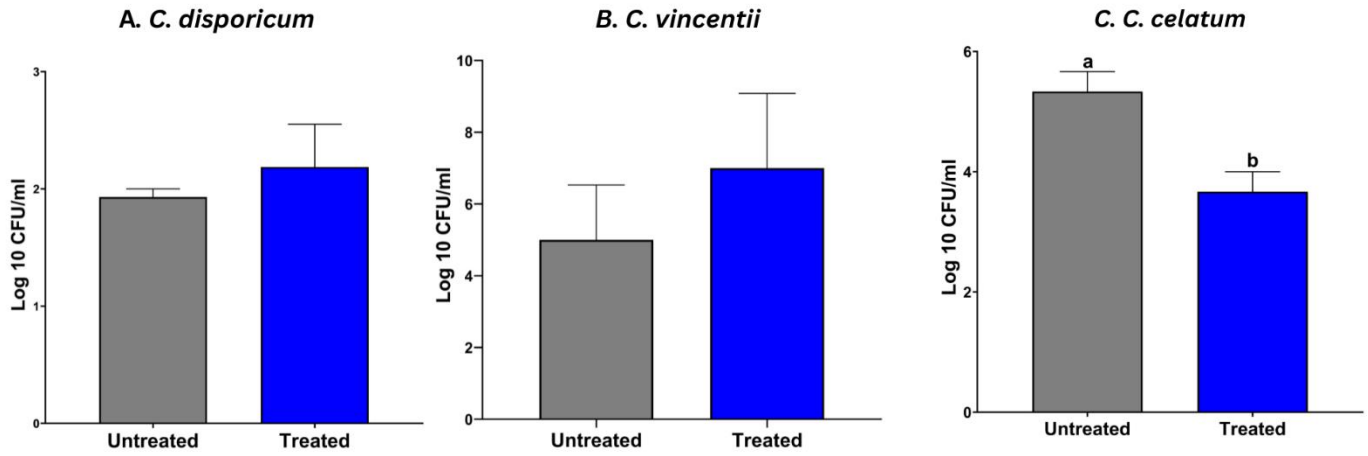


Figure 4.2: Impact of caffeine on bacterial sporulation.
Bacteria were inoculated in RCM media with or without Caffeine for 14 days. Supplementation of RCM media with caffeine had **(A)** no effect on sporulation of *C. disporicum* **(B)** no effect on sporulation of C. *vincentii*, (**C)** reduced the sporulation of *C. celatum* (P < 0.05, n = 3).

4.1.3 Resilience to Environmental Conditions

Survival After Exposure to Oxygen

After 21 days, spores from all 3 bacteria retained viability. Survival rates were 62.8% 55.7%, 49.5% for *C. celatum*, C. *disporicum*, and *C. vincentii* respectively. *C. celatum* exhibited a continuous decrease in survival rate, although the decrease between intervals showed no significant difference (P<0.05). Both *C. disporicum* and *C. vincentii* also displayed insignificant decreases between intervals, except for the decrease between day 3 and day 6 which was significant as shown in figure 4.3.

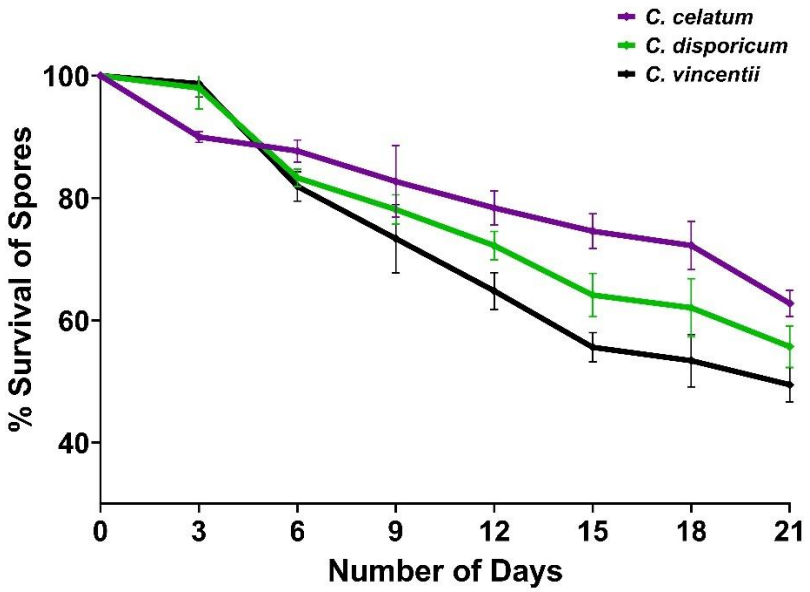


Figure 4.3: Survival rates of spores after exposure to Oxygen
Spores isolated from cultures were placed on petri dishes and left exposed to atmospheric oxygen for 21 days. Spores were then spread on agar media in the anerobic hood and colony forming units determined after 3 days. 62.8% of *C. celatum* spores, 55.7% of *C. disporicum* and 49.5% of C. *vincentii* survived and germinated to form CFUs. There was a significant decrease in survival from day 1 to day 21 ($P < 0.05$, $n = 3$).

Survival Across Different Temperatures

None of the bacteria exhibited the capability to withstand heat shock at temperatures above 70° C as shown in figure 4.4. *C. celatum* demonstrated resilience to temperatures up to 60°C. The quantity of spores remaining after heat shock exhibited a significant increase ($P<0.05$) at 50°C and 40°C. Both *C. vincentii* and *C. disporicum* displayed the ability to endure temperatures of 50°C and below, with the spore count significantly increasing ($P<0.05$) as the temperature was lowered to 40°C.

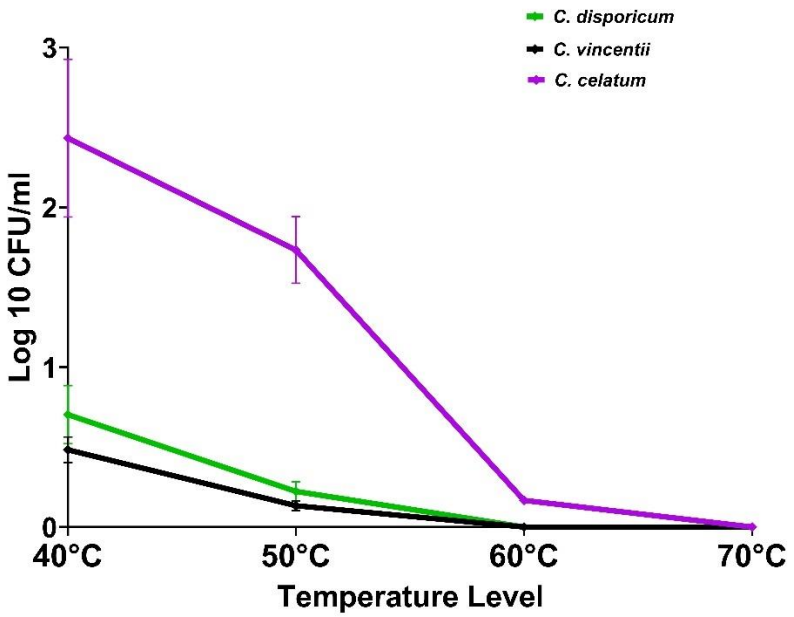


Figure 4.4: Tolerance of spores to temperature after heat shock
Spore suspensions were exposed to wet heat up to 100°C for 10 minutes and CFUs were quantified. None of the spores survived above 70°C. *C. celatum* showed significant survival at 50°C and 40°C. *C. vincentii* and *C. disporicum* survived up to 50°C, with significant survival at 40°C ($P < 0.05$, $n = 3$).

Survival after Exposure to Gastric Acids

All three bacteria demonstrated the ability to survive gastric acids after the 3 hours of exposure (*C. celatum* 86.7%, *C. disporicum* 64.8%, *C. vincentii* 25.64%) as shown in figure 4.5. *C. celatum* exhibited a significant decrease ($P<0.05$) in survival rate after 1 hour of exposure; however, there was an insignificant increase after 2 hours, and then the survival rate remained constant after the third hour. Both *C. disporicum* and *C. vincentii* showed a significant decline ($P<0.05$) in the survival rate after 1 hour of exposure. Additionally, the rate significantly dropped after the second hour, but the subsequent decrease after the third hour was insignificant.

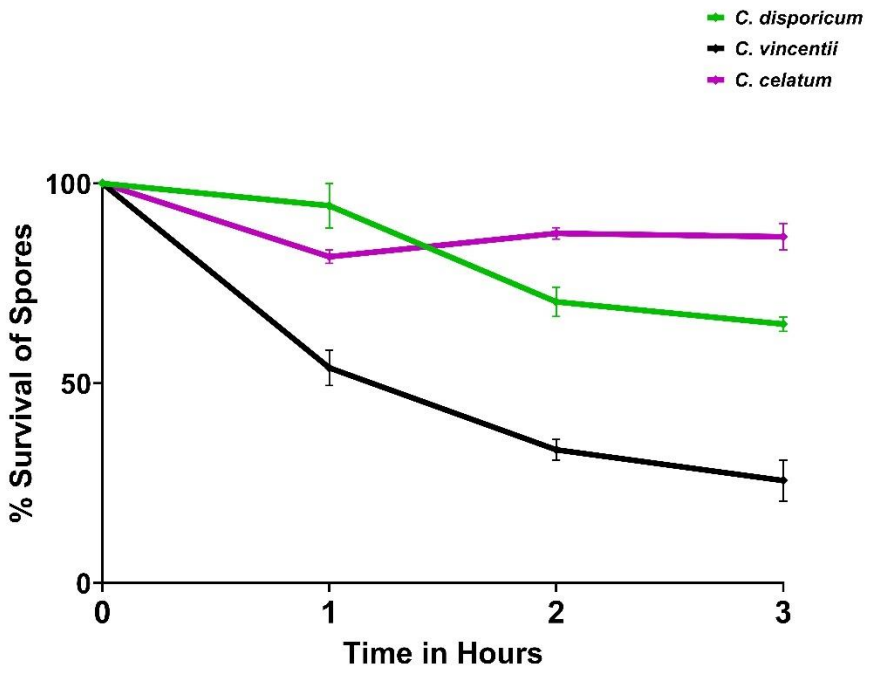


Figure 4.5: Survival rates of spores after exposure to gastric acid
Spore suspensions were incubated in gastric acid and evaluated for viability by plating on RCA. *C. celatum* spores had a survival rate of 86.7% and showed a decrease after 1 hour. *C. disporicum* spores had a survival rate of 64.8%, with a decrease after 1 and 2 hours, and no additional significant decrease after 3 hours. *C. vincentii* spores had a survival rate of 25.64%, with a decrease after 1 and 2 hours, and significant decrease after 3 hours ($P < 0.05$, $n = 3$).

Survival after Exposure to Bile Salts

All the three species exhibited survival against the bile salts, with percentage survival at the end of 4 hours as follows: *C. disporicum* 65%, *C. vincentii* 50.7%, and *C. celatum* 57.8% as shown in figure 4.6. In all three species, there was a significant drop ($P<0.05$) in survival rates after 1 hour of exposure. Subsequent drops in the second, third, and fourth hours remained insignificant. Notably, in *C. disporicum*, there was an insignificant increase after the third hour, followed by a reduction in the fourth hour. The spores also retained viability after a 4-hour exposure to bile salts.

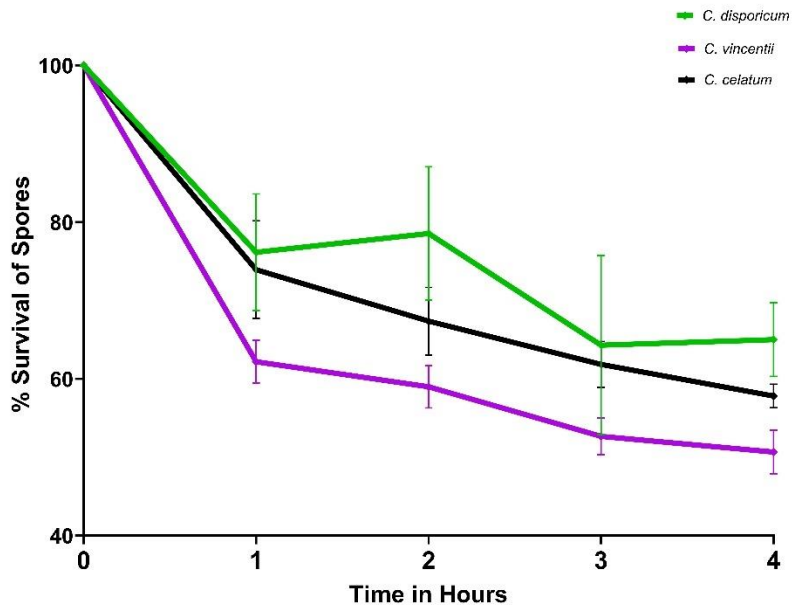


Figure 4.6: Survival rates of spores after exposure to bile salts
After spore suspensions were incubated in RCB medium supplemented with bile salts and viability of remaining spores assessed, C. *disporicum* had a survival rate of 65%, *C. vincentii* 50.7%, and *C. celatum* 57.8%. All species showed a significant drop in survival after 1 hour ($P < 0.05$, $n = 3$), with no significant decreases thereafter.

4.2 Determine the colonization potential, safety and health impacts of

4.2.1 Colonization Potential of the Bacteria

Adhesion Ability

Hydrophobicity

The percent hydrophobicity of the bacteria to hydrocarbons, in this case xylene has been presented graphically in Figure 4.7. Statistical analysis revealed significant differences in the percentage hydrophobicity among the bacteria ($P < 0.05$). *C. vincentii* exhibited the highest hydrophobicity (34.51%), followed by *C. celatum* (24.64%), with *C. disporicum* demonstrating the lowest (5.78%)

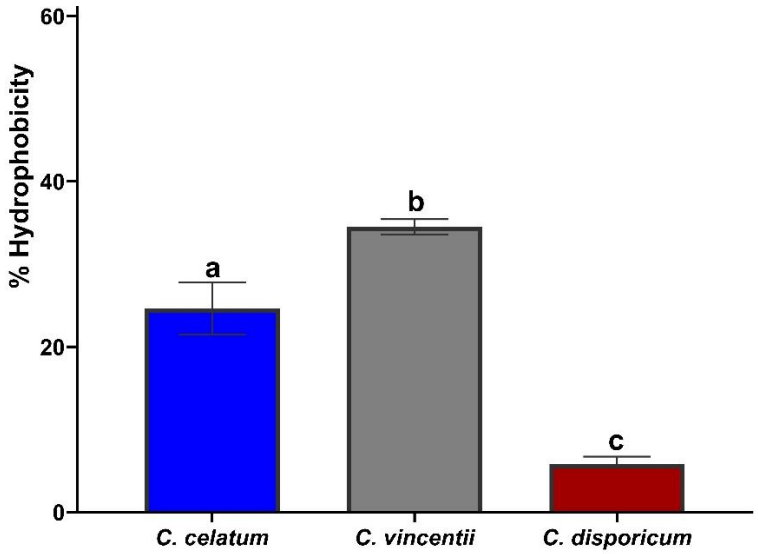


Figure 4.7: Percent hydrophobicity of bacteria
Hydrocarbons (xylene) was mixed with Intact Cell Solution (ICS), incubated and % hydrophobicity determined. *C. vincentii* exhibited the highest % hydrophobicity at 34.51%, followed by *C. celatum* at 24.64%, and *C. disporicum* at 5.78%. Significant differences among the bacteria were observed ($P < 0.05$, $n = 3$).

Auto Aggregation

The percentage of cell auto-aggregation of the bacteria, as depicted in Figure 4.8, indicates that there was no significant difference ($P < 0.05$) in the aggregation property among the bacteria. *C. vincentii* exhibited the highest aggregation percentage (54.87%), followed by *C. celatum* (43.58%), while *C. disporicum* demonstrated the lowest aggregation percentage (30.85%)

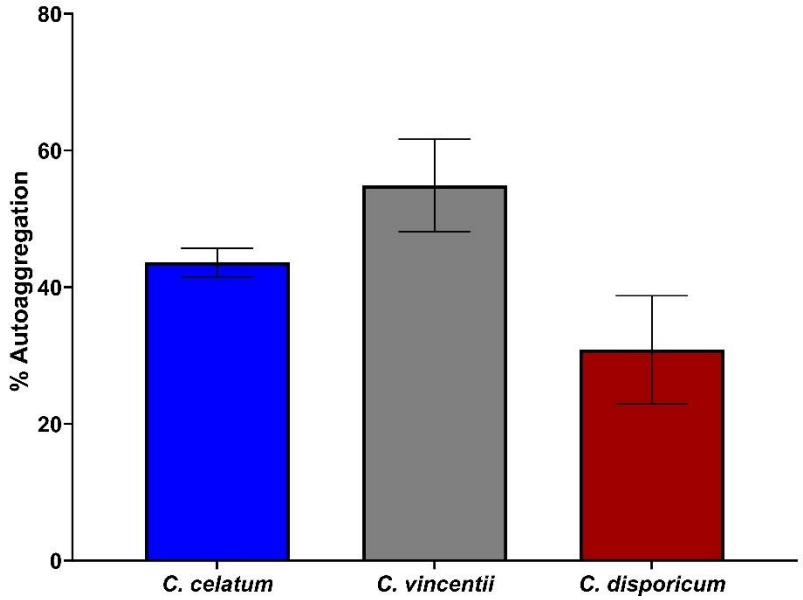


Figure 4.8: Percentage auto-aggregation in bacteria
Bacteria cells were allowed to auto-aggregate for 24 hours, and then the auto-aggregation index was calculated. *C. vincentii* exhibited the highest auto-aggregation at 54.87%, followed by *C. celatum* at 43.58%, and *C. disporicum* at 30.85%. Statistical analysis showed no significant differences among the bacteria ($P < 0.05$, $n = 3$).

4.2.2 Safety

4.2.2.1 Antibiotic Susceptibility

The antibiotic susceptibility to 3 different antibiotics (ciprofloxacin, tetracycline, and ceftriaxone) of the 3 selected species was assessed according to Clinical and Laboratory Standards Institute (CLSI) recommendations from the diameters of zones of inhibitions shown in table 4.1

Table 4.1: Antibiotic susceptibility of the selected *Clostridium* sp.

Antibiotic	***C. celatum***	***C. vincentii***	***C. disporicum***
Tetracycline	58(S)	61(S)	45(S)
Ceftriaxone	51(I)	59(I)	50(I)
Ciprofloxacin	37(S)	32(S)	31(S)

A B

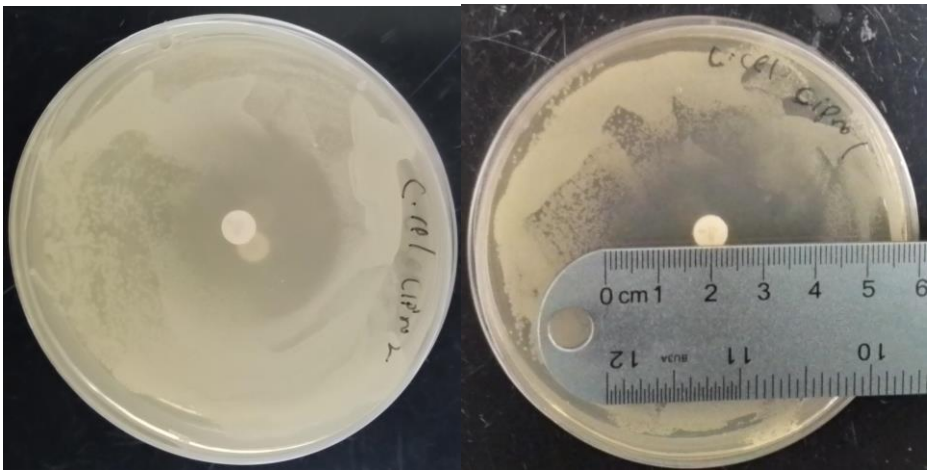


Figure 4.9: Antibiotic susceptibility Zones of inhibition
After evenly spreading bacteria on RCA media, and antibiotic discs placed on the surface followed by incubation, all the bacteria were sensitive to the antibiotics **(A)** Plate showing the zone of inhibition for *C. celatum* to ciprofloxacin and **(B)** Determination of the diameter of the zone of inhibition. Antibiotic susceptibility was categorized as **S (susceptible/sensitive)** or **I (intermediate)**, with diameters measured in millimeters according to CLSI breakpoints.

4.2.2.2 Hemolytic activity

None of the examined bacteria revealed β-hemolytic (i.e., red blood cell lysis) activity when grown in Columbia sheep blood agar as shown in figure 4.10. All the 3 bacteria were γ-hemolytic (i.e., no hemolysis) *Staphylococcus aureus* (positive control) showed hemolytic activity.

Table 4.2: The Hemolytic Activity of *Clostridium* sp.

Bacteria	Hemolytic Activity
C. celatum	-
C. vincentii	-
C. disporicum	-
S. aureus	+

Note "-"means gamma hemolysis, "+" means beta hemolysis

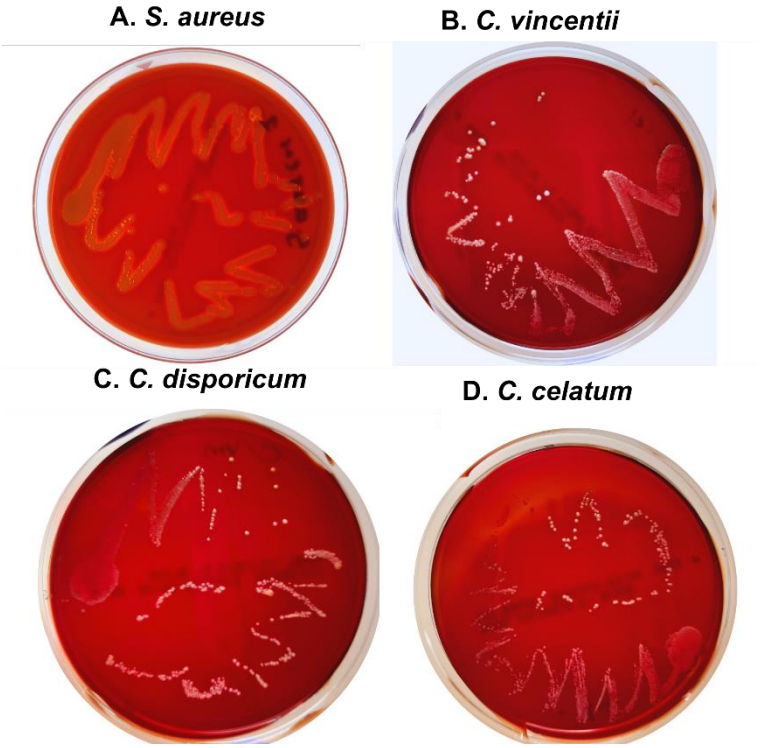


Figure 4.10: Hemolytic activity on Columbia sheep blood agar
Bacteria cells were streaked on Columbia blood agar plates and incubated. **(A)** *Staphylococcus aureus* (the positive control) showed β-hemolysis with gold zones around colonies, indicating complete hemolysis. **(B)** *C. vincentii*, **(C)** *C. disporicum*, and **(D)** *C. celatum* showed no color change, indicating γ-hemolysis (no hemolysis).

4.2.3 Determining the Health Impacts

4.2.3.1 Bile salt hydrolase activity

Among the three bacteria screened on plates for BSH activity, none yielded positive results against bile salt taurodeoxycholate acid as shown in figure 4.11. There were no zones of precipitation around the colonies of the bacteria, indicating negative results.

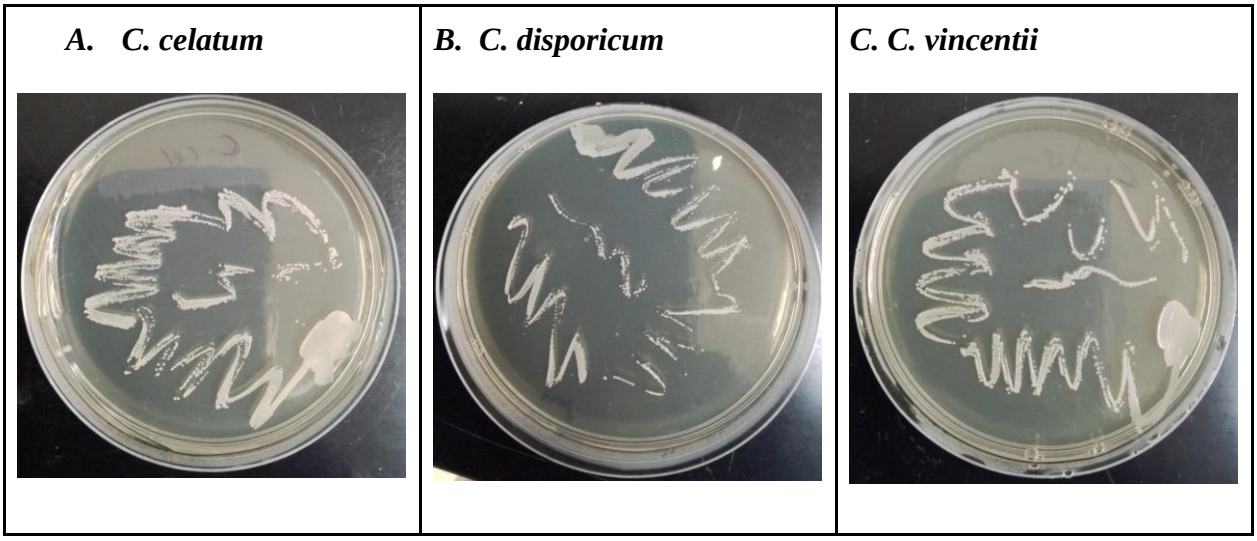


Figure 4.11: Bile salt hydrolase (BSH) activity on plates with Tauro deoxycholate acid
Bacteria were streaked on TSA plates supplemented with Tauro deoxycholic acid and incubated. None of the three bacteria showed BSH activity, as indicated by the absence of zones of precipitation around the colonies, signifying negative BSH activity.

4.2.3.2 Antioxidant activity

The DPPH-radical scavenging activity of the bacteria metabollites is illustrated in Figures 4.12 and 4.13. Results indicated that the cell-free supernatant (CFS) (Fig. 4.12) of the three bacteria exhibited the highest average for radical scavenging activity, reaching 82.1%, compared to bacteria extracellular vesicles (BEVs) (Fig. 4.13), which showed 46.28%. Nevertheless, in the CFS, the bacteria did not show any significant difference in % scavenging compared to the negative control

RCM). Meanwhile, the average DPPH scavenging rate conducted using BEVs for the bacteria demonstrated a significant increase in % scavenging activity compared to the negative control, except in *C. celatum*. However, there were no significant differences among the three bacteria. The highest percentage scavenging in BEVs was observed in *C. vincentii* (56.26%), followed by *C. celatum* (37.53%), and the least was seen in *C. disporicum* (27.28%)

Cell Free Supernatant

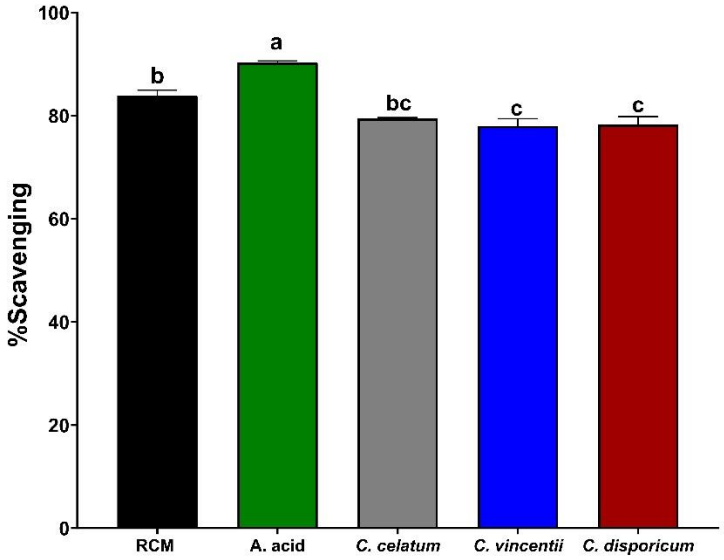


Figure 4.12: DPPH-radical scavenging activity of cell-free supernatants (CFS)
The CFS of each bacteria was tested for antioxidant activity using the DPPH-radical scavenging assay. Ascorbic acid was the positive control and RCM media was a negative control. The average scavenging activity across the treatment groups was 82.1%, with no significant difference compared to the negative control (RCM) ($P < 0.05$, $n = 3$).

Bacteria Extracellular Vesicles

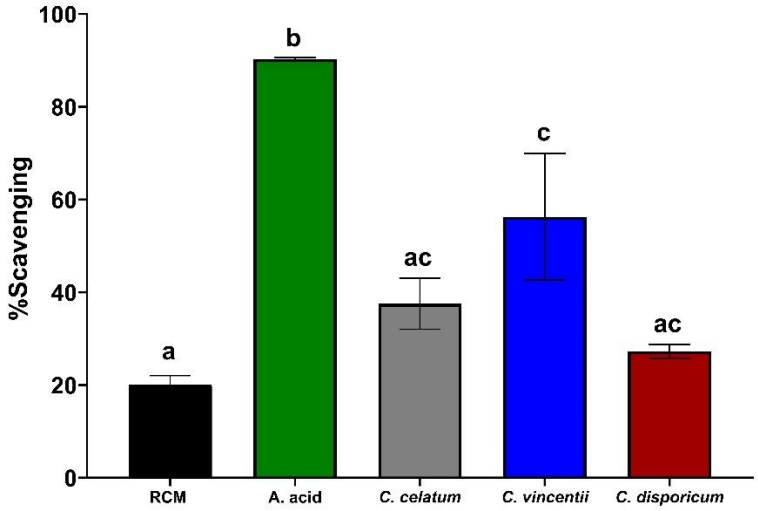


Figure 4.13: DPPH-radical scavenging activity of bacterial extracellular vesicles (BEVs)
The BEVs from each bacteria were tested for antioxidant activity using the DPPH-radical scavenging assay. Ascorbic acid was the positive control and BEVs from RCM media was the negative control. The % scavenging activity was 56.26% for *C. vincentii*, showing a significant increase compared to the negative control (RCM). *C. celatum* and *C. disporicum* did not have an effect in % scavenging.

Antibacterial Activity

The antibacterial activity test of the three *Clostridium* bacteria against the tested pathogens (*Escherichia coli, Staphylococcus aureus, Shigella flexneri, Pseudomonas aeruginosa, Listeria innocua, and Vibrio parahaemolyticus*) showed no effect. There was no observed zone of inhibition for each of them. This observation was consistent across all sample plates as shown in sample plates in Figure 4.14.

Table 4.3: Antibacterial activities of selected *Clostridium* spp. against 6 pathogenic bacteria

Pathogenic Bacteria	C. celatum	C. disporicum	C. vincentii
Listeria innocua	-	-	-
Staphylococcus aureus	-	-	-
Escherichia coli	-	-	-
Pseudomonas aeruginosa	-	-	-
Shigella flexneri	-	-	-
Vibrio parahaemolyticus	-	-	-

- **Means no zones of inhibition seen.**

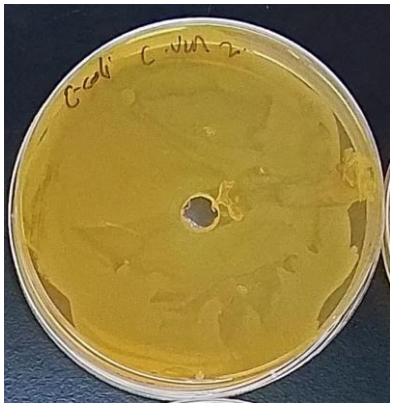
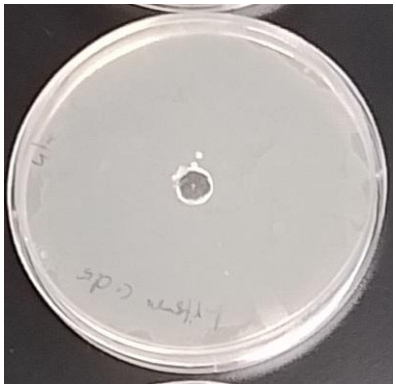
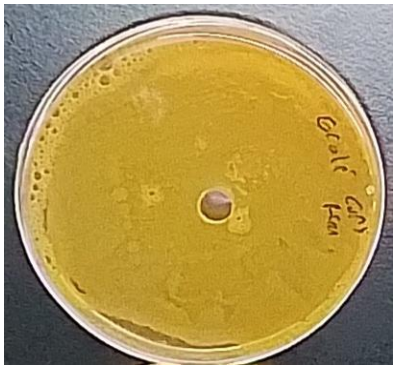
A. E. coli	*B. L. innocua*	*C. E. coli*
		

Figure 4.14: Antibacterial activity against tested pathogens
CFS from each test bacteria was tested for antibacterial activity using the agar well diffusion method on TSA plates pre-seeded with pathogen bacteria. No zones of inhibition were observed on any of the three species, indicating no antibacterial effect. Sample plates for **(A)** and **(C)** *E. coli*, **(B)** *L. innocua*.

4.2.3.4 Anti Inflammatory Properties

Interleukin-6 (IL-6) gene expression

The results indicated a significant reduction ($P < 0.05$) in IL-6 gene expression for *C. celatum*. Although IL-6 downregulation was observed in *C. disporicum*, it was not statistically significant ($P < 0.05$). Interestingly, IL-6 expression was significantly upregulated by *C. vincentii*, as illustrated in Figure 4.15.

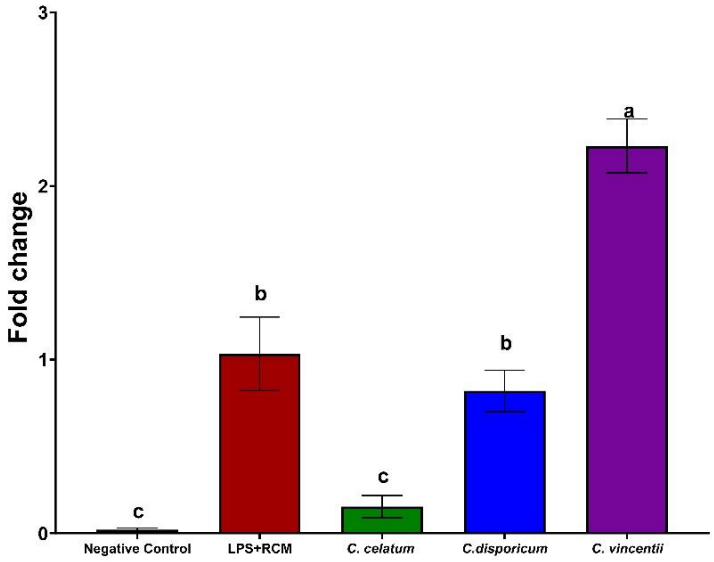


Figure 4.15: Gene expression of IL-6 in RAW 264.7 macrophages
RAW 264.7 macrophages were treated with 10 µl CFS for 18 hours, followed by LPS for 6 hours; The gene expression of IL-6 was determined by qPCR . *C. celatum* significantly downregulated IL-6 expression, *C. disporicum* had no effect, and *C. vincentii* upregulated IL-6 expression ($P < 0.05$, $n = 3$).

Transforming growth factor beta (TGF-β)

The results indicated a significant increase ($P < 0.05$) in TGF-β gene expression for *C. celatum.* Although TGF-β upregulation was observed in both *C. disporicum* and *C. vincentii*, these increases were not statistically significant ($P < 0.05$) as shown in figure 4.16.

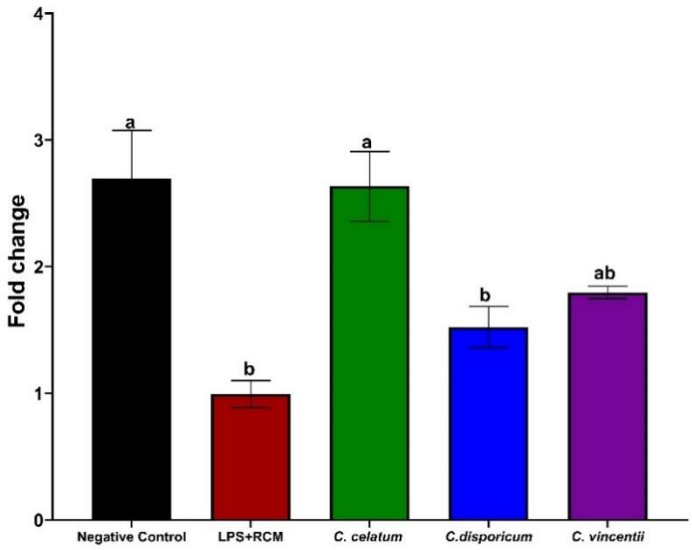


Figure 4.16: Gene expression of TGF-β in RAW 264.7 macrophages
RAW 264.7 macrophages were treated with 10 µl CFS for 18 hours, followed by LPS for 6 hours. The gene expression of TGF-β was determined by qPCR. *C. celatum* significantly increased TGF-β expression, while *C. disporicum* and *C. vincentii* had no effect ($P < 0.05$, $n = 3$).

Inducible nitric oxide synthase (iNOS)

For iNOS expression, the results indicated that *C. celatum* significantly reduced its levels ($P < 0.05$). In contrast, both *C. disporicum* and *C. vincentii* significantly increased iNOS expression ($P < 0.05$) as shown n figure 4.17.

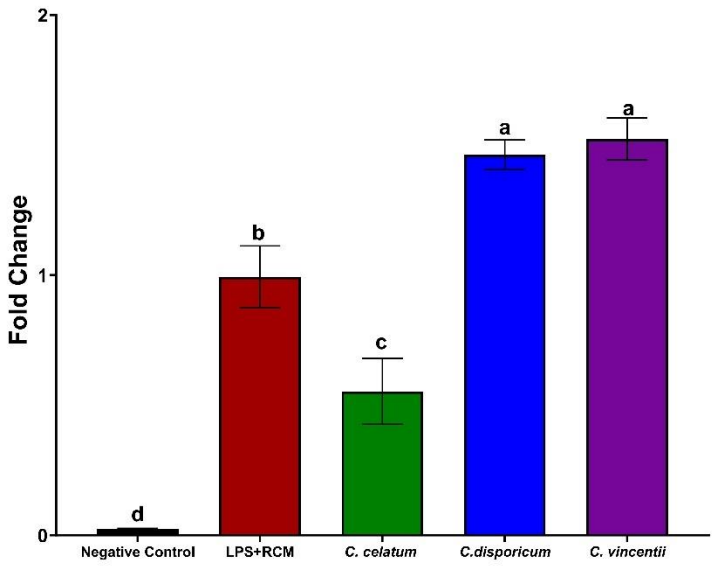


Figure 4.17: Gene expression of iNOS in RAW 264.7 macrophages
RAW 264.7 macrophages were treated with 10 µl CFS for 18 hours, followed by LPS for 6 hours; The gene expression of iNOS was determined by qPCR. *C. celatum* significantly reduced iNOS gene expression, whereas *C. disporicum* and *C. vincentii* increased the expression. ($P < 0.05$, $n = 3$).

Interleukin 1 (IL-1)

For IL-1 the results indicated that *C. celatum* and *C. disporicum* reduced its expression, though these reductions were not statistically significant. In contrast, *C. vincentii* significantly increased IL-1 expression ($P < 0.05$) as shown in figure 4.18.

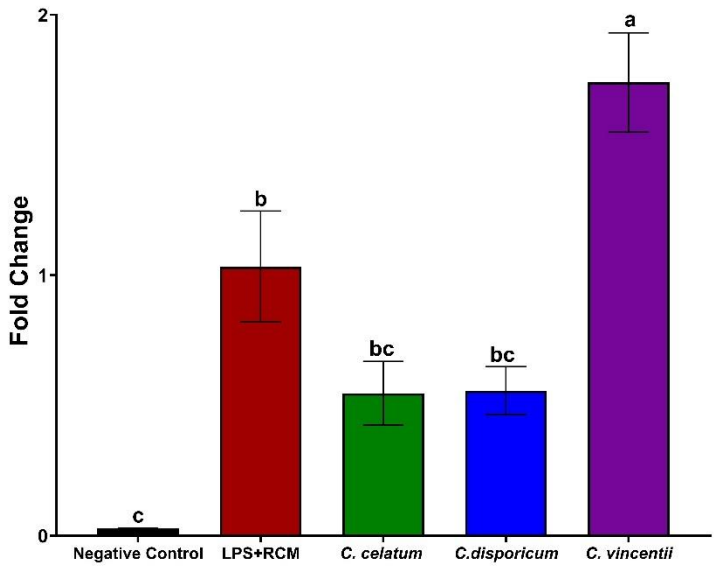


Figure 4.18: Gene expression of IL-1 in RAW 264.7 macrophages
RAW 264.7 macrophages were treated with 10 µl CFS for 18 hours, followed by LPS for 6 hours. The gene expression of IL-1 was determined by qPCR. *C. celatum* and *C. disporicum* had no effect on IL-1 expression, while *C. vincentii* increased IL-1 expression ($P < 0.05$, $n = 3$).

4.2.3.5 TLR Pathway Screening

Screening of the TLR pathway revealed that metabolites from *C. celatum* significantly upregulated one gene: TLR10 ($P < 0.05$). In contrast, *C. celatum* did not induce any significant changes in the expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, or TLR9. Additionally, no significant alterations were observed in the expression of cytokines and protein genes associated with the TLR pathway in response to the bacterial molecular patterns.

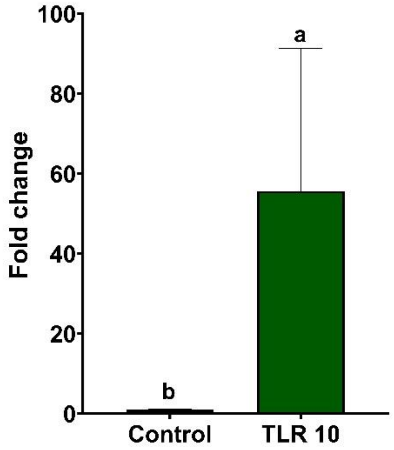


Figure 4.19: TLR10 expression in response to *C. celatum* metabolites
Caco-2 cells were treated with 10 µl of CFS from *C. celatum* for 24 hours. The relative gene expression of 84 genes associated with TLR pathway were determined. Only TLR10 expression was significantly upregulated in response to *C. celatum* metabolites. No significant change was observed for all other TLRs and associated genes ($P < 0.05$, $n = 3$).

4.4 *In silico* characterization of Clostridium gene pathways

4.4.1 Genomic analysis of safety aspects of the bacteria

Virulence Factor Genes

After screening the three bacteria against thousands of genes in the virulence factor database, none of the bacteria was shown to have any virulence factor gene as shown in table 4.4.

Table 4.4: Virulence Factor Number of Genes Found in the *Clostridium* sp.

Bacteria	Number of Virulence Factor Gene Found

C. celatum	0
C. disporicum	0
C. vincentii	0

Antimicrobial Resistance Genes

After screening for antimicrobial resistance genes in the three bacteria, *C. celatum*, and C. *vincentii* demonstrated no antimicrobial resistance genes. However, *C. disporicum* demonstrated two antimicrobial resistance genes as shown in table 4.5.

Table 4.5: Antimicrobial Resistance Number of Genes found in the *Clostridium* sp

Bacteria	Number of Virulence Factor Gene Found
C. celatum	0
C. disporicum	2
C. vincentii	0

The genes found in *C. disporicum* shown in Table 4.6 were (i) ant(6)-la_3 with accession number KF864551 which is responsible for the resistance against Streptomycin and (ii) tetA(P)_2 with accession number L20800 which is responsible for the resistance against Doxycycline/Tetracycline.

Table 4.6: Identity of Antimicrobial Resistance Genes found in *C. disporicum*.

Gene Found	Accession Number	Resistance
ant(6)-la_3 tetA(P)_2	KF864551 L20800	Streptomycin Doxycycline/Tetracycline

5.0 DISCUSSION

5.1 Sporulation of *Clostridium* sp. and resilience of the spores to environmental and gastrointestinal conditions.

5.1.1 Spore Formation and Purification

Phylum *Firmicutes* are well-known spore formers. The analysis of the genome sequence of these bacteria has provided insight into their evolution and physiological characteristics related to spore formation (Galperin, 2013). In our study, the three *Clostridium* species formed spores, which is a natural characteristic of Class *Clostridia*. In the laboratory, sporulation in a culture occurs due to the exhaustion of nutrients during growth (Serra et al., 2014). The three species under study, *C. celatum* was isolated from human feces, *C. disporicum* from rat caecum, and *C. vincentii* was isolated from low-salinity pond on the McMurdo Ice Shelf, Antarctica (Mountfort et al., 1997). With the three species having grown and multiplied in the same medium, all the nutrients were exhausted, leading the bacterial cells to sporulate as a survival mechanism in the nutrient-deprived environment.

5.1.2 Effect of Caffeine on Sporulation

Research has shown that some methylxanthines, including caffeine, 3-isobutyl-1-methylxanthine, and theophylline, increase spore yield in *C. perfringens*, a member of the genus *Clostridium* (Labbe & Nolan, 1981). In our study, caffeine was used to induce sporulation and increase spore formation in the three bacteria under investigation. However, our results showed that caffeine had no significant effect on the formation of spores. These results contradict previous research where caffeine-treated medium increased spore formation in *C. perfringens* (Labbe & Nolan, 1981). It is

not well understood why caffeine significantly reduced sporulation in *C. celatum*. Since caffeine did not show any statistically significant effect in inducing sporulation compared to natural sporulation by the bacteria, all the tests in this study were conducted using spores from the natural sporulation of the bacterial species.

5.1.3 Spores Resilience to Environmental Conditions

Spores exhibit resistance to a variety of agents and environments, including both dry and wet temperatures, as well as acids and bases. For obligate anaerobes, spores are considered for use in next-generation probiotics, prompting exploration of their resilience in harsh atmospheric and intestinal conditions (Setlow, 2014b). Despite being dormant, spores cannot function as probiotics themselves. However, their ability to withstand environmental and intestinal conditions allows them to reach the lower gut, where they can germinate and become vegetative cells, thereby exerting probiotic effects. In the case of *C. difficile*, its spores' resilience to environmental stressors and low stomach pH enables them to survive in foods and become pathogenic in the lower gut (Setlow, 2014b).

Our findings indicated that none of the spores from the three bacteria exhibited the capability to withstand heat shock at temperatures above 70°C. The survival of the spores across the temperature range varied among the three bacteria. Significant evidence has shown variability in wet heat resistance among spore populations, although the reasons for this heterogeneity are not fully understood. A previous study by Wells-Bennik et al. (2019) demonstrated that different sporulation conditions may affect the spores' resistance to heat. Other factors influencing spore

resistance to *wet heat* include DNA saturation by *α/β*-type small, acid-soluble spore protein (*α/β*-type SASP), low core water content, and sporulation temperature optimum (Setlow, 2014).

More knowledge about gut *Clostridium* commensals is continually being discovered, and exploring these bacteria as probiotics or biotherapeutics poses challenges due to their strict *anaerobic* nature. In our study, we subjected spores from the three *Clostridium* species to oxygen exposure to test their resilience to environmental oxygen. The results showed varying survival rates among the species (*C. celatum* 62.8%, *C. disporicum* 55.7%, and *C. vicentii* 49.5%). The mechanism of survival of spores from *anaerobic* bacteria when exposed to oxygen is not entirely clear, but several factors can be attributed to it. Firstly, this resilience can be attributed to the spore's structure, which consists of different layers that play distinct roles in making the spore resistant to various environmental conditions. These layers include exosporium, coat, outer membrane, cortex, germ cell, inner membrane, and the core (Setlow, 2014b). Secondly, these mechanisms may involve molecular defenses employed by anaerobic bacteria to protect their metabolic pathways from impairments caused by oxygen (Lu & Imlay, 2021).

As probiotics, the next hurdle for ingested spores is to withstand harsh conditions in the stomach (pepsin, gastric acid,) and the intestines (bile salts, trypsin, and high pH). This necessitates that probiotics must tolerate acids and bile to survive in the gut (Zhang et al., 2022).

After subjecting the spores to gastric acid for 4 hours, they exhibited resilience to gastric acids with varying survival rates. *C. celatum* showed the highest survival rate at 86.7%, followed by *C. disporicum* (64.8%) and *C. vincentii* (25.64%). A study by Madana and Sathiavelu (2024) demonstrated that *L. lactis*, one of the most studied probiotics, had a survival rate of 75% after exposure to gastric acids. Conversely, a study by Dai et al. (2024) on *Bacillus subtilis* as a probiotic

showed lower survival rates (15%-30%) in gastric acid pH between 2 and 3, similar to the findings for *C. vincentii* in our study.

After passing through the stomach, probiotics move to the duodenum where they encounter bile, which has a higher pH. In our study, all three species survived exposure to bile salts (*C. disporicum* 65%, *C. vincentii* 50.7%, and *C. celatum* 57.8%). These results showed lower survival rates compared to findings on isolated bacterial strains studied for intestinal survival assessment by Liang et al. (2020b). After the resilience assessment we subjected the bacteria spores to favorable growth environment and within three days the spores germinated back to vegetative cells,

5.2 I*n vitro* characterization of colonization potential, safety and Health Impacts

5.2.1 Colonization Potential

Adhesion Ability

The ability to adhere to the intestinal epithelium serves as one of the criteria for selecting probiotics. While possessing this trait does not guarantee health benefits, it enhances the bacteria's chances of survival in the intestines, allowing it to exert its health impacts. Additionally, attachment to the intestinal epithelium creates competition with harmful bacteria, thereby playing a protective role against these pathogens (Krausova et al., 2019).

In our study, we assessed adhesion ability using hydrophobicity. Bacteria cells' hydrophobicity strength has been associated with them being able to easily attach to the epithelia lining of the lower gut (De Wouters et al., 2015). We subjected the bacteria to xylene, a hydrocarbon, and observed varying percentages of hydrophobicity among the three bacteria (*C. vincentii* 34.51%, *C. celatum* 24.64%, *C. disporicum* 5.78%). These results show lower hydrophobicity percentages compared to those reported by Mallappa et al. (2019) in their study on *Lactobacillus* strains, but

the decreasing trend aligns with findings from a study by Krausova et al. (2019b) on other *Lactobacillus* strains, indicating variability in adhesion ability even within the same taxonomic group. From our findings, *C. vincentii* exhibited the highest hydrophobicity potential.

Secondly, we assessed adhesion ability through bacterial cell auto aggregation. Similar to hydrophobicity, auto aggregation varied across different species. The three bacteria under study exhibited varying percentages of auto aggregation (*C. vincentii* 54.87%, *C. celatum* 43.58%, *C. disporicum* 30.85%). *C. vincentii* showed the highest percentage of auto aggregation. Notably, auto aggregation was higher across all three bacteria compared to hydrophobicity, a trend similar to observations in studies on *Lactobacillus* strains (Krausova et al., 2019b; Mallappa et al., 2019).

5.2.2 Safety

5.2.2.1 Antibiotic Susceptibility

Antibiotic resistance presents a significant challenge in clinical settings, complicating the treatment of bacterial infections when bacteria have developed resistance to available antibiotics (Nwobodo et al., 2022). Therefore, bacteria possessing antibiotic-resistant genes should not be used as probiotics, as they could potentially transfer these genes to pathogenic bacteria in the gut. In our study, we subjected the bacteria to antibiotics, and the results indicated that all the bacteria were susceptible to the antibiotics. These findings are crucial in affirming the safety of the bacteria under study for potential use as probiotics. Similar findings were reported by Yang et al. (2022) in their study on *Clostridium tyrobutyricum*.

5.2.2.2 Hemolytic activity

Hemolytic activity is a critical safety assessment that must be conducted when screening bacteria for use as probiotics (Pahumunto et al., 2021). In our study, hemolytic analysis of the bacteria was performed using Columbia Blood Agar. The results indicated that all three bacteria do not exhibit hemolytic activity. These findings align with studies conducted on potential probiotic bacteria (Yang et al., 2022; Pahumunto et al., 2021). Our results suggest that these bacteria, if used as probiotics, would not cause hemolysis if they get in contact with red blood cells.

5.2.3 Health Impacts

5.2.3.1 Bile salt hydrolase activity

Bile Salt Hydrolase (BSH) enzymes have been a major focus in probiotic studies due to their importance in weight loss and cholesterol-lowering effects (Kusada et al., 2022). In our study, we screened the bacteria for BSH activity using taurodeoxycholic acid (TDCA). None of the bacteria exhibited BSH activity, which contrasts with findings from many studies assessing bacterial strains for their probiotic potential. In a study by Wei et al. (2022b), where TDCA was used, all three bacteria studied exhibited BSH activity. However, in a study by Hernández-Gómez et al. (2021b), where microorganisms were plated on TDCA-supplemented plates, *S. boulardii* did not show any BSH activity, similar to our findings for all three bacteria.

5.2.3.2 Antioxidant activity

Oxidative stress, which damages cells by causing imbalance in the body, arises from the continuous production of reactive oxygen species (ROS). Probiotic bacteria that exhibit antioxidant activity to counteract these ROS are believed to have beneficial health impacts (Kim

et al., 2022). In our study, we investigated antioxidant activity using the DPPH scavenging method, employing both cell-free supernatant (CFS) and bacterial extracellular vesicles (BEVs). The results from the cell-free supernatant showed negative control which was RCM having antioxidant activity which can be attributed to the antioxidant properties of the Reinforced *Clostridium* media. This is evidenced by the high antioxidant activity observed in the control, which is media without any bacteria, likely due to the presence of L-cysteine, a proven antioxidant (Plaza et al., 2018; USDA, 2007).

Isolating bacterial extracellular vesicles allowed us to remove the media, which initially exhibited antioxidant activities, thereby obtaining a clearer picture of the antioxidant activities of the bacteria under study. The findings showed varied scavenging percentages across the species (*C. vincentii* 56.26%, *C. celatum* 37.53%, *C. disporicum* 27.28%). Although not fully understood, the literature indicates that BEVs possess various therapeutic properties, including antioxidative proteins (Krzyżek et al., 2023).

5.2.3.3 Antibacterial Activity

Probiotics have been proposed as alternatives to the excessive use of antibiotics, which contributes to antibiotic resistance. Bacteria's antibacterial activity arises from compounds they produce, such as organic acids, hydrogen peroxide, and bacteriocins (S et al., 2020).

The three species were cultured against six pathogenic bacteria, and the results showed that none of them were able to inhibit the growth of these pathogens. These findings differ from those reported by Zaghloul and Halfawy (2024), who studied the antibacterial activity of *Pediococcus pentosaceus* against similar pathogenic bacteria. Bacteriocins, known for their role in inhibiting bacterial growth, are primarily produced by Lactic Acid Bacteria (LAB). The ability of these

bacteria to produce organic acids enables them to inhibit the growth of pathogenic bacteria (Verma et al., 2014; Darbandi et al., 2021). Similarly, a wide range of *Clostridium* species have been associated with bacteriocin production (Pahalagedara et al., 2020). Although the ability of the bacteria in our study to produce organic acids using substrates from the growth media is not well-documented in the literature, their ability not to form organic acids may explain why they did not inhibit the growth of the pathogenic bacteria.

5.2.3.4 Anti-Inflammatory Properties

Inflammation in the gut is a major factor in various diseases such as cancers, diabetes, obesity, and irritable bowel syndrome. Evidence suggests that gut dysbiosis may lead to increased gut-derived lipopolysaccharide (LPS), which interacts with macrophages in the gut, causing chronic low-grade inflammation that can contribute to the development of these diseases (Khanna et al., 2020).

We exposed macrophages to LPS to induce inflammation and assessed the effects of the three bacteria under study on inflammation. Gene expression of different cytokines associated with inflammation was evaluated. The proinflammatory cytokine IL-6 was downregulated by *C. celatum* and *C. disporicum*, consistent with findings reported by Khanna et al. (2020). Additionally, these bacteria showed upregulation of TGF-β, an anti-inflammatory cytokine. TGF-β plays a crucial role in immune homeostasis and, in chronic inflammation, is responsible for suppressing inflammation in later stages (Kok, 2023).

Evaluating the expression of iNOS, which is primarily expressed in response to inflammatory signals, revealed that *C. celatum* significantly reduced its expression. Similar results were observed by Hao et al. (2023), who evaluated the anti-inflammatory properties of *L. plantarum*. Additionally, the results for IL-1, an inflammatory cytokine, indicated that both *C. celatum* and *C.*

disporicum reduced its expression; however, these reductions were not statistically significant. Hao et al. (2023) also reported that *L. plantarum* reduced IL-1 expression, although this reduction was not statistically significant.

5.2.3.5 TLR Pathway Screening

Toll-like receptors (TLRs) are among the most extensively studied pattern recognition receptors (PRRs). Primarily, TLRs function to recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Upon recognition, they initiate a cascade of immune responses that facilitate the recruitment of phagocytes to the site of infection. In our study, TLR10 was found to be upregulated. TLR10 is the most recently discovered TLR, and further research is ongoing to elucidate the functions of this novel receptor. The gene encoding TLR10 is clustered with the genes encoding TLR1 and TLR6 on the same chromosome (Fore et al., 2020).

The precise function of TLR10 remains unclear. Some studies suggest that TLR10 has inflammatory properties by activating NF-κB, while others indicate an anti-inflammatory role. TLR10 has been shown to modulate inflammation by exhibiting anti-inflammatory properties, potentially through its ability to inhibit both MyD88-dependent and -independent pathways. Some studies have observed that TLR10 inhibition of these pathways is associated with a reduction in IL-6 and TNF-α production (Hess et al., 2017). TLR10 is unique among TLRs in its ability to toggle downstream pathways on and off depending on the levels of inflammatory cytokines (Jiang et al., 2016).

Further studies have shown that TLR10 can bind to TLR2, and the resulting dimer can recruit MyD88. This interaction is one of the proposed mechanisms by which TLR10 modulates inflammation. Additionally, TLR10 plays a role in the PI3K/Akt-mediated induction of anti-

inflammatory cytokines such as IL-8 and IFN-β, which are believed to attenuate inflammation (Su et al., 2020).

5.3 *In silico* screening for the safety of the bacteria as probiotics

The safety of bacteria intended for use as probiotics is assessed to ensure they are non-pathogenic and do not harbor antimicrobial resistance genes. We conducted *in silico* assessments of the three bacteria for both virulence factors and antimicrobial resistance genes. Screening for virulence factors was performed against genes in the Virulence Factor Database (VFDB), which revealed no virulence factor genes, indicating that our bacteria of interest do not possess pathogen-related factors. Regarding antimicrobial resistance genes, *C. celatum* and *C. vincentii* did not exhibit any detected genes. However, *C. disporicum* showed genes conferring resistance to streptomycin and tetracycline rendering it unsafe for use as a probiotic.

6.0 CONCLUSION AND RECOMMENDITIONS

Based on our hypothesis that resilient spores germinate into bacterial cells and produce protective molecular patterns with antioxidant and anti-inflammatory effects, all three bacterial strains demonstrated potential for use as probiotics. These bacteria were able to sporulate naturally, indicating that they do not require an inducer to sporulate.

Regarding objective one, which aimed to evaluate the resilience of the formed spores, all bacterial strains were capable of forming spores resistant to oxygen, high temperatures up to 60°C, gastric acids, and bile salts. *C. celatum* exhibited the highest resilience to oxygen, temperature, and gastric acids, while *C. disporicum* showed the greatest resistance to bile salts. This indicates that these bacteria can produce resilient spores capable of surviving atmospheric and intestinal stresses.

In Objective Two, *C. vincentii* proved to be the most effective in adhesion ability, as evidenced by its percentage of hydrophobicity and auto aggregation. All three bacteria were found to be safe, as they did not exhibit hemolytic activity and were susceptible to the selected antibiotics. However, *C. disporicum* was identified to have antibiotic resistance genes for streptomycin and tetracycline. It is recommended that *in vitro* testing for antibiotic susceptibility of *C. disporicum* to these antibiotics be conducted.

None of the three bacterial strains demonstrated bile salt hydrolase activity or antibacterial activity. In terms of antioxidant properties, bacterial extracellular vesicles from *C. vincentii* exhibited the highest antioxidant activity compared to *C. celatum* and *C. disporicum*. Analysis of anti-inflammatory properties revealed that *C. celatum* demonstrated the greatest anti-inflammatory

potential by reducing the expression of pro-inflammatory cytokines IL-6, IL-1, and iNOS, and upregulating TGF-β, which is an anti-inflammatory cytokine.

Overall, the three bacterial strains have demonstrated potential for use as probiotics. Their ability to form resilient spores suggests that, despite being strict anaerobes, their spores could be incorporated into food products. Their survival in gastric acid and bile salts indicates that they can successfully traverse the upper gut and reach the lower gut, where they can exert their health benefits. These bacteria, particularly *C. celatum*, have shown promising probiotic potential due to their adhesion ability, antioxidant actvity and anti-inflammatory effects.

Building on the findings of this thesis, further tests to elucidate the probiotic potential of *Clostridium* species and their impact on human health will be conducted. Firstly, extended testing of spore resilience under prolonged oxygen exposure will be conducted to assess the long-term stability and viability of the spores, which is crucial for their potential application as probiotics. This will provide insights into how these spores might perform in varied environmental conditions and storage scenarios. Additionally, antibiotic resistance testing will be done, focusing specifically on *C. disporicum*.

In addition, further *in vivo* studies will be undertaken to evaluate the probiotic potential and health impacts of *C. celatum*. Using diet-induced obesity models, specifically C57BL/6J mice fed a high-fat diet, the protective effects of *C. celatum* spores on diet induced obesity will be investigated. Additionally, an inflammation model involving C57BL/6J mice will be employed, where inflammation and dysbiosis are induced using Dextran Sodium Sulfate (DSS). This will enable the investigation of how *C. celatum* spores influence gut health under inflammatory conditions.

Lastly, future *in vitro* experiments will utilize human intestinal immune cells rather than epithelial cell lines, such as Caco-2 cells.

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