Induction and elimination of viable but non-culturable *Campylobacter jejuni* in agri-food systems

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Abstract

Numerous bacterial species possess the ability to enter a state known as viable but non-culturable (VBNC) in response to adverse environmental conditions. Although pathogenic bacteria are unlikely to cause diseases in the VBNC state, they can regain virulence upon resuscitation under favorable conditions, thereby posing a substantial threat to food safety and public health. Moreover, the inability to detect VBNC bacteria using conventional microbiological culture-based methods further accentuates the risk. Therefore, it is imperative to inactivate pathogenic bacteria in the VBNC state before they regain culturability. Due to the high antimicrobial tolerance exhibited by VBNC bacterial pathogens, current inactivation methods may be ineffective, necessitating the development of novel strategies to control these pathogens in the VBNC state.

Campylobacter jejuni is a leading cause of human gastroenteritis worldwide and is commonly found in both food production and environmental settings. However, the mechanisms by which this microaerophilic microbe survives in the aerobic environment and disseminates throughout the food supply chain remain incompletely understood. The factors involved in inducing *C. jejuni* to enter the VBNC state during food processing and the persistence of VBNC *C. jejuni* to antimicrobials are largely unknown and require further investigation. This dissertation aims to address the aforementioned knowledge gaps and enhance our understanding of the persistence of *C. jejuni* in the agri-food system, with the ultimate goal of developing more effective intervention strategies.

C. jejuni encounters various stressors that can trigger the induction of the VBNC state during food processing and within food products. Notably, when exposed to chlorine, *C. jejuni* completely lost

its ability to form colonies, but a fraction of the bacteria (1-10%) maintained their viability. In contrast, under aerobic and low temperatures conditions, ~10% of *C. jejuni* entered the VBNC state after 24 h and 20 days, respectively. Furthermore, the strain *C. jejuni* ATCC 33560 showed a higher propensity for entering the VBNC state in both refrigerated UHT and pasteurized milk compared to other strains such as F38011, NCTC 11168, and 81116. These observations of heterogeneous behavior among different strains suggest the existence of strain-specific variations in response to stressors. By conducting the time-kill assay, notable variations were observed in the response of VBNC *C. jejuni* towards plant-based antimicrobials and metal oxide nanoparticles (NPs). Specifically, the bacterium exhibited a remarkable persistence against carvacrol or diallyl sulfide, whereas it displayed susceptibility to aluminum oxide NPs. Moreover, the interactions among these antimicrobials were thoroughly investigated. The combination of carvacrol and diallyl sulfide resulted in an additive antimicrobial effect. Furthermore, synergistic effects were observed when either carvacrol or diallyl sulfide was combined with Al_2O_3 NPs. Remarkably, the ternary combination of carvacrol, diallyl sulfide, and Al_2O_3 NPs demonstrated a synergistic effect, enabling reduced concentrations of these antimicrobials to achieve effective inactivation. Subsequently, these antimicrobial treatments were employed to inactivate VBNC *C. jejuni* under poultry processing conditions. However, relatively lower effectiveness was observed, possibly attributed to the presence of lipids and proteins in chicken juice. These components have the potential to interact with antimicrobials, resulting in reduced antimicrobial availability and compromised efficacy. The interactions among the antimicrobials were first assessed using the time-kill assay, revealing additive effects in all combinations tested. To ensure accurate assessment without overestimation or underestimation, a novel mathematical model was developed to further investigate these antimicrobial interactions. The application of this computational approach

revealed that combinations of plant-based antimicrobials and Al_2O_3 NPs, whether in binary or ternary form, demonstrated synergistic effects and were effective in combating VBNC *C. jejuni*. Moreover, this high-throughput computational approach has the potential to screen and optimize the selection of antimicrobial combinations for combating VBNC *C. jejuni* effectively.

In summary, the findings of this thesis project provide valuable insights into the survival and distribution of *C. jejuni* and facilitate the development of effective intervention strategies to reduce the prevalence of this significant foodborne pathogen. In addition, this research holds significant implications for the control and prevention of foodborne illnesses caused by bacterial pathogens in the VBNC state. It emphasizes the imperative need to comprehend and tackle the challenges associated with this dormant state. Ultimately, the knowledge will make substantial contributions to the improvement of food safety and public health, thereby bringing overall benefits to society.

Résumé

De nombreuses espèces bactériennes ont la capacité d'entrer dans un état dit viable mais non cultivable (VBNC) en réponse à des conditions environnementales défavorables. Bien qu'il soit peu probable que les bactéries pathogènes provoquent des maladies dans l'état VBNC, elles peuvent redevenir virulentes lorsqu'elles sont réanimées dans des conditions favorables, ceci constitue une menace importante pour la sécurité alimentaire et la santé publique. De plus, l'impossibilité de détecter les bactéries VBNC à l'aide des méthodes microbiologiques conventionnelles basées sur la culture accentue encore le risque. Il est donc impératif d'inactiver les bactéries pathogènes à l'état de VBNC avant qu'elles ne redeviennent cultivables. En raison de la grande tolérance antimicrobienne dont font preuve les bactéries pathogènes VBNC, les méthodes d'inactivation actuelles peuvent s'avérer inefficaces, ce qui nécessite le développement de nouvelles stratégies pour contrôler ces pathogènes à l'état VBNC.

Campylobacter jejuni est l'une des principales causes de gastro-entérite chez l'Homme dans le monde entier et on le trouve couramment dans la production alimentaire et dans l'environnement. Cependant, les mécanismes par lesquels ce microbe microaérophile survit dans l'environnement aérobie et se dissémine tout au long de la chaîne d'approvisionnement alimentaire restent incompris. Les facteurs impliqués dans l'induction de *C. jejuni* à entrer dans l'état VBNC au cours de la transformation des aliments et la persistance aux antimicrobiens à l'état VBNC sont largement inconnus et nécessitent des recherches plus approfondies. Cette thèse vise à combler les lacunes de connaissances susmentionnées et à améliorer notre compréhension de la persistance de *C. jejuni* dans le système agroalimentaire, dans le but ultime de développer des stratégies d'intervention plus efficaces.

C. jejuni est confronté à divers facteurs de stress qui peuvent déclencher l'induction de l'état VBNC au cours de la transformation des aliments et à l'intérieur des produits alimentaires. Notamment, lorsqu'il est exposé au chlore, *C. jejuni* perd complètement sa capacité à former des colonies, mais une fraction des bactéries (1-10%) maintient sa viabilité. En revanche, dans des conditions aérobies et à basse température, ~10 % de *C. jejuni* sont entrés dans l'état VBNC après 24 heures et 20 jours, respectivement. En outre, la souche *C. jejuni* ATCC 33560 a montré une plus grande propension à entrer dans l'état VBNC à la fois dans le lait UHT réfrigéré et dans le lait pasteurisé par rapport à d'autres souches telles que F38011, NCTC 11168, et 81116. Ces observations de comportement hétérogène entre les différentes souches suggèrent l'existence de variations spécifiques aux souches en réponse aux facteurs de stress. En effectuant l'essai time-kill, des variations notables ont été observées dans la réponse de la VBNC *C. jejuni* aux antimicrobiens d'origine végétale et aux nanoparticules d'oxyde métallique (NPs). Plus précisément, la bactérie a montré une persistance remarquable contre le carvacrol ou le sulfure de diallyle, alors qu'elle s'est montrée sensible aux NP d'oxyde d'aluminium. En outre, les interactions entre ces antimicrobiens ont fait l'objet d'une étude approfondie. La combinaison du carvacrol et du sulfure de diallyle produit un effet antimicrobien additif. En outre, des effets synergiques ont été observés lorsque le carvacrol ou le sulfure de diallyle étaient combinés avec des NP d'Al_2O_3. Remarquable, la combinaison ternaire de carvacrol, de sulfure de diallyle et de nanoparticules d'Al_2O_3 a démontré un effet synergique, permettant de réduire les concentrations de ces antimicrobiens afin d'obtenir une inactivation efficace. Par la suite, ces traitements antimicrobiens ont été utilisés pour inactiver le VBNC *C. jejuni* dans des conditions de transformation de la volaille. Cependant, une efficacité relativement faible a été observée, ce qui est probablement attribuée à la présence de lipides et de protéines dans le jus de poulet. Ces composants peuvent interagir avec les antimicrobiens, ce qui

réduit leurs disponibilités et compromet leur efficacité. Les interactions entre les antimicrobiens ont d'abord été évaluées à l'aide du test "time-kill", qui a révélé des effets additifs dans toutes les combinaisons testées. Pour garantir une évaluation précise sans surestimation ni sous-estimation, un nouveau modèle mathématique a été mis au point pour étudier plus efficacement ces interactions antimicrobiennes. L'application de cette approche computationnelle a révélé que les combinaisons d'antimicrobiens à base de plantes et de NP d'Al_2O_3, que ce soit sous forme binaire ou ternaire, ont démontré des effets synergiques et ont été efficaces dans la lutte contre la VBNC *C. jejuni*. En outre, cette approche informatique à haut débit a le potentiel de cribler et d'optimiser la sélection des combinaisons antimicrobiennes pour lutter efficacement contre la VBNC *C. jejuni*.

En résumé, les résultats de ce projet de thèse fournissent des informations précieuses sur la survie et la distribution de *C. jejuni* et facilitent le développement de stratégies d'intervention efficaces pour réduire la prévalence de cet important agent pathogène d'origine alimentaire. En outre, cette recherche a des implications significatives pour le contrôle et la prévention des maladies d'origine alimentaire causées par des pathogènes bactériens dans l'État du VBNC. Elle souligne la nécessité impérieuse de comprendre et de relever les défis associés à cet état de dormance. En fin de compte, les connaissances acquises contribueront de manière substantielle à l'amélioration de la sécurité alimentaire et de la santé publique, apportant ainsi des avantages globaux à la société.

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ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CFU	Colony-forming unit
CI	Combination index
Ct	Cycle threshold
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DVC	Direct viable count
ECDC	European Centre for Disease Prevention and Control
EMA	Ethidium monoazide
EOs	Essential oils
EPS	Extracellular polymeric substance
FICI	Fractional inhibitory concentration index
LOD	Limit of detection
MCBT	Multiple-combination bactericidal antimicrobial testing
MH agar/broth	Mueller-Hinton agar/broth
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
NPs	Nanoparticles
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PMA	Propidium monoazide
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sRNA	Small ribonucleic acid
TA system	Toxin-antitoxin system
UHT	Ultra-high-temperature
USDA	United States Department of Agriculture
VBNC	Viable but non-culturable

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Contribution of authors

This thesis is structured in the manuscript format and consists of six chapters. Chapter 1 serves as a general introduction, outlining the hypothesis and research objectives of the thesis. In Chapter 2, a comprehensive literature review is presented, covering essential topics necessary for understanding the research. Chapters 3 to 5 are presented as individual manuscripts, arranged sequentially with interconnected text. Among these manuscripts, Chapter 3 has already been published in *Applied Environmental Microbiology*, while Chapter 4 and Chapter 5 are soon to be submitted for publication. Lastly, Chapter 6 offers a comprehensive conclusion to the thesis, highlighting the scientific contributions made and providing valuable recommendations for future research.

All chapters of this study were conducted by Jingbin Zhang, a Ph.D. candidate, under the guidance of her supervisor, Dr. Xiaonan Lu. Jingbin Zhang was responsible for conceptualizing and designing the experiments, carrying out the experimental work, collecting and analyzing the results, and drafting both the thesis and scientific manuscripts for presentation and publication. Dr. Xiaonan Lu served as the thesis supervisor, providing direct advisory support for all aspects of the experimental design, offering guidance and supervision to the Ph.D. candidate throughout the research process, and reviewing and editing the manuscripts.

Publications

Articles included in this thesis:

1. Zhang, J., Lu, X., 2023. Susceptibility of *Campylobacter jejuni* to stressors in agrifood systems and induction of viable-but-nonculturable state. *Applied and Environmental Microbiology*, 89, e00096-23. (IF: 5.005)

2. Zhang, J., Konkel, M., Gölz, G., Lu, X., 2022. *Campylobacter*-associated food safety. *Frontiers in Microbiology*, 13, 1038128. (IF: 6.064)

Articles not included in this thesis:

3. Zhang, J., Wang, Y., Lu, X., 2021. Molecular imprinting technology for sensing foodborne pathogenic bacteria. *Analytical and Bioanalytical Chemistry*, 413, 4581-4598. (IF: 4.478)

4. Chen, J., Zhang, J., Yang, Z., Niu, Y., Cai, Z., Wang, J., Yin, L., Lin, Y., Lu, X., 2022. Characterization of indigenous coagulase-negative staphylococci isolated from Chinese spontaneously fermented meat products. *Microbiological Research*, 263, 127160. (IF: 5.07)

5. Koshani, R., Zhang, J., van de Ven, T. G., Lu, X., Wang, Y., 2021. Modified hairy nanocrystalline Cellulose as photobactericidal nanofillers for food packaging application. *ACS Sustainable Chemistry & Engineering*, 9, 10513- 10523. (IF: 9.224)

6. Ma, L., Feng, J., Zhang, J., Lu, X., 2022. *Campylobacter* biofilms. *Microbiological Research*, 264, 127149. (IF: 5.07)

Conference presentations

1. Zhang, J., Lu, X., Induction of viable-but-non-culturable *Campylobacter jejuni* under different food processing conditions. International Association for Food Protection 2023 Annual Meeting, Toronto, Ontario, Canada. July 16-19. (Oral presentation)

2. Zhang, J., Lv, R., Lu, X., Optimization of a propidium monoazide-quantitative PCR method for quantification of viable-but-non-culturable *Campylobacter jejuni* in poultry products. International Association for Food Protection 2023 Annual Meeting, Toronto, Ontario, Canada. July 16-19. (Poster presentation)

3. Zhang, J., Lv, R., Lu, X., Determination of viable-but-non-culturable *Campylobacter jejuni* in chicken using quantitative PCR combined with propidium monoazide pretreatment. 2022 *Campylobacter*, *Helicobacter* and Related Organisms Conference, Yangzhou, Jiangsu, China. November 14-19. (Oral presentation)

4. Zhang, J., Lv, R., Lu, X., Detection and quantification of viable-but-non-culturable *Campylobacter jejuni* in poultry products. 2022 Canadian Food Summit, Guelph, Ontario, Canada. June 1-3, 2022. (Poster presentation)

Chapter 1. Introduction

1.1. General introduction

Campylobacter jejuni is a significant cause of foodborne gastrointestinal illnesses worldwide, commonly manifesting as acute gastroenteritis accompanied by symptoms such as fever, headache, vomiting, and bloody diarrhea (Blaser and Engberg, 2008). However, the impact of *Campylobacter* infections can extend beyond the acute phase, potentially leading to long-term complications such as Guillain-Barré syndrome and Crohn's disease (Kaakoush et al., 2015). The primary sources of human campylobacteriosis are the consumption of raw milk, undercooked poultry products, and contaminated drinking water (Silva et al., 2011). Among these sources, contaminated poultry and poultry products represent a significant proportion, accounting for ~50-70% of the reported cases (Keener et al., 2004).

Despite its strict growth requirements including microaerophilic conditions and a temperature range of 32-47°C, *C. jejuni* has demonstrated the ability to survive in various environments (Farrar et al., 2011). It can persist in atmospheric conditions and disseminate throughout the food-processing and storage environment. This paradox can be attributed to the ability of *C. jejuni* to either form biofilms or enter a viable but non-culturable (VBNC) state as a response to the adverse conditions (Farrar et al., 2011; Svensson et al., 2008). The VBNC state serves as a survival strategy employed by various bacteria to cope with environmental stresses (Li et al., 2014; Oliver, 2010). Foodborne bacteria have been identified to enter the VBNC state due to their exposure to complex environmental conditions encountered during various stages of food processing and preservation (Li et al., 2020; Oliver et al., 2005; Zhang and Lu, 2023; Zhao et al., 2013). While pathogenic bacteria in the VBNC state may not actively induce diseases, they retain their viability and possess the ability to resuscitate and regain virulence when provided with favorable conditions (Baffone

et al., 2006; Cappelier et al., 2007; Fu et al., 2020). In addition, the association of VBNC pathogenic bacteria (*e.g.*, Shiga toxigenic *Escherichia coli* and *Salmonella enterica* serotype Oranienburg) with various foodborne outbreaks underscores the significance of this dormant state in relation to food safety and public health (Asakura et al., 2002; Aurass et al., 2011; Makino et al., 2000). Moreover, conventional microbiological culture-based methods are inadequate for the detection of VBNC bacteria, further complicating their control and prevention.

In comparison to their culturable counterparts, bacteria in the VBNC state exhibit greater resistance to chemical and physical stresses as well as antimicrobials (Nowakowska and Oliver, 2013; Ramamurthy et al., 2014). Moreover, the complex composition of food matrices, including lipids and proteins, may reduce the effectiveness of antimicrobials, thereby offering protection to VBNC bacterial pathogens. This poses a substantial challenge in effectively controlling VBNC bacteria, especially in food products. In recent years, there has been a growing interest in employing combinational antimicrobial treatments as a strategy to improve antimicrobial efficacy, minimizing the risk of toxicity, and mitigating the emergence of antimicrobial resistance (Chou, 2006). The rationale behind combining antimicrobials lies in the potential synergistic effects that can lead to enhanced antimicrobial activity. In this regard, combination of essential oils and nanoparticles has emerged as a promising approach (Babapour et al., 2021; Hakeem et al., 2019; Xue et al., 2018). The synergistic antimicrobial effects observed with these combinations have the potential to contribute to the development of novel strategies for effectively controlling VBNC bacterial pathogens including *C. jejuni*.

The investigation of synergistic interactions between antimicrobials has traditionally relied on various approaches, including disk diffusion, time-kill assay, and the checkboard method (Doern, 2014). However, in recent years, the emergence of mathematical models has revolutionized the quantitative evaluation of synergy and facilitated high-throughput screening of drug combinations (Duarte and Vale, 2022; Meyer et al., 2020). By employing computational approaches, it becomes feasible to identify additional antimicrobial combinations that exhibit both synergistic effects and effectiveness against VBNC *C. jejuni*. This innovative approach holds great promise for reducing the incidence of *Campylobacter* infections by enabling the discovery of novel antimicrobial combinations capable of effectively controlling VBNC *C. jejuni*. Thus, it contributes significantly to the overarching objective of enhancing public health and ensuring food safety.

1.2. Research hypotheses

The present study was conducted with the hypotheses that:

(1) *C. jejuni* cells have the potential to enter the VBNC state when subjected to food processing conditions and within food products. This hypothesis recognizes the influence of specific factors encountered during food processing and the potential for *C. jejuni* to adapt to such conditions through VBNC state induction.

(2) Combination of plant-based antimicrobials and metal oxide nanoparticles can effectively inactivate VBNC *C. jejuni* by exerting a synergistic effect. This hypothesis builds upon the previous findings that have demonstrated the enhanced antimicrobial activity resulting from the combined application of plant-based antimicrobials and metal oxide nanoparticles against various pathogens, suggesting its potential effectiveness against VBNC *C. jejuni*.

(3) We propose the development of a mathematical model to evaluate the synergistic interactions among the aforementioned antimicrobial components in their actions against VBNC *C. jejuni* specifically under poultry processing conditions. This hypothesis recognizes the value of mathematical modeling in assessing complex interactions and optimizing the efficacy of antimicrobial treatments targeting VBNC *C. jejuni* within the poultry processing environment.

1.3. Research objectives

The primary objective of this research is to gain valuable insights into the interactions between *C. jejuni* and the agroecosystem, with a specific focus on comprehending the unique response of VBNC *C. jejuni* to different antimicrobials. The knowledge acquired from this study has the potential to significantly contribute to the development of innovative mitigation strategies that effectively control VBNC *C. jejuni* in the poultry industry and enhance food safety. Furthermore, this research holds broader implications for the management and prevention of foodborne illnesses caused by VBNC bacterial pathogens, emphasizing the critical importance of understanding and addressing the challenges associated with this dormant state. The research objectives of this study are as follows:

(1) To induce VBNC *C. jejuni* under conditions that mimic food processing, and to monitor the dynamics of both culturable and viable bacterial populations. This investigation aims to provide a comprehensive understanding of the interaction between *C. jejuni* and the food processing environment, shedding light on the factors influencing VBNC state.

(2) To investigate the efficacy of plant-based antimicrobials and metal oxide nanoparticles in inactivating VBNC *C. jejuni*. Furthermore, to explore the synergistic interactions among different

combinations of antimicrobials to assess their collective effectiveness. This research will contribute to the identification of novel antimicrobial strategies that can effectively combat VBNC *C. jejuni*.

(3) To assess the efficacy of the aforementioned antimicrobials in inactivating VBNC *C. jejuni* under simulated poultry processing conditions. Additionally, to develop a mathematical model that can elucidate the synergistic interactions among various antimicrobial combinations. This comprehensive approach aims to provide a quantitative understanding of the antimicrobial efficacy and optimize the utilization of antimicrobial combinations against VBNC *C. jejuni*.

Chapter 2. Literature review

2.1. *Campylobacter*

Campylobacter species are non-spore forming, gram-negative bacteria that belong to the family *Campylobacteraceae* (Kaakoush et al., 2015). These bacteria have a slender, spiral rod shape, measuring between 0.2 to 0.8 μm in width and 0.5 to 5 μm in length. They exhibit a distinctive corkscrew pattern of movement, which is facilitated by unipolar or bipolar flagella at one or both ends of the cell. Optimal growth conditions for *Campylobacter* include a microaerophilic environment with 10% CO_2, 5% O_2, and 85% N_2, with temperatures between 37 and 42°C (Silva et al., 2011).

Campylobacter is a significant cause of foodborne gastrointestinal illness worldwide, with the majority of cases caused by *C. jejuni* (80-90%), while *C. coli* (4%) and *C. lari* (<1%) are less commonly reported (Huang et al., 2015). These thermotolerant *Campylobacter* species are commonly found in the intestinal tracts of warm-blooded animals that are used for food production, such as poultry, cattle, sheep, and swine (Hansson et al., 2018). Although a significant number of *Campylobacter* organisms are excreted in feces, they are vulnerable to environmental stress, and the health risk usually arises from consuming undercooked poultry and dairy products or untreated drinking water (Donnison and Ross, 2014).

Campylobacteriosis is among the most commonly reported foodborne illnesses in the US, with an estimated 1.5 million cases occurring annually, despite strict control measures in the food supply chain (CDC, 2019). In Canada, the incidence of *Campylobacter* infection surpassed that of *Listeria*, *Salmonella*, and *Escherichia coli* combined (Kalmokoff et al., 2006). Similarly, the report from the European Centre for Disease Prevention and Control (ECDC) indicated that human

campylobacteriosis has been the most frequently reported zoonotic infection in Europe since 2005 (European Food Safety Authority, European Centre for Disease Prevention and Control, 2012). Moreover, the economic burden of treating campylobacteriosis is substantial, with healthcare expenses amounting to $1.56 billion in the USA, $80 million in Canada, and €2.4 billion in the European Union annually (Devleesschauwer et al., 2017). *Campylobacter* infection is usually characterized by an acute, self-limiting gastroenteritis with symptoms such as fever, inflammation, nausea, abdominal pain, and bloody diarrhea (Sahin et al., 2015). Notably, a mere 500 bacterial cells have been shown to be sufficient to initiate an infection (Robinson, 1981). Although most cases of campylobacteriosis resolve spontaneously within 2–5 days, up to 10% of patients may experience a more extended and recurrent diarrheal illness that requires medical intervention and can persist for several weeks (Galanis, 2007). Additionally, *Campylobacter* infections are closely associated with the development of immune-mediated disorders such as Guillain-Barré syndrome, which can lead to respiratory muscle weakness, flaccid paralysis, and even death (Nachamkin et al., 1998).

Campylobacter infections are typically sporadic although rare community outbreaks have been associated with the consumption of raw milk, contaminated water, and contact with pets and farm animals (Galanis, 2007). *C. jejuni* may contaminate milk via fecal contamination during milking or udder infection, making raw milk a source of human campylobacteriosis (Chon et al., 2020). However, the consumption of poultry and poultry products contaminated with *C. jejuni* is recognized as the primary cause of human infections, accounting for 50 to 70% of the cases (Keener et al., 2004). The frequent association of various serotypes of *C. jejuni* isolated from chicken carcasses with human campylobacteriosis cases confirms the important role of poultry in

the epidemiology of this disease. Poultry flocks have high levels of colonization with *Campylobacter*, ranging from 30% to 100%, with up to 9 log CFU/g present in their cecal contents (Sahin et al., 2015). Contamination can occur during the processing of poultry carcasses as a result of leaking or ruptured intestinal content, leading to introduction of this microorganism due to high contamination levels in carcass rinse (5-8 log CFU/mL) (Muhandiramlage et al., 2020). *C. jejuni* is commonly identified in the poultry products with contamination levels ranging from 2 to 5 log CFU/carcass in around 70 to 90% of commercially available raw poultry products in Europe and North America (Jacobs-Reitsma et al., 2008; Jorgensen et al., 2002; Willis and Murray, 1997).

2.2. *Campylobacter* survival strategies

C. jejuni is highly prevalent in the environment and can persist within the food supply chain, posing a significant challenge to its control in the agri-food systems. Although many foodborne bacterial pathogens are generally robust and can withstand the harsh conditions encountered during food processing and preservation, *C. jejuni* has specific growth requirements and is highly vulnerable to environmental stresses. In addition, *C. jejuni* lacks many of the well-characterized adaptive factors typically associated with stress resistance in other bacteria, including the sigma factor (RpoS) involved in stress response during stationary phase and the regulatory factors (SoxRS, OxyR) that aid in counteracting oxidative stress (Svensson et al., 2008).

Given these unique characteristics, there is a great interest in understanding how *C. jejuni* can survive in unfavorable conditions. Although the underlying mechanisms by which *C. jejuni* survives in the environment are not fully elucidated, the bacteria employ a range of strategies to

cope with environmental stress. These strategies include the activation of stress response systems, formation of bacterial biofilms, and entry into a dormant state.

2.2.1. Bacterial stress response system

Bacteria thrive in a constantly changing environment characterized by fluctuations in temperature, nutrient availability, oxygen level, and the presence of diverse chemical compounds. Over time, bacteria have developed intricate mechanisms to detect and assess these external conditions. In response, they modulate their gene expression patterns by activating a specific set of genes that enhance their survival capabilities while simultaneously suppressing genes whose products are deemed unnecessary in a given environment (Chowdhury et al., 1996). This adaptive response allows bacteria to effectively navigate and adapt to their surroundings.

In general, bacterial stress response systems can be categorized into four distinct regulatory patterns: (1) Stress response mediated by sigma factors (σ factor): Sigma factors serve as essential transcription factors capable of reversibly binding to RNA polymerase. By facilitating promoter recognition, σ factors play a crucial role in regulating gene transcription under stress conditions. Bacteria employ a large variety of sigma factors to optimize their stress response, with each species of sigma factors specifically recognizing promoter elements associated with a particular set of genes (Davis et al., 2017). For instance, σ^s (RpoS) serves as the principal regulator of the general stress response in *E. coli*. Its expression is significantly upregulated during the transition into the stationary phase and/or under various stress conditions, such as osmotic pressure and oxidative stress. Moreover, RpoS plays a crucial role in the expression of ~10% of the *E. coli* genome, thereby demonstrating its essential contribution to cellular adaptation and survival (Weber et al.,

2005). (2) Stress response mediated by DNA repressors: DNA repressors can bind to the controlling element of DNA, impeding the attachment of RNA polymerase to the promoter region and thereby preventing gene transcription. The binding affinity of the repressor to DNA is intricately regulated through the interaction between an effector molecule and the repressor protein (Govindarajan and Amster-Choder, 2019). This interaction modulates the repression of gene expression, allowing for precise control over cellular processes under different conditions. For example, CtsR serves as the primary transcriptional repressor for class III heat shock genes in *Bacillus subtilis*. When exposed to increased temperatures, the binding of McsA and McsB to CtsR induces its dissociation from DNA, enabling the expression of heat shock genes, such as *clpC*, *clpE*, and *clpP* (Schumann, 2003). (3) Stress response mediated by intracellular proteolysis. Proteolysis facilitates rapid and immediate regulation of cellular processes, particularly in stress responses where the need for prompt intervention is crucial. For example, the Clp protease system in *B. subtilis* facilitates precise control of the timing and magnitude of the heat-shock response through regulatory proteolysis. This system effectively degrades the misfolded or aggregated proteins that accumulate during stress conditions, thereby mitigating their toxicity to the cell itself (Kruger et al., 2000). (4) Stress response mediated by small RNAs. Small RNAs (sRNAs) are a class of RNA regulators characterized by their relatively short transcripts, typically ranging from ~50 to 300 nucleotides in length. Bacteria harbor a diverse array of these RNA regulators, which selectively interact with specific target mRNAs, thereby modulating their translation efficiency and/or stability (Storz et al., 2011). The expression of small RNAs plays a crucial role in facilitating cellular adaptation to environmental stress by modulating cellular metabolism. Several widely acknowledged stress response regulons in *E. coli* are known to be regulated by small RNAs. One notable example is the sRNA RyhB, which serves as a suppressor of translation for a cluster

of genes involved in iron storage and usage when the availability of iron is limited. This regulatory mechanism is of utmost significance for *E. coli* to adapt and survive under conditions of iron starvation (Masse and Gottesman, 2002).

While stress response systems exhibit a notable level of similarity across bacteria, there exist significant variations in the specific regulations of these systems among different bacterial species. For instance, the oxidative stress response in microaerophilic bacteria demonstrates a higher level of complexity compared to aerobic bacteria. In the case of *C. jejuni*, although not all well-established stress response operons have been identified, its bacterial stress response system encompasses several distinct elements. These include the heat-shock response (*dnaK*) (Thies et al., 1999), cold-shock response, oxidative response (*csrA* and *ahpC*) (Fields and Thompson, 2008; van Vliet et al., 1999), and stringent response (*spoT*) (Gaynor et al., 2005). The survival of *C. jejuni* in the agri-food processing environment is significantly affected by high oxygen levels, low temperatures, and nutrient deficiency. Consequently, *C. jejuni* relies on specific adaptive mechanisms, including oxidative stress response, cold response, and starvation response, to ensure its persistence and adaptability within the agri-food systems.

Table 2.1 Summary of key stress response genes identified in two model bacterial species but absent in *C. jejuni* (Park, 2002).

Genes	Functions	*C. jejuni*	*E. coli*	*B. subtilis*
Oxidative stress				
soxRS	Positive regulators of the response to superoxide stress	−	+	−
oxyR	Positive regulator of the response to peroxide stress	−	+	−
SodA	Manganese cofactor of superoxide dismutase in response to superoxide stress	−	+	+
katG	Catalase-peroxidase in response to peroxide stress	−	+	−
Osmotic stress				
proU or *opuC*	High-affinity osmoregulatory uptake of compatible solutes	−	+	+
osAB	Osmoregulatory trehalose synthesis	−	+	−
betAB or *gbsAB*	Osmoregulatory choline-glycine betaine synthesis pathway	−	+	+
Starvation/stationary phase				
rpoS	General stress/stationary phase sigma factor in Gram-negative bacteria	−	+	−
relA	Stringent response regulator in response to amino acid, glucose, and oxygen starvation	−	+	+
Heat and cold shock				
rpoH	Alternative sigma factor regulating the heat shock response	−	+	−
cspA	Major cold shock protein	−	+	+
Global regulation				
lrp	Global regulator of metabolism	−	+	+

2.2.1.1. Response of *C. jejuni* to oxidative stress

C. jejuni is commonly recognized as a microaerophilic microorganism with a high susceptibility to oxygen. Consequently, oxygen present in the surrounding environment and within the food processing chain constitutes a primary stressor affecting the survival of *C. jejuni* (Pokhrel et al., 2022). When bacterial cells are exposed to aerobic conditions, there is a substantial increase in the intracellular levels of reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radicals, and superoxide anion radicals (Farrar et al., 2011). This increase in ROS significantly affects the intracellular redox balance and subsequently causes damage to intercellular biomacromolecules, such as lipids, nucleic acids, and proteins (Imlay, 2013). Bacteria possess specific genes associated with oxidative stress, enabling the regulation of ROS-detoxifying enzymes, such as alkyl hydroperoxide reductase, catalase, and superoxide dismutase (Pokhrel et al., 2022). In contrast to various Gram-negative pathogenic bacteria, such as Shiga-toxigenic *E. coli* and *S. enterica*, which harbor oxidative regulators (*i.e.*, OxyR and SoxR) to thrive in oxygen-rich environments (Chiang and Schellhorn, 2012; Imlay, 2008), *C. jejuni* lacks these regulators and primarily relies on PerR (peroxide resistance regulator) typically found in Gram-positive bacteria to counteract oxidative stress (Kim et al., 2015). Moreover, *C. jejuni* harbors singular copies of genes involved in oxidative stress defense, namely *aphC, sodB*, and *katA*. Exposing *C. jejuni* to aerobic conditions significantly enhances the transcription levels of these genes. Notably, deletion of these genes renders C. *jejuni* more susceptible to atmospheric oxygen, indicating their crucial role in protecting the bacteria against oxidative stress (Oh et al., 2015).

2.2.1.2. Response of *C. jejuni* to cold stress

Refrigeration and freezing are commonly used methods for food preservation. Rapid temperature decrease triggers the synthesis of cold shock proteins in various bacteria, including *E. coli*, *B. subtilis*, and *S.* Typhimurium (Ermolenko and Makhatadze, 2002). These proteins facilitate membrane adaptation, sensing of cold signals, and modification of translation machinery, all of which contribute to bacterial growth under suboptimal temperature conditions (Ultee et al., 2019). As a thermophilic microorganism, *C. jejuni* has strict growth requirements and exhibits optimal replication within a narrow temperature range of approximately 37-42℃. Consequently, low temperatures present significant challenges for *C. jejuni*. Nevertheless, the cold shock genes, including *cspA* and its homologs, were not detected in the genomes of *C. jejuni*, which may explain its inability to thrive at temperatures below 30℃. However, metabolic activities such as oxygen consumption, catalase activity, ATP generation, and protein synthesis were still observed in *C. jejuni* even when the temperature was decreased to 4℃ (Hazeleger et al., 1998). Although the survival mechanism of *C. jejuni* under cold exposure remains unclear, transcriptomic studies conducted at refrigeration temperatures suggest that genes related to energy metabolism, motility, and chemotaxis may contribute to the adaption of this microorganism to a cold environment. Specifically, the transcription levels of genes involved in chemotaxis, flagellin biosynthesis, and flagellar motility are reduced, while genes associated with energy metabolism, particularly the tricarboxylic acid cycle, oxidative phosphorylation, glycolysis, and gluconeogenesis, are upregulated (Moen et al., 2005; Stintzi and Whitworth, 2003).

2.2.1.3. Response of *C. jejuni* to starvation

Foodborne pathogens may encounter nutrient-limited conditions throughout different stages of food processing and preservation. Starvation triggers a series of physiological adaptations in bacteria, including reduced metabolic activity, decreased growth rate, and induction of proteolysis (Shi et al., 2021). These adaptations contribute to the persistence of bacteria under different stress conditions such as heat shock, oxidative stress, and osmotic stress (Rees et al., 1995). The regulation of starvation response in several gram-negative bacteria involves sigma factor RpoS, which is associated with the stationary phase, and homologs of RelA/SpoT responsible for mediating the stringent response (Loewen et al., 1998). However, the genome of the *C. jejuni* model strain NCTC 11168 lacks both *rpoS* and the *relA* homolog, potentially contributing to the increased susceptibility of *C. jejuni* to heat shock and oxidative stress under conditions of nutrient deprivation (Kelly, 2001; Parkhill et al., 2000). In *C. jejuni*, the stringent response is primarily regulated by the well-characterized general regulator SpoT (Gaynor et al., 2005). Additionally, studies have demonstrated the importance of *ppk1*-dependent accumulation of polyphosphate (poly-P) within *C. jejuni* cells for survival during starvation, osmotic stress, and biofilm formation (Candon et al., 2007). Furthermore, *C. jejuni* has limited capacity to utilize sugars as an energy source and instead relies on amino acids, specifically aspartate, glutamate, serine, and proline, as well as organic acids for growth and energy production (Stahl et al., 2012). Peptides derived from *in vivo* sources are likely critical reservoirs of amino acids for *C. jejuni*. One of the genes involved in peptide utilization, *Cj0917*, shares homology with carbon starvation protein A (CstA) in *E. coli* and shows significant upregulation during starvation conditions, indicating its potential role in regulating the response to starvation (Rasmussen et al., 2013).

2.2.2. Biofilms

In addition to the stress response system, *C. jejuni* can also form biofilms to protect the encased bacteria from environmental stressors and therefore aid in its survival under challenging environmental conditions. Biofilms refer to clusters of single or multiple bacterial species that are attached and embedded in an extracellular polymeric substances (EPS) containing polysaccharides, proteins, lipids, and extracellular DNA (Flemming and Wingender, 2010). *C. jejuni* can form biofilms on various abiotic surfaces in water systems within animal husbandry facilities as well as in the food processing plants (Teh et al., 2014). Studies indicate that *C. jejuni* biofilm formation is promoted by low nutrient conditions and aerobic environments (Bronowski et al., 2014). For example, *C. jejuni* exhibited a faster rate of biofilm formation under aerobic conditions compared to microaerobic conditions (Reuter et al., 2010). Reeser and co-workers identified that the biofilm formation of *C. jejuni* in MHB was significantly higher than in the more nutrient-rich brucella and Bolton broths (Reeser et al., 2007).

In comparison to planktonic cells, bacteria residing within biofilms have a high stress tolerance due to changes in metabolism and the protective effects of EPS. EPS provides various advantages, including water retention, nutrient storage, and prevention of antimicrobial penetration (Karygianni et al., 2020). For instance, when exposed to temperature fluctuations, *C. jejuni* cells within biofilms were observed to exhibit longer survival times (~1 week) compared to their planktonic counterparts (Trachoo et al., 2002). Additionally, studies also indicated that biofilm cells of *C. jejuni* were relatively less susceptible to commonly used sanitizers such as tetrasodium phosphate, quaternary ammonia compounds, peracetic acid, and chlorine-based disinfectants than planktonic cells (Somers et al., 1994; Trachoo and Frank, 2002).

2.2.3. Bacterial dormancy state

Bacterial cells in suboptimal environments undergo transitions between various physiological states ranging from active growth to deep dormancy and eventual death. When entering a dormancy state, bacteria reduce their metabolic activity and cease to grow, which helps them conserve energy and cope with various forms of stresses (Rittershaus et al., 2013). Bacterial persisters and bacterial “viable but non-culturable state” (VBNC) are two related forms of dormancy. Ayrapetyan and coauthors proposed a hypothesis that these states constitute a "dormancy continuum", with VBNC cells exhibiting a deeper level of dormancy than persister cells (Ayrapetyan et al., 2018). It has been suggested that bacterial cells may first become persisters in response to short-term stress, and then enter the VBNC state if the stress persists for an extended period (Chen et al., 2021). Figure 2.1 summarizes the relationship between active cells, persister cells, and VBNC cells.

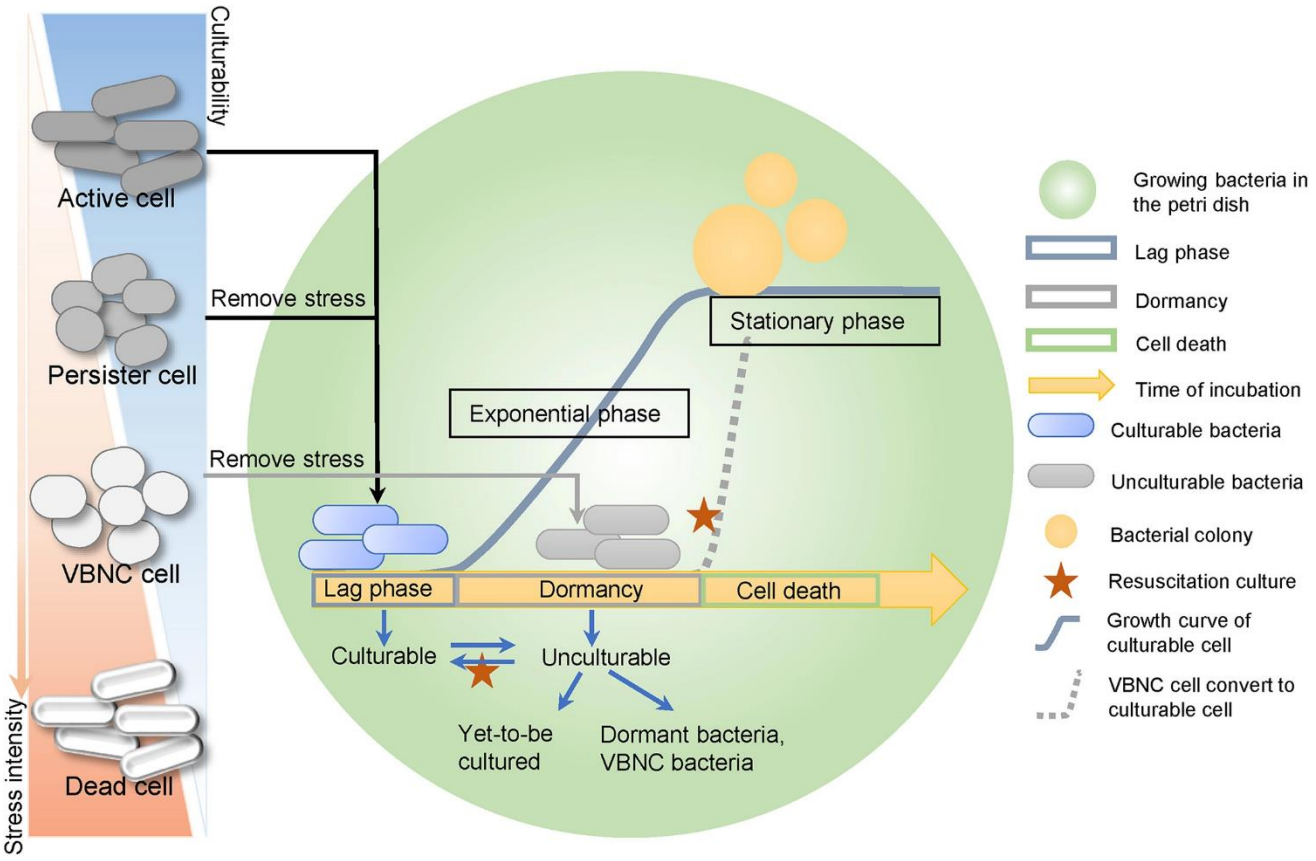

Figure 2.1 Physiological transitions of bacterial cells based on the dormancy continuum hypothesis. Environmental stresses induce changes in cellular processes, resulting in reduced cellular metabolism and growth inhibition. During the initial stage of dormancy, persisters are generated and they can enter the lag and exponential phases upon removal of the stress. However, if stress conditions persist or intensify, these cells may enter a deeper dormancy state known as the viable but non-culturable (VBNC) state. When VBNC cells are transferred to a growth medium, they cannot form colonies due to inadequate metabolic activity. Moreover, they require sufficient time and favorable conditions for resuscitation and adaptation to the cultural environment. Once resuscitated, these cells regain their capacity to grow on the media (Mu et al., 2021).

In the presence of antibiotics, some bacterial cells are capable of surviving without acquiring resistance, and they are commonly regarded as persister cells. Typically, persister cells represent a minor fraction (~1%) of the bacterial community in the stationary phase and biofilms. In contrast to antibiotic-resistant cells, which are capable of active growth even in the presence of antibiotics, persister cells remain in a dormant state and do not undergo multiplication (Wood et al., 2013). Persister cells in biofilms have been found to contribute to the persistence of chronic infections, as they remain viable even when the majority of cells are killed by antibiotics (Defraine et al., 2018). As a result, persister cells can grow and repopulate the biofilm once the antibiotic concentration decreases.

2.3. VBNC state

2.3.1. Overview of VBNC state

The VBNC state is a physiological state that bacteria can enter when subjected to environmental stresses. In this state, bacteria are unable to grow on the conventional culture media, but they maintain their metabolic activity and can resuscitate to the culturable state when favorable conditions are present (Li et al., 2014). This phenomenon was first identified in *Escherichia coli* and *Vibrio cholerae* by Colwell and colleagues in 1982 (Xu et al., 1982). Since then, researchers have identified 85 species of bacteria that can enter the VBNC state under stress conditions (Li et al., 2014).

VBNC cells exhibit distinct characteristics that differentiate them from dead cells, despite their inability to grow on the conventional culture media. VBNC cells have an intact membrane that retains both chromosomal and plasmid DNA, while dead cells have a damaged membrane that

leads to the loss of genetic information (Cook and Bolster, 2007; Heidelberg et al., 1997). In addition, VBNC cells maintain metabolic activity and are capable of respiration, with some species retaining high levels of ATP even after prolonged dormancy (Lindback et al., 2010). Unlike dead cells, VBNC cells continue to transcribe genes and produce mRNA (Lleo et al., 2000). Moreover, VBNC cells actively take up and incorporate amino acids into proteins, in contrast to dead cells that no longer utilize nutrients (Lleo et al., 1998).

Although VBNC bacterial cells share many common features with their culturable counterparts, a range of physiological changes such as cellular morphology, metabolism, stress tolerance, gene expression and potential virulence occur when bacteria transit from normal state to VBNC state (Zhao et al., 2017). When entering the VBNC state, bacterial cells maintain apparent cell integrity but exhibit dwarfing or cell rounding. These changes in cellular morphology are attributed to gene regulation and are closely linked to modifications in cell wall and membrane components, such as peptidoglycan cross linking, lipoprotein, glycan strands, and fatty acids (Day and Oliver, 2004; Linder and Oliver, 1989; Signoretto et al., 2002; Signoretto et al., 2000). For example, after incubation at 4°C for 60 days, *V. cholerae* transformed into coccoid cells in an aquatic microcosm (Chaiyanan et al., 2007). In another study, Zeng and coworkers discovered that the morphology of VBNC *Salmonella* Typhi changed from rod shape to spherical shape (Zeng et al., 2013). Except for morphological changes, the respiration rate, nutrient transport, and macromolecular synthesis of bacteria decreased greatly in the VBNC state (Zhao et al., 2016). Due to these changes, VBNC bacterial cells have greater resistance to chemical stresses, physical stresses, and antimicrobials than their culturable counterparts (Nowakowska and Oliver, 2013; Ramamurthy et al., 2014; Signoretto et al., 2000).

2.3.2. The mechanisms of VBNC formation

The formation mechanisms of the VBNC state have not been fully elucidated. Currently, three possible mechanisms have been proposed, including the stringent response, the general stress response systems and the toxin-antitoxin (TA) systems (Figure 2.2) (Cai et al., 2022).

Bacteria activate stringent response when experiencing a shortage of amino acids, resulting in decreased growth and increased amino acid synthesis. This response is initiated by the production of guanosine tetraphosphate and guanosine pentaphosphate [collectively known as (p)ppGpp], which act as alarmone molecules that inhibit energy-consuming cellular processes such as transcription, translation, and cell division (Traxler et al., 2008). Numerous studies have provided evidence for the involvement of the stringent response in the induction of the VBNC state. For instance, Boaretti and coworkers demonstrated that *E. coli* mutants deficient in ppGpp production exhibited an accelerated induction of the VBNC state (Boaretti et al., 2003). Furthermore, the expression of *relA* gene was found to be significantly up-regulated in *V. cholerae* O1 when it entered the VBNC state. As part of the stringent response signaling pathway, the enzyme RelA facilitates the synthesis of ppGpp, leading to its accumulation within the cell, which subsequently inhibits the synthesis of DNA, RNA, and proteins and result in growth arrest of bacterial cells (Mishra et al., 2012).

The induction of VBNC state in bacteria is also influenced by the general stress response system, which involves the regulation of various genes and pathways. Two key regulators within this system are RNA polymerase sigma S (RpoS) and LysR-type transcriptional regulator (OxyR), which are involved in stress regulation and oxidative stress response, respectively (Dong et al.,

2020). In response to oxidative stress caused by reactive oxygen species, OxyR can regulate the expression of various antioxidant genes such as alkyl hydroperoxide reductase subunit C (*ahpC*), glutathione *S*-transferase (*GST*), catalase *katA* and *katG* and superoxide dismutase (*sodA*). The presence of RpoS in bacterial cells enhances their adaptability to the environment, thereby impeding the formation of the VBNC state. Accordingly, depletion of *rpoS* in *E. coli* and *Salmonella* resulted in a more rapid induction of the VBNC state (Boaretti et al., 2003; Kusumoto et al., 2012). In addition, the transcription and translation of *rpoS* is positively regulated by (p)ppGpp, and the elevated level of (p)ppGpp will lead to a several-fold increase in the amount of RpoS (Magnusson et al., 2005).

It has been recently reported that the toxin-antitoxin (TA) systems are crucial in inducing the VBNC state (Gupta et al., 2016; Zhang et al., 2020). In brief, the expression of toxins in the TA systems inhibits bacterial growth, leading to the entry of bacteria into the VBNC state. The TA systems typically consist of two fundamental elements: a toxin that interferes with an essential cellular process and an antitoxin that counteracts the action of its corresponding toxin (Jurenas et al., 2022). However, under conditions of environmental stress such as amino acid or carbon source deprivation, the antitoxins are degraded by specific proteases (*e.g.*, Lon, ClpXP, or ClpAP) (Brzozowska and Zielenkiewicz, 2013). As a result, the released toxins affect various cellular processes, including DNA replication, mRNA stability and protein synthesis, leading to rapid growth arrest, VBNC formation, and even cell death (Yamaguchi and Inouye, 2011; Yamaguchi et al., 2011).

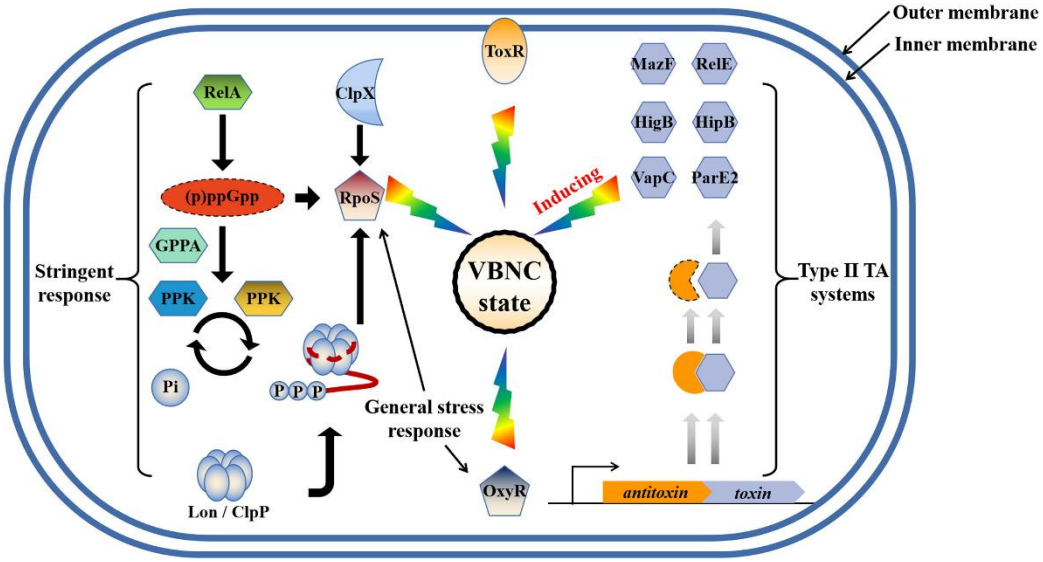

Figure 2.2 Formation of viable but nonculturable (VBNC) bacteria involves three primary pathways: the stringent response, the general stress response system, and the toxin-antitoxin (TA) system. The stringent response pathway regulates VBNC bacteria formation by modulating the activity of the alarmone molecule (p)ppGpp, which is under the regulation of the protein RelA. Various toxins present in type II TA systems including MazF, RelE, HigB, HipB, VapC, and ParE2 can induce the VBNC state in bacteria. Furthermore, the TA systems themselves are regulated by the stringent response pathway due to the phosphohydrolase activity of polyphosphokinase (PPK) and phosphohydrolase (GPPA), which leads to the generation of ppGpp and subsequent accumulation of PolyP. As a result, Lon protease/ClpP protease are activated, facilitating the degradation of antitoxins and promoting the transition of bacteria into the VBNC state. Moreover, the general stress response system components, including RNA polymerase σS (RpoS) and LysR transcription regulator (OxyR), play significant roles in VBNC bacterial formation. Similarly, RpoS is regulated by the stringent response pathway and ClpX protease. Furthermore, the inner membrane-associated toxin transcription activator (ToxR) is involved in inducing the VBNC state, potentially serving as a responder to the environmental signals (Cai et al., 2022).

2.3.3. Induction of VBNC state

Bacteria can enter the VBNC state in response to stressors commonly found in agri-food systems. The presence of VBNC bacteria has been identified in a diverse range of food products, including fresh produce, fruit juice, dairy products, poultry, meat, and seafood (Anvarian et al., 2018; Cao et al., 2019; Highmore et al., 2018; Lindback et al., 2010; Lv et al., 2021; Nicolo et al., 2011; Purevdorj-Gage et al., 2018). Numerous physical and chemical factors during food processing and storage can trigger foodborne pathogens to enter the VBNC state. Physical factors, such as low/high temperature, drying, irradiation, oxidative stress, and starvation have been shown to induce the VBNC state in bacteria (Barron and Forsythe, 2007; Dinu and Bach, 2013; Lothigius et al., 2010; Oh et al., 2015; Zhang et al., 2015). Similarly, chemical factors including food preservatives, disinfectants, osmotic pressure, and low/high pH, also induce the VBNC state (Arvaniti et al., 2021; Cunningham et al., 2009; Lv et al., 2019). The classical study of VBNC induction is shown in Figure 2.3.

To investigate the effect of these stress factors on inducing VBNC bacteria, numerous simulation studies have been conducted. For instance, low-temperature conditions have been validated to induce major foodborne pathogens such as *E. coli* O157:H7, *C. jejuni*, *L. monocytogenes*, and *Staphylococcus aureus* into the VBNC state (Giao and Keevil, 2014; Masmoudi et al., 2010; Patrone et al., 2013). *C. jejuni* and *V. vulnificus* have also been confirmed to enter the VBNC state under oxidative stress (Abe et al., 2006; Oh et al., 2015). Moreover, UV disinfection can induce *E. coli* O157:H7 to enter the VBNC state (Zhang et al., 2015). Chemical stress factors such as low salinity (0.04%) and sublethal salinity (0.12%) could also induce bacteria to enter the VBNC state. For example, NaCl was used by Wong and colleagues to induce *V. vulnificus* into the VBNC state

(Wong and Liu, 2008). Additionally, Cunningham and coworkers reported that *L. monocytogenes* cells grown in the presence of potassium sorbate at pH 4.0 entered the VBNC state within 24 h (Cunningham et al., 2009). Furthermore, it has been observed that excessive use of disinfectants induces pathogenic bacteria such as *L. monocytogenes* to enter the VBNC state (Arvaniti et al., 2021).

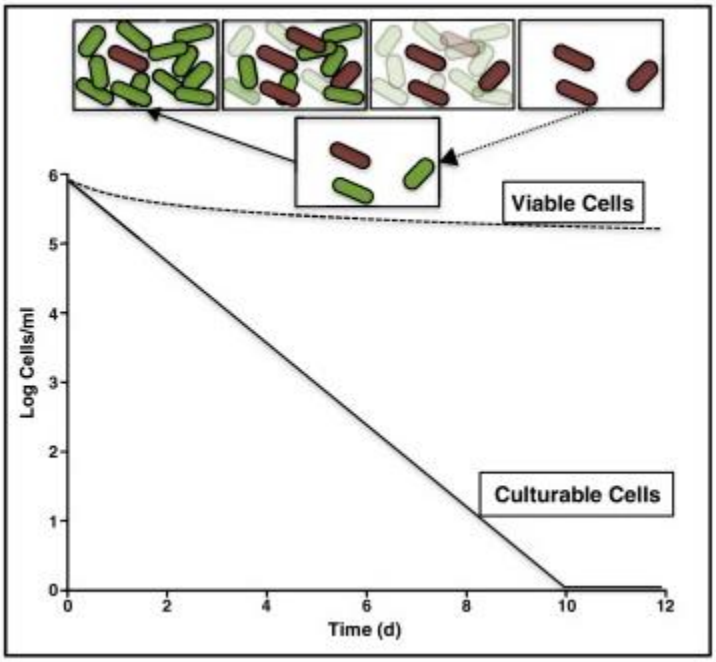

Figure 2.3 Classical study of viable but non-culturable (VBNC) induction. The process of inducing VBNC cells (depicted in red) involves the incubation of a cell suspension (depicted in green) under conditions of environmental stresses, such as low temperature and starvation. As time progresses, the cells gradually become undetectable on nutrient media (represented by the solid line), while a considerable proportion of the population still maintains viable (represented by a dashed curve). Subsequent to the removal of the inducing stress, the population undergoes a resuscitation period (indicated by the broken arrow), leading to the generation of resuscitated cells that exhibits a comparable level of tolerance to the stress conditions as the original population (as depicted by the unbroken arrow) (Ayrapetyan and Oliver, 2016).

2.3.4. Potential food safety risks associated with VBNC pathogens

While the ability to enter into a VBNC state can be advantageous for the survival of bacteria, it also poses potential threats to food safety and public health. This is because it cannot be detected by the conventional culture-based methods, yet it retains the capability of producing virulence factors and has the potential to resuscitate under suitable conditions (Ayrapetyan and Oliver, 2016). Many virulence genes can be expressed normally in the VBNC state of various pathogens including *E. coli*, *L. monocytogenes*, *S.* Thompson, and *Legionella pneumophila* (Alleron et al., 2013; Highmore et al., 2018; Liu et al., 2010). For instance, Dinu and Bach discovered that toxin genes (*hly*, *stx1*, and *stx2*) were stably expressed in VBNC *E. coli* O157:H7, resulting in the production of enterotoxins and indicating the potential for virulence (Dinu and Bach, 2011).

In addition, VBNC cells have the potential to regain their pathogenicity and initiate disease by resuscitating under favorable conditions. For example, VBNC *L. monocytogenes* was successfully resuscitated and regained virulence identical to that of the culturable cells after incubation in embryonated eggs (Cappelier et al., 2007). In fact, several foodborne outbreaks have been associated with VBNC pathogenic bacteria such as *E. coli* and *S.* Oranienburg (Asakura et al., 2002; Aurass et al., 2011; Makino et al., 2000). Moreover, VBNC cells have the capacity to survive in harsh environments encountered during food processing and exposure to different antimicrobials. This could potentially trigger a relapse of the disease in patients previously considered to be cured.

Even after being induced into the VBNC state, *C. jejuni* exhibits a reduced but detectable expression of its virulence-associated genes, including *flaA*, *flaB*, *cadF*, *ciaB*, *cdtA*, *cdtB*, and *cdtC*.

Despite this, these VBNC cells can still invade human intestinal epithelial cells, as demonstrated by Chaisowwong and coworkers (Chaisowwong et al., 2012). As a result, it is important to identify the presence of VBNC *C. jejuni* in food products.

2.3.5. Detection methods of VBNC cells

The inability to detect pathogens in the VBNC state with reliable techniques can increase the risk of contaminated food and water, leading to potential health hazards. It is therefore critical to employ appropriate methods to detect VBNC bacteria and assess their viability. Bacteria in the VBNC state typically exhibit two distinct features. Specifically, they have metabolic activity but do not generate colonies on the conventional culture media. Since the conventional microbiological detection methods are ineffective in detecting VBNC cells due to their nonculturable nature, alternative detection approaches have been developed primarily focusing on assessing their viability.

Methods used for detecting VBNC bacterial cells can be categorized into two main groups. The first category consists of methods that directly detect viable cells using microscopic enumeration and staining procedures. These methods include the direct viable count (DVC) based on substrate absorption ability (Kogure et al., 1979) as well as respiration detection methods that rely on the electron transport system. Two commonly used respiration detection assays are the 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) assay and the *p*-iodonitrotetrazolium violet (INT) assay (Winding et al., 1994). Another method in this category is the LIVE/DEAD BacLight^{TM} assay, which assesses cytoplasmic membrane integrity using two fluorescent stains (*i.e.*, SYTO 9 and propidium iodide) (Cunningham et al., 2009). In recent years, stained cells have been analyzed

using fluorescence microscopy or flow cytometry (FCM), which are superior to DVC or CTC staining methods and serve as powerful tools for rapidly analyzing bacterial populations on a cell-by-cell basis (Zhao et al., 2017).

While staining methods are commonly used, recent progress in molecular biology has made molecular diagnostic approaches a more effective choice for assessing bacterial viability. The approaches within the second category allow for selective detection of gene expression or amplification, including techniques such as reverse transcription polymerase chain reaction (RT-PCR), quantitative PCR (qPCR), and loop-mediated isothermal amplification (LAMP). However, direct use of PCR for differentiation of viable and dead bacterial cells is not possible as DNA extracted from both can be amplified by PCR. To overcome this issue, bacterial samples can be treated with DNA intercalating dyes, such as propidium monoazide (PMA) and ethidium monoazide (EMA), prior to PCR analysis. These dyes can only penetrate bacterial cells with compromised cell membrane and covalently bind to double-stranded DNA upon photoactivation. As the amplification signal from dead cells is blocked, only DNA from viable cells can be amplified during the subsequent qPCR process, enabling quantification of VBNC cells. This method has been widely used to detect and quantify various VBNC cells such as *E. coli*, *S. aureus*, and *S. enterica* (Fu et al., 2020; Li et al., 2020; Zhang et al., 2023). For example, Dinu and Bach successfully detected VBNC cells of *E. coli* O157:H7 on the surface of lettuce and spinach plants using PMA-qPCR, with a detection limit of 3 log CFU/g (Dinu and Bach, 2013).

Recent advancements in detection methods have provided novel techniques for detecting VBNC cells within bacterial populations. Several studies have demonstrated the effectiveness of

bacteriophages in this regard (Ben Said et al., 2010; Fernandes et al., 2014). Another emerging detection method utilizes biosensors to convert biological molecules into measurable electric or optical signals. Additionally, since VBNC bacteria retain metabolic activity and continue to produce ATP, the generation of ATP has also been employed as another indicator in the detection of VBNC bacteria (Robben et al., 2019). A comparison between different detection methods is presented in Figure 2.4.

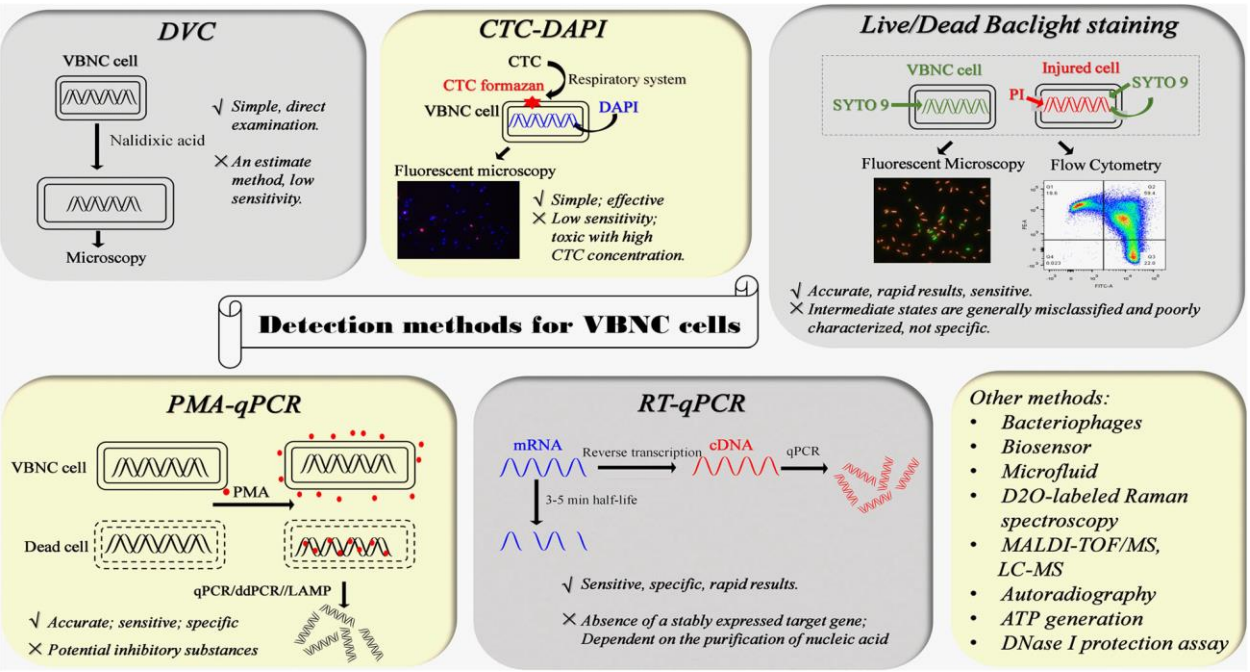

Figure 2.4 Schematics of the major detection methods for VBNC bacterial cells (Dong et al., 2020).

2.4. Alternative strategies to control *C. jejuni* in agri-food systems

2.4.1. Current situation of *C. jejuni* persistence

The prevalence of antimicrobial resistant *Campylobacter* in animals and foods has raised significant concerns in recent years (Možina et al., 2011). Numerous studies have identified two primary factors contributing to the increasing resistance of *Campylobacter* strains against

antibiotics (Maćkiw et al., 2012). Firstly, the frequent and inappropriate use of antibiotics in the treatment of human infections, particularly in cases where the infections are self-limiting, has been identified as a significant cause. Secondly, the excessive administration of these compounds in veterinary medicine for infection prevention and treatment has emerged as another significant factor. Notably, the administration of antibiotics in poultry to serve as the primary reservoir for *Campylobacter* can facilitate the selection of resistant strains and their subsequent transmission to humans via contaminated food products.

While culturable cells develop antibiotic resistance through specific resistance genes, the resistance of VBNC cells is primarily attributed to a non-specific stress response that slows down metabolic activity (Lin et al., 2017). Numerous studies have demonstrated the high resistance of VBNC cells to multiple antimicrobials, as observed in pathogens such as *Enterococcus faecalis*, *Helicobacter pylori*, and *Haemophilus influenzae* (Lleo et al., 2007; Oliver, 2010). Similarly, in another study conducted by Robben and coworkers, bacteria in the VBNC state (*E. coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *L. monocytogenes*) showed resistance to all tested antibiotics (*i.e.*, ampicillin, imipenem, ciprofloxacin, and gentamicin). Furthermore, these VBNC pathogens were also insensitive to disinfectants (*e.g.*, benzalkonium chloride and trioctylmethylammonium chloride) and preservatives (*e.g.*, bronopol and sodium azide) (Robben et al., 2019). These findings emphasize the inadequacy of current methods for inactivating VBNC bacterial pathogens due to their strong antimicrobial tolerance. Thus, it is crucial to develop innovative strategies to effectively address the challenges posed by bacterial pathogens in the VBNC state. Additionally, the understanding of the persistence of VBNC *C. jejuni* in the presence of antimicrobials remains largely unknown and necessitates further investigation.

2.4.2. Plant-based antimicrobials

Plant-derived compounds have been widely used in the food and pharmaceutical industry as they harbor a broad spectrum of biological activities, such as antibacterial, antiviral, and antioxidant properties (Chouhan et al., 2017). Essential oils (EOs) are volatile secondary metabolites synthesized by aromatic plants in response to microbial infections and can be obtained through distillation from various plant parts (*e.g.*, leaves, buds, fruits, flowers, etc.) (Bassole and Juliani, 2012). These oils comprise a diverse array of chemical compounds, typically ranging from around 20 to 80 different constituents. Notably, the major constituents responsible for antimicrobial activity are terpenes, terpenoids, and phenols (Burt, 2004). Numerous studies have demonstrated the high efficacy of essential oils in low concentrations in inhibiting bacterial pathogens commonly found in food products (Oussalah et al., 2006; Oussalah et al., 2007). The use of essential oils is of particular significance due to the growing public concern regarding the use of antibiotics in livestock, driven by the emergence of antibiotic-resistant bacteria and the potential transmission of such bacterial strains from livestock to humans. Therefore, essential oils emerge as promising antimicrobial candidates in the food industry, offering a potential alternative to traditional antibiotics.

Carvacrol ($C_{10}H_{14}O$) constitutes a major component of essential oils derived from oregano, thyme, marjoram, and summer savory (Knowles et al., 2005). Its use as a preservative in a wide range of foods (*e.g.*, rice, grape tomatoes, apple juice, and fresh-cut kiwifruit) has been well-documented (Kisko and Roller, 2005; Lu and Wu, 2010; Roller and Seedhar, 2002; Ultee et al., 2000). This monoterpenoid phenolic compound possesses a broad-spectrum antimicrobial effect and exhibits remarkable efficacy against major foodborne pathogens such as *B. cereus*, *S. aureus*, *S.*

Typhimurium, *E. coli*, and *L. monocytogenes* (Knowles et al., 2005; Perez-Conesa et al., 2011; Ultee et al., 2000). The antimicrobial efficacy of carvacrol can be attributed primarily to the presence of a free hydroxyl functional group and its specific molecular configuration (Ben Arfa et al., 2006). Disruption of cell membrane is the most commonly reported mechanism of action, resulting in the leakage of intracellular contents. Additionally, carvacrol has been proposed to inhibit efflux pumps involved in antibiotic resistance, disturb ATP-mediated cellular activities, and interfere with protein synthesis and quorum sensing (Kachur and Suntres, 2020).

Diallyl sulfide ($C_6H_{10}S$) is a naturally occurring organosulfur compound primarily derived from allium plant species (Suman and Shukla, 2016). It is highly reactive with the free sulfhydryl groups present in proteins, leading to the formation of disulfide bonds. This bonding process results in protein denaturation, enzyme inactivation and subsequent bacterial death. Furthermore, diallyl sulfide can interact with bacterial cell membrane, compromising its integrity and resulting in the leakage of cellular contents (Bhatwalkar et al., 2021). Numerous studies have documented the robust antimicrobial properties of diallyl sulfide. For instance, it effectively inhibited both the planktonic and sessile cells of *C. jejuni* (Lu et al., 2012). Moreover, diallyl sulfide exhibited strong bactericidal effects against various microorganisms, such as *Klebsiella pneumoniae*, *H. pylori*, *E. coli*, *L. monocytogenes*, and methicillin-resistant *S. aureus* (Chen et al., 1999; Lu et al., 2011; O'Gara et al., 2000; Tsao et al., 2003).

2.4.3. Metal oxide nanoparticles

Metal oxides, particularly in their nano-forms, have gained significant attention as antibacterial agents in diverse fields, such as agriculture and environmental protection (Stanić and Tanasković,

2020). The antimicrobial activities of zinc oxide, titanium dioxide, copper oxide, and other metal oxide nanoparticles have recently been extensively investigated (Mendes et al., 2022; Naseem and Durrani, 2021). Among these, aluminum oxide nanoparticles (Al_2O_3 NPs) have emerged as a promising candidate due to their inherent inertness and low cytotoxicity (Radziun et al., 2011; Sliwinska et al., 2015). Moreover, their distinctive electrical, chemical, and physical properties render them commercially viable and of particular interest for antimicrobial investigations (Mukherjee et al., 2011). In fact, Al_2O_3 NPs have demonstrated effective inhibition against various bacterial pathogens, including *C. jejuni*, *E. coli*, and *S. aureus* (Gudkov et al., 2022; Xue et al., 2018). The antimicrobial effect of Al_2O_3 NPs primarily arises from their electrostatic interaction with bacterial outer membrane/cell wall and aggregation of Al_2O_3 NPs on bacterial cell surface. Furthermore, the generation of aluminum cations triggers the production of reactive oxygen species, resulting in oxidation of biopolymers and ultimately leading to bacterial cell death (Gudkov et al., 2022).

2.5. Synergism

2.5.1. Overview of synergism

The application of antimicrobial combinations has played a pivotal role in treating diseases and alleviating human suffering since ancient times, exemplified by the use of traditional Chinese herbal medicine for therapeutic purposes. The escalating incidence of antibiotic resistance in recent years has prompted scientists to develop alternative therapeutic strategies. Furthermore, it has been observed that bacteria exhibit increased tolerance to antimicrobial agents and stress while in the VBNC state. In light of these challenges, combination antimicrobial treatments present a

promising approach to enhance antimicrobial efficacy, minimize side effects, and impede the development of antimicrobial resistance (Chou, 2006).

The inherent complexity of biological systems and dose-effect models has led to the development of numerous models, approaches, hypotheses, and theories in the field of synergism analysis over the past century. It is crucial to differentiate between synergistic effects and additive effects. Synergy refers to the phenomenon wherein the combined effect of two or more antimicrobial agents exceeds the sum of their individual effects. On the other hand, an additive effect refers to the cumulative effect of individual agents, where each antimicrobial agent does not affect the others (Figure 2.5). It is important to note that the combined effect of antimicrobial agents A and B being higher than each of their individual effects (*i.e.*, $A + B > A$ or $A + B > B$) does not provide evidence or indication of synergism. Moreover, it is incorrect to assume that the additive effect of two antimicrobial agents is simply the arithmetic sum of their effects. For example, if antimicrobials A and B exhibit inhibitory effects of 60% and 80% respectively, at certain concentrations, it would be incorrect to state that their additive effect at these concentrations is 140%. Determination of additive effect necessitates the use of more intricate mathematical algorithm models, as it involves factors beyond simple summation (Chou, 2006).

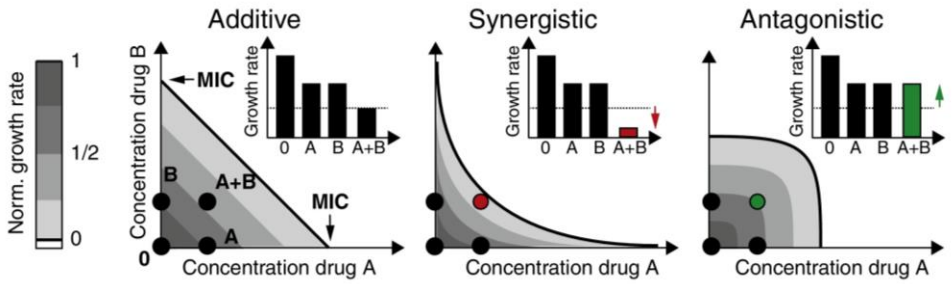

Figure 2.5 Drug interactions are characterized by the configuration of lines representing equal effect in the concentration space of a two-drug combination. Illustrations present the growth rate (represented by grayscale) and minimal inhibitory concentration (MIC) (represented by a black line indicating zero growth) within the two-dimensional concentration space of drugs A and B. The additive reference is determined by linearly interpolating the MICs of the individual drugs. Synergistic and antagonistic drug interactions are identified when the MIC line lies below or above this additive reference, respectively. Insets within the diagrams provide additional information on the growth rates under different conditions, such as the absence of drugs ('0'), fixed concentrations of drugs A and B individually, and their combined effect ('A+B'). A dashed horizontal line is included as an inset to indicate the expected additive effect (Bollenbach, 2015).

2.5.2. Synergy testing method

Synergy testing employs various susceptibility testing techniques to assess the combined effects of antimicrobials and evaluate their collective efficacy. Several *in vitro* methods have been utilized to investigate antimicrobial synergism, including disk diffusion, time-kill curve assay, and checkboard method (Doern, 2014). Disk diffusion method is a visually straightforward test that involves comparing inhibition zones of bacterial growth resulting from the diffusion of single and combined antimicrobial agents on the agar plates (Zhou et al., 2016). The time-kill curve assay examines the response of a bacterial population to antimicrobial treatment at different time intervals. Synergistic action in the time-kill assay is determined by observing a reduction of ≥2 log CFU/mL in bacterial growth in combination compared to the most potent single agent (Laishram et al., 2017). The checkerboard assay constructs a two-dimensional array of two antimicrobial combinations in serial 2-fold dilutions, either in microbroth or macrobroth. The minimum inhibitory concentrations (MIC) of single and combined treatments are compared to calculate the fractional inhibitory concentration index (FICI). Synergy is indicated by a four-fold or greater reduction in the MIC of both antimicrobials combined (*i.e.*, an FICI value of ≤ 0.5) (Odds, 2003).

However, conventional methods of synergy testing that rely on growth inhibition, such as disk diffusion and checkboard assay, are not suitable for testing VBNC bacteria. This is due to the fact that VBNC bacteria are in a dormant state and no longer capable of growing on the agar plates or in the broth. To address this limitation, several mathematical models have been developed to facilitate the quantitative evaluation of synergy and enable high-throughput screening of drug combinations.

The combination index (CI) model is derived from Loewe additivity and offers a robust approach that has gained prominence in synergy testing (Duarte and Vale, 2022). This model evaluates drug interactions by calculating the CI value, considering doses and individual effects. A CI value < 1 indicates synergy, while a value = 1 suggests additivity, and a value >1 indicates antagonism (Chou, 2006). Notably, the CI model enables accurate quantification of synergistic effects, providing a reliable method to interpret the outcomes of drug combinations. Its application extends to scenarios where multiple drugs with varying potencies are being considered, offering insights into the optimal dosage ratios.

While the CI model takes a prominent role in synergy assessment, other mathematical models also contribute to the field. Isobolographic analysis presents an alternative approach by providing a graphical depiction of dose-response relationships. While visually insightful, its interpretative subjectivity and limited precision for complex interactions can be challenging. In contrast, the Bliss independence model predicts expected effects by assuming no interaction between drugs, thereby facilitating high-throughput analysis (Greco et al., 1995). However, its assumption of equal interaction strengths and absence of dose-response information limit its accuracy in characterizing intricate synergistic patterns. As an advancement, the Zero interaction potency (ZIP) model amalgamates the principles of Loewe additivity and Bliss independence, enabling a comprehensive synergy assessment (Yadav et al., 2015). It offers quantification of the interaction strength but may not fully account for individual drug effects, potentially oversimplifying complex scenarios. The Highest single agent (HSA) model assesses combination effects relative to the most potent single agent, accommodating varying potencies. However, it solely focuses on potency comparison and disregards dose-response relationships. Further expanding the spectrum, surface

and three-dimensional response analysis provide a multidimensional view of drug interactions, presenting a comprehensive perspective (Ma and Motsinger-Reif, 2019). However, they necessitate sophisticated computational tools and may pose interpretation challenges.

In conclusion, while various mathematical models contribute insights into antimicrobial synergy, the combination index model's quantitative rigor and dose-response consideration make it a powerful tool for precise interpretation. By leveraging the strengths of the CI model and acknowledging the merits and limitations of other approaches, researchers can gain deeper insights into optimal combination therapies.

Connecting text

In the previous chapter, a comprehensive review of the relevant literature was conducted, providing an extensive overview of *C. jejuni*, including its characteristics, prevalence, and the challenges that it poses to food safety in agri-food systems. Additionally, the bacterial stress response system and the concept of the VBNC state were discussed in details. This review also explored various alternative strategies that have been proposed to control and mitigate the risks associated with *C. jejuni* contamination.

Chapter 3 focused on investigating the mechanisms by which food processing practices can induce *C. jejuni* to enter the VBNC state. The chapter examined how factors such as chlorine, aerobic stress and low temperature encountered during food processing can impact the viability and culturability of *C. jejuni*. The potential role of different dairy products in inducing the VBNC state in *C. jejuni* was thoroughly investigated.

The main objective of Chapter 3 was to advance our understanding of the complex interaction between *C. jejuni* and the agro-ecosystem. By elucidating the specific conditions that triggered the transition of *C. jejuni* to the VBNC state within the context of food processing, this research aimed to provide valuable insights into the persistence and survival of *C. jejuni* in agri-food systems. These findings can contribute to the development of innovative intervention strategies and control measures to mitigate the food safety risks associated with this pathogenic bacterium. Chapter 3 has been published in *Applied Environmental Microbiology*: Zhang, J., Lu, X., 2023. Susceptibility of *Campylobacter jejuni* to stressors in agrifood systems and induction of viable-but-nonculturable state. *Applied and Environmental Microbiology*, 89, e00096-23.

Chapter 3. Susceptibility of *Campylobacter jejuni* to stressors in agri-food systems and induction of viable-but-non-culturable state

3.1. Abstract

Many bacteria can become viable-but-non-culturable (VBNC) in response to stressors commonly identified in agri-food systems. *Campylobacter* is able to enter the VBNC state to evade unfavorable environmental conditions, but how food processing can induce *C. jejuni* to enter the VBNC state and the potential role of foods in inducing VBNC *C. jejuni* remains largely unknown. In this study, culturability and viability of *C. jejuni* were investigated under chlorine treatment (25 ppm), aerobic stress (atmospheric condition), and low temperature (4°C) conditions that mimic food processing. In addition, the behaviors of *C. jejuni* in ultra-high-temperature (UHT) and pasteurized milk were also monitored during refrigerated storage. The number of viable and culturable *C. jejuni* in both pure bacterial culture and food matrices was separately determined by propidium monoazide (PMA)-quantitative PCR and the plating assay. *C. jejuni* lost its culturability but partially remained its viability (1-10%) once mixed with chlorine. In comparison, ~10% *C. jejuni* was induced to the VBNC state after 24 h and 20 days under aerobic and low temperature condition, respectively. Viability of *C. jejuni* remained stable during induction process in UHT (>10%) and pasteurized (>10%) milk. The number of culturable *C. jejuni* decreased quickly in pasteurized milk, but culturable cells could still be detected in the end (day 21). In contrast, culturable *C. jejuni* slowly decreased and became undetectable after >42 days in UHT milk. *C. jejuni* responded differently to various stress conditions and survived in high numbers in the VBNC state in agri-food systems.

Importance

VBNC state of pathogens can pose risks to food safety and public health because they cannot be detected by the conventional microbiological culture-based methods, but can resuscitate under

favorable conditions to develop virulence. As a leading cause of human gastroenteritis worldwide, *C. jejuni* can enter the VBNC state to survive in the environment and food processing chain with high prevalence. In this study, the effect of food processing conditions and food products on the progress of VBNC *C. jejuni* formation was investigated, providing a better understanding on the interaction between *C. jejuni* and the agro-ecosystem. The knowledge elicited from this study can aid in developing novel intervention strategies to reduce food safety risks associated with this microbe.

Keywords

Campylobacter, viable-but-non-culturable, induction, survival, food products

3.2. Introduction

Many bacteria can enter a viable-but-non-culturable (VBNC) state to combat adverse environmental conditions, such as nutrient starvation, osmotic stress, and shifts in temperature and pH (1, 2). VBNC bacteria fail to form colonies on the conventional culture media, but they maintain membrane integrity and low metabolic activity (2). Although pathogenic bacteria are unlikely to cause diseases in the VBNC state, they are potentially virulent after resuscitation under favorable conditions, posing a threat to food safety and public health (3-5). The risk is particularly accentuated considering that VBNC bacteria cannot be detected by the conventional microbiological culture-based methods.

Foods are frequently exposed to complex environmental conditions before, during and after processing and preservation, thus providing stress to induce bacteria to enter the VBNC state. Several major foodborne pathogens have been reported to enter the VBNC state under the conditions that mimic food processing. For example, non-thermal processing such as high-pressure CO_2 could induce *Escherichia coli* to enter the VBNC state (6). In addition, *E. coli* and *Salmonella enterica* serotype Typhimurium were reported to survive in the VBNC state after chlorination treatment of drinking water (7). *Staphylococcus aureus* was able to enter the VBNC state under strong acid conditions along with adequate nutrients, which is similar to the condition in foods with acidic additives (8).

Campylobacter is the major cause of foodborne bacterial gastroenteritis in developed countries (9). Consumption of raw milk, undercooked poultry products, and contaminated drinking water has been identified as the potential sources of human campylobacteriosis (10). Although

Campylobacter infections typically cause self-limiting human gastroenteritis including diarrhea, fever, and abdominal cramps, it can also trigger prolonged postinfectious complications such as Guillain-Barré syndrome, Crohn's disease and septicemia in the immunocompromised individuals (11). According to the Centers for Disease Control, *Campylobacter* genus [mainly *C. jejuni* (> 85%) and *C. coli* (5-10%)] causes an estimated 1.5 million cases of foodborne diseases annually (12, 13), accounting for 5% deaths and 15% hospitalizations due to common foodborne pathogens (14, 15). The economic cost of *Campylobacter* infections was estimated to be $2.2 billion in 2018 by the U.S. Department of Agriculture Economic Research Service (16).

Although *C. jejuni* has fastidious growth requirements (*e.g.*, microaerobic, 32-47°C) and an unusual sensitivity towards environmental stresses (17), it can survive under aerobic condition with high prevalence by either forming biofilms or entering the VBNC state (17, 18). The formation of VBNC *C. jejuni* in response to adverse conditions has been described previously. For example, a large percentage of *C. jejuni* was induced into the VBNC state after 30 days of incubation at 4°C in the microcosm water (19). In addition, exposing *C. jejuni* to high osmotic pressure (7% NaCl) induced its VBNC state after 24 h (20). Moreover, *C. jejuni* inoculated into acidic Mueller-Hinton (MH) broth became non-culturable but remained viable after 2 h of incubation (21).

However, little is known about how food processing can induce *C. jejuni* to enter the VBNC state. The potential role of foods in inducing VBNC *C. jejuni* also remains largely unknown. In the current study, the culturability and viability of *C. jejuni* were monitored under the conditions mimicking food processing, including chlorine treatment, aerobic stress, and low temperature. In

addition, the effect of food product (*i.e.*, milk) on the progress of VBNC *C. jejuni* formation was also investigated. The findings from this study could provide a better understanding of the induction of VBNC *C. jejuni* in the agro-ecosystem and aid in the development of innovative mitigation strategies to reduce the health risks associated with this microbe.

3.3. Materials and methods

3.3.1. Bacterial strains and growth conditions

Two *C. jejuni* human clinical isolates (F38011 and 81-116) and two reference strains (ATCC 33560^T originated from bovine and NCTC 11168 originated from human) were used in this study. All the strains were routinely cultivated on MH agar (BD Difco, Franklin Lakes, NJ, USA) containing 5% (v/v) defibrinated sheep blood (Cedarlane, Burlington, ON, Canada) in microaerobic condition (85% N_2, 10% CO_2, 5% O_2) at 37°C. A single colony of each strain from the agar plates was transferred to MH broth (BD Difco) and cultivated in microaerobic condition at 37°C with constant shaking at 175 rpm. The bacterial suspension was diluted by MH broth to obtain a final optical density (OD_{600}) value of 0.3 (~9 log CFU/mL) after incubation for 16-18 h to late-log phase.

3.3.2. Induction of VBNC *C. jejuni* under food processing conditions

Different food processing conditions including chlorine treatment, aerobic stress, and low temperature were used to induce *C. jejuni* into the VBNC state. For chlorine treatment, overnight *C. jejuni* culture was centrifuged at 15,000 ×*g* for 5 min at room temperature and washed with phosphate-buffered saline (PBS). The obtained cell pellet was then re-suspended in 25 ppm NaClO solution (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 8 ± 0.5 log CFU/mL and

incubated in microaerobic condition at 37°C. For aerobic stress and low temperature treatment, overnight *C. jejuni* culture was adjusted to a final concentration of 8 ± 0.5 log CFU/mL in MH broth and incubated under atmospheric condition at either 37°C or 4°C. All the bacterial samples were under constant shaking at 175 rpm during the process of VBNC induction.

The dynamics of culturable and viable cells in the bacterial population were separately monitored by the plating assay and PMA-qPCR at a pre-determined sampling schedule, namely 1) chlorine treatment for 0, 3, 6, 9, 12 and 24 h, 2) aerobic stress for 0, 4, 8, 12, 24 and 36 h, and 3) low temperature every 4 days until day 24. The population of VBNC *C. jejuni* was calculated by subtracting culturable cell counts from viable cell counts. Bacterial culture (2 mL) was transferred onto each MH blood agar plate to confirm the absence of culturable cells. Thus, the detection limit (LOD) was determined to be 0.5 CFU/mL. When the concentration of culturable cells was below the LOD, all viable cells were considered to be in the VBNC state.

3.3.3. Induction of VBNC *C. jejuni* in milk

Milk was selected as a representative food model to investigate its role in inducing VBNC state of this microbe because the consumption of contaminated milk is one of the major routes of human campylobacteriosis (22). Considering that induction of VBNC *C. jejuni* is relatively long under low temperature condition, ultra-high-temperature (UHT) milk was used due to its long shelf life through which the transition of *C. jejuni* from culturable state to VBNC state can be monitored. However, UHT milk is considered as sterile, and there is a relatively low possibility of *Campylobacter* contamination during storage. To better mimic the real scenario, pasteurized milk

was also selected to evaluate bacterial VBNC induction process as it may carry microorganisms including *C. jejuni* caused by inadequate pasteurization or preservation.

Both UHT (2% fat) milk and pasteurized milk (3.8% fat) were purchased from a local grocery store in Montreal. *C. jejuni* culture (10 mL) was separately added into each milk sample (90 mL) to obtain a final concentration of 8.0 ± 0.5 log CFU/mL, followed by storage at 4℃ to facilitate the induction of VBNC bacteria. To be consistent with the induction process using pure bacterial culture under low temperature condition, UHT milk samples were incubated with constant shaking at 175 rpm. In the meanwhile, pasteurized milk samples were kept static to mimic the real storage condition for milk. The number of culturable and viable *C. jejuni* in milk samples was separately monitored by the plating assay and propidium monoazide (PMA)-quantitative polymerase chain reaction (qPCR). The sampling time for UHT milk was set as every 7 days until day 63 and the sampling time for pasteurized milk was set as every 3 days until day 21. Considering the existence of background microbiome in pasteurized milk, Campy-Cefex agar was prepared and used to selectively cultivate *C. jejuni* (23), and the agar plates were incubated at 37°C under microaerobic condition for 48 h before enumeration.

3.3.4. PMA treatment

PMA-qPCR has been widely used for detection and quantification of VBNC bacteria (4, 8, 24). PMA is a DNA intercalating dye that can only penetrate bacterial cells with compromised cell membrane and covalently bind to double-stranded DNA upon photoactivation. As the amplification signal from dead cells is blocked, only DNA from viable cells can be amplified during the subsequent qPCR process, enabling quantification of VBNC cells.

The number of viable cells in the bacterial population was determined by PMA-qPCR and the procedures were modified based on the method developed by Lv and others (20). Briefly, 1 mL of each bacterial suspension under different induction conditions was centrifuged at 15,000 ×*g* for 5 min and resuspended in PBS with the same volume. A total of 395 μL of the bacterial suspension was first mixed with 100 μL of PMA enhancer for Gram-negative bacteria (Biotium Inc., Hayward, CA, USA) to improve the affinity of PMA to the DNA of dead cells. Afterwards, PMA solution (Biotium Inc., Hayward, CA, USA) was added to cell mixture at a final concentration of 15 μM in a transparent microcentrifuge tube (Froggabio, Concord, ON, Canada). Following incubation in the dark at room temperature for 10 min, the samples were exposed to a 600-w halogen lamp (Smith victor, Bartlett, IL, USA) for another 10 min. During light exposure, all the samples were horizontally placed on ice at a distance of 20 cm away from light to avoid excessive heating. The complete mixing and cross-linking of PMA and DNA of dead cells were achieved by constant agitation during the entire PMA treatment. Before DNA extraction, the PMA-treated samples were centrifuged at 15,000 ×*g* for 5 min and washed once with double-deionized water (ddH_2O) to remove the unbounded PMA.

3.3.5. DNA extraction and qPCR

Thermal treatment was used for rapid extraction of bacterial DNA from non-food samples. In brief, genomic DNA was released from bacterial cells by boiling at 100°C for 10 min, followed by cooling down on ice for another 10 min. To obtain DNA samples with high yield and purity for downstream analysis, *C. jejuni* genomic DNA in spiked milk was extracted using the Qiagen Powerfood microbial kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until qPCR amplification.

The primer set (forward: 5'-GAGTAAGCTTGCTAAGATTAAAG-3', reverse: 5'-AAGAAGTTTTAGAGTTTCTCC-3') targeting the DNA-directed RNA polymerase of *C. jejuni* was selected for amplification, and its specificity was validated in a previous study performed by Lv and others (20). The qPCR was performed on an Agilent Mx3005P Real-Time PCR System (Agilent, Santa Clara, CA, USA) with a total volume of 20 μL: 2 μL of DNA temple, 10 μL of SensiFAST SYBR Lo-ROX Kit (Bioline, Memphis, TN, USA), 0.2 μL of each primer (100 nM), and 7.6 μL of sterile ddH_2O. The amplification program was developed as follows: one cycle of 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. At the end of each extension step, the fluorescence density of SYBR green was measured and used to calculate the cycle threshold (Ct) value of each sample. Samples were tested in triplicate and DNase-free sterile ddH_2O was included as the negative control in each run of the experiment.

The sensitivity of PMA-qPCR assay was evaluated in a live-dead bacterial mixture to mimic the practical conditions where bacterial cells exist in different states. The bacterial mixture was prepared by adding dead *C. jejuni* cell culture (6 log CFU/mL, heat inactivated at 90°C for 5 min) to 10-fold serially diluted live cell culture (ranging from 2 to 8 log CFU/mL). The aforementioned PMA treatment was applied to the mixture, and bacterial genomic DNA was extracted using the boiling method. A standard curve was obtained by correlating Ct values against known concentrations of *C. jejuni* cells and used to quantify the number of viable cells. The LOD of this assay is defined as the lowest concentration of bacterial cells that generate Ct value > 35.

3.3.6. Statistical analysis

All the experiments were performed in at least three biological replicates with 2-4 technical replicates. The data were expressed as the mean value of 3 independent replicates ± standard deviation. The comparison among different groups was conducted by the analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) software and the significance level was set as $P < 0.05$.

3.4. Results and discussion

The overall schematic workflow of this study is shown in Figure 3.1.

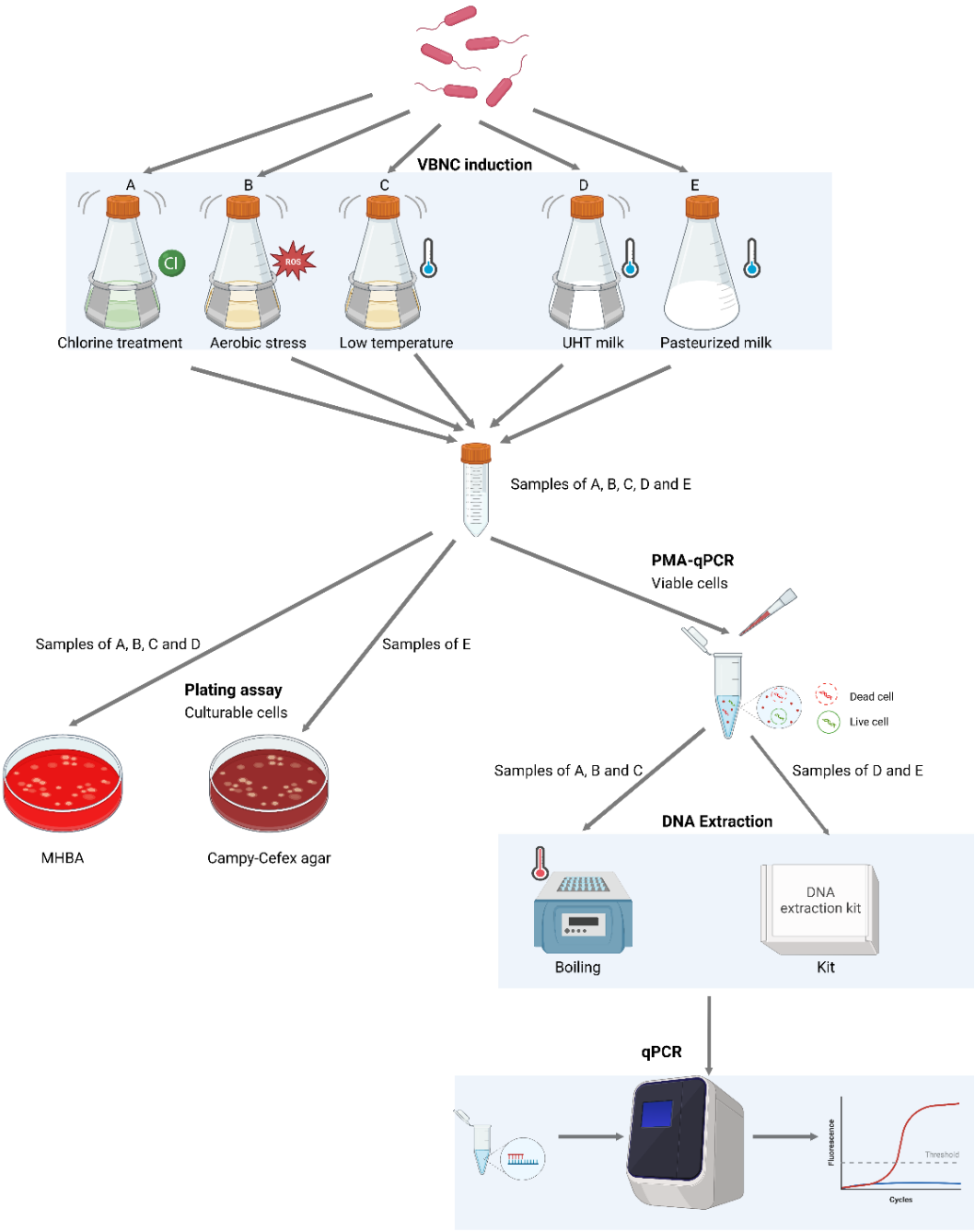

Figure 3.1 Schematic workflow of this study. PMA-qPCR and the plating assay were performed to monitor the viability and culturability of *C. jejuni* under conditions that mimic food processing and in foods [*i.e.*, chlorine treatment (A), aerobic stress (B), low temperature (C), UHT milk (D) and pasteurized milk (E)]. For samples of A, B, C, and D, the plating assay was conducted on MHBA as no background microflora existed. For sample of E, Campy-Cefex agar was used to inhibit microbiota in pasteurized milk and selectively cultivate inoculated *C. jejuni*. All the samples

were pretreated with PMA, followed by DNA extraction by either boiling (for A, B, and C) or a commercial DNA extraction kit (for D and E). The number of viable cells was obtained by qPCR analysis of the extracted DNA.

3.4.1. VBNC induction by chlorine treatment

Chlorine-based sanitizers have been widely used in the food industry for decontamination of agricultural products and agri-food processing equipment (25). However, chlorine treatment may not only inactivate bacterial cells but also lead to the induction of VBNC bacteria in some cases (26). For example, VBNC *E. coli, S. aureus,* and *Listeria monocytogenes* were detected in the process water of chlorine-disinfected fresh produce (27, 28). Chicken meat is usually treated with chlorine to mitigate the load of *Campylobacter* and other foodborne pathogenic bacteria. Therefore, *Campylobacter* might be induced into the VBNC state during chlorine treatment of poultry products. An initial chlorine concentration of 50 ppm in chlorinated water is recommended by USDA for poultry carcass wash applications and this concentration was used as the upper limit in various studies (29-32). Therefore, NaClO solution with 25 ppm of free chlorine was selected to induce VBNC *C. jejuni* in the current study.

The initial concentration of *C. jejuni* was 8.23 log CFU/mL, and the dynamics of culturable and viable cells under chlorine treatment over 24 h are shown in Figure 3.2. *C. jejuni* was susceptible to chlorine treatment and all four strains quickly lost their culturability (< 0.5 log CFU/mL) upon the addition of chlorine. The number of viable cells decreased by 1-2 log CFU/mL once bacterial suspension was added to the NaClO solution, and it remained constant during the induction. Therefore, around 1-10% of *C. jejuni* population was induced to the VBNC state under chlorine treatment. Similarly described in a previous study, *L. monocytogenes* and *S.* Thompson quickly

transited to the VBNC state as no culturable cells could be detected after 2-minute exposure to either 3 or 12 ppm chlorine (28). In another study, Chen and coauthors treated *E. coli* with increased concentrations of chorine and investigated the impact of chlorine disinfection on the culturability and viability of *E. coli* (26). Approximately 3-5 log CFU/mL *E. coli* was induced to the VBNC state after 5 min-2 h of chlorine treatment. Longer exposure to chlorine over 24 h did not cause a significant reduction in viable *E. coli*.

HOCl is the active content in NaClO solution, and it can inactivate bacteria by interacting with membrane components and changing membrane permeability, finally leading to the leakage of cellular contents including DNA and proteins (26). Bacteria develop various defense systems against reactive chlorine species. For example, chaperone Hsp33 and the HOCl-sensing transcription factor YjiE were upregulated when *E. coli* encountered HOCl stress (33). In addition, stress resistance genes (*rpoS*, *marA*, *ygfA*, and *relE*) and antibiotic resistance genes expressed at higher levels in chlorine-induced VBNC *E. coli* compared with culturable counterparts (34). As for *C. jejuni*, genes related to bacterial-type flagellum-dependent cell motility, tricarboxylic acid cycle, cellular respiration, and membrane proteins were upregulated when it was treated with chlorine (35). However, mechanisms in the formation of chlorine-induced VBNC *C. jejuni* remain unknown and need to be investigated in future studies.

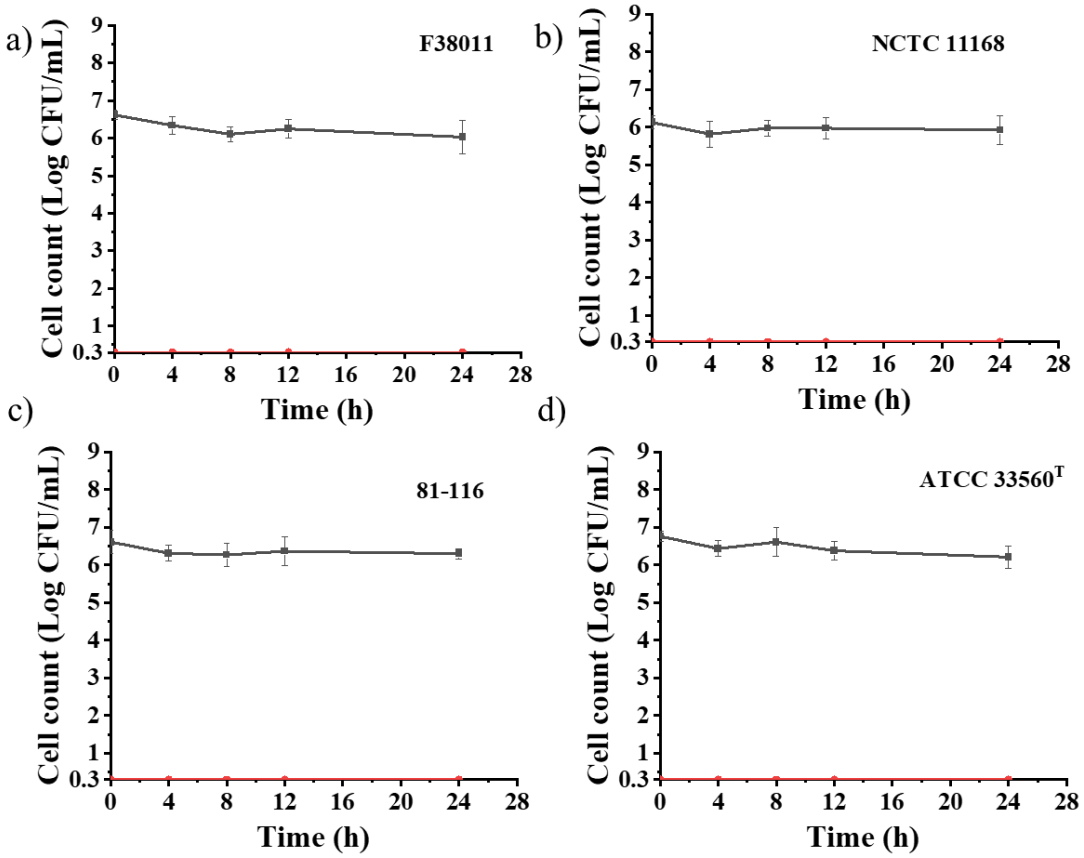

Figure 3.2 Culturable and viable cell counts of *C. jejuni* under chlorine treatment (25 ppm): a) F38011; b) NCTC 11168; c) 81-116; d) ATCC 33560^{T}. Black squares represent viable cell counts determined by PMA-qPCR and red circles represent culturable cell counts determined by the plating assay. Each data point represents three replicates.

3.4.2. VBNC induction under aerobic condition

C. jejuni is generally considered as a microaerophilic microbe and is highly susceptible to oxygen and its reduction products such as superoxide anion radical, hydroxyl radical and hydrogen peroxide (17, 36). Therefore, oxygen in the environment and food processing chain is a primary stress to the survival of *C. jejuni* (37). Entering into the VBNC state has been recognized as one of the strategies for *C. jejuni* to counteract oxidative stress (38). In this study, the effect of oxygen

on the induction of VBNC for four *C. jejuni* strains was investigated, and the response of *C. jejuni* under aerobic condition is shown in Figure 3.3. *C. jejuni* strains F38011, NCTC 11168, 81-116 and ATCC 33560^{T} gradually lost their culturability and could not be detected after 24 h of incubation. Viability of all four strains was retained, and >10% *C. jejuni* was induced into the VBNC state in the end. To compare the effect of different oxygen levels on the induction of VBNC *C. jejuni*, Yagi and coauthors monitored the number of culturable cells under aerobic, microaerobic, and anaerobic conditions (39). No colony was detected after 45, 60, and 60 days of incubation under aerobic, microaerobic, and anaerobic conditions, respectively, indicating that aerobic condition was the most effective in inducing VBNC *C. jejuni*. The difference in the progress of VBNC formation of *C. jejuni* under aerobic stress between our current study and this pervious study might be due to the use of different bacterial strains. In addition, constant shaking used in the current study could dissolve more oxygen in the media to facilitate induction process.

Exposure to aerobic condition increases the level of intracellular reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and superoxide anion radicals in bacterial species (17). Bacteria have specific oxidative stress genes to regulate ROS-detoxification enzymes (*e.g.*, alkyl hydroperoxide reductase, catalase, and superoxide dismutase) (37). For example, various Gram-negative pathogenic bacteria including *E. coli* and *S. enterica* harbor oxidative regulators (*e.g.*, OxyR and SoxR) to survive in oxygen-rich conditions (40, 41). However, *C. jejuni* lacks these regulators and defends against oxidative stress mainly using PerR (peroxide resistance regulator) that usually found in Gram-positive bacteria (42). In addition, *C. jejuni* only possesses single copies of oxidative stress defense genes (*aphC*, *sodB*, and *katA*). The transcription levels of these genes were increased when *C. jejuni* encountered aerobic condition, and deletion of these

genes increased the sensitivity of *C. jejuni* towards atmospheric oxygen (38). Further study is needed to identify the potential roles of these genes and investigate the underlying mechanisms in inducing VBNC *C. jejuni* under aerobic condition.

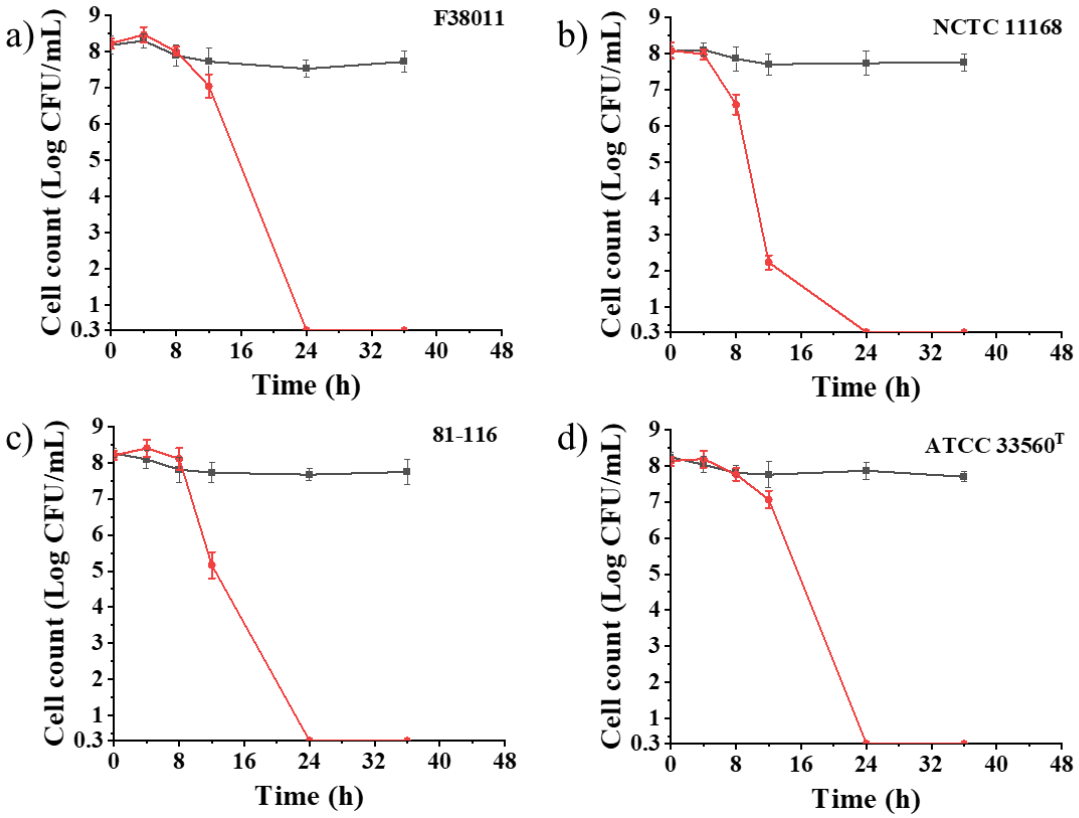

Figure 3.3 Culturable and viable cell counts of *C. jejuni* under aerobic condition: a) F38011; b) NCTC 11168; c) 81-116; d) ATCC 33560^{T}. Black squares represent viable cell counts determined by PMA-qPCR and red circles represent culturable cell counts determined by the plating assay. Each data point represents three replicates.

3.4.3. VBNC induction under low temperature condition

Low temperature is a common method for food preservation, and it has been validated as a major cause of inducing various pathogenic bacteria such as *E. coli*, *Staphylococcus*, and *Vibrio* into the VBNC state (8, 43, 44). In the current study, refrigeration temperature (4°C) used for processing, transportation, and storage of animal food products was used to induce VBNC *C. jejuni*.

The culturability and viability of *C. jejuni* were evaluated to monitor the response of *C. jejuni* to low temperature condition (Figure 3.4). Four *C. jejuni* strains showed similar trends during induction. The number of culturable cells gradually decreased and reached to detection limit on day 20, indicating that all viable cells entered the VBNC state. In comparison, although ~1 log CFU/mL decrease was observed in the viable cells, it maintained its viability in the end. The induction of VBNC *C. jejuni* under low temperature condition was reported previously. Baffone and coauthors monitored the culturability and viability of ten *C. jejuni* strains in artificial seawater at 4°C (5). Different induction periods (12-15 days, 26 days, and 35 days) were required for these strains to lose their culturability, indicating the strain-specific difference. The difference in the induction period under low temperature condition among these studies might be due to the variation in bacterial strains, nutrient levels, and atmospheric conditions (39). In the current study, the induction time that *C. jejuni* required to fully enter into the VBNC state under low temperature condition (20 days) was much longer than that required under chlorine (immediately) and aerobic stress (24 hours) conditions. Similar results were observed for other bacteria such as *E. coli* and *Vibrio*. In a study conducted by Falcioni and collaborators, successful induction of VBNC *Vibrio parahaemolyticus* was achieved by incubating culturable cells in artificial seawater at 4°C for 69 days (45). Wei and Zhao used low temperatures (4 and -20°C) to induce VBNC *E. coli* O157:H7

in saline, distilled water, and LB broth (43). The culturability of *E. coli* maintained at a high level even after being incubated in LB broth and distilled water for over 180 days.

Many bacteria such as *E. coli*, *Bacillus subtilis*, and *S.* Typhimurium produce cold shock proteins in response to rapid temperature decrease (46). Cold shock proteins mediate membrane adaptation, cold signal sensing, and translation-device alteration, all of which facilitate the growth of bacteria below optimal growth temperature (47). However, cold shock genes such as *cspA* and the homologs were not detected in *C. jejuni* genomes, possibly explaining the inability of *C. jejuni* to grow under temperature below 30℃. However, metabolic activities such as oxygen consumption, catalase activity, ATP generation, and protein synthesis were still observed in *C. jejuni* even when the temperature was decreased to 4℃ (48). Further study is required to investigate cold shock-related genes in *C. jejuni* for elucidating the formation mechanisms of VBNC state under low temperature condition.

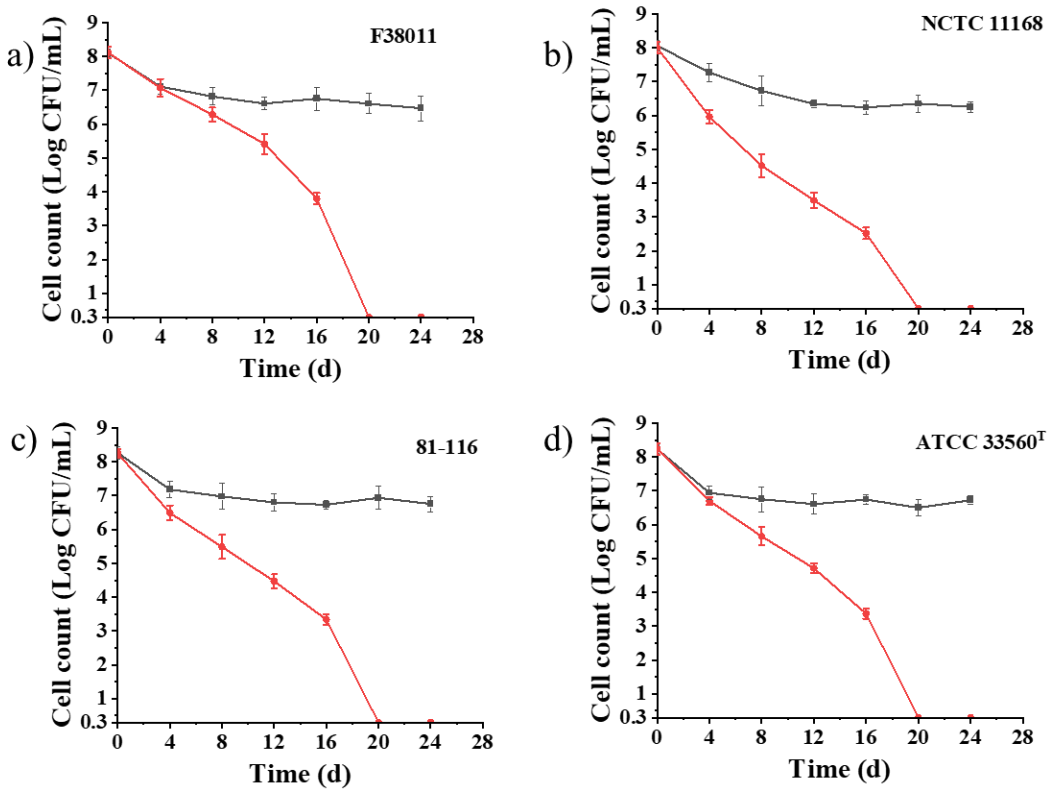

Figure 3.4 Culturable and viable cell counts of *C. jejuni* under low temperature (4°C): a) F38011; b) NCTC 11168; c) 81-116; d) ATCC 33560^{T}. Black squares represent viable cell counts determined by PMA-qPCR and red circles represent culturable cell counts determined by the plating assay. Each data point represents three replicates.

3.4.4. VBNC induction in milk

Raw milk should be free of microorganisms if it is produced by a healthy cow (49). However, microorganisms can be transmitted to raw milk via milking equipment, mastitis, and fecal contamination (50). Numerous *C. jejuni* outbreaks have been linked with the consumption of unpasteurized or inadequately pasteurized milk, and these products are identified as important vehicles for *C. jejuni* infections (51-53). The survival of *C. jejuni* in raw/pasteurized milk under low temperatures has been studied (54, 55). However, these studies mainly used culture-based methods to quantify the number of *C. jejuni*, resulting in potential under-estimation of the risks of

VBNC *C. jejuni*. To evaluate whether *C. jejuni* can be induced to the VBNC state in milk at low temperature and understand the role of milk in the induction process, our current study investigated the dynamics of culturable and viable *C. jejuni* in both UHT and pasteurized milk during refrigerated storage.

The number of viable *C. jejuni* cells in UHT milk remained stable over the entire course of nine weeks with <1 log CFU/mL reduction in the end. In contrast to rapid population reduction observed in MHB where no culturable *C. jejuni* was detected after 20 days of induction, the population decreased gradually in refrigerated UHT milk, in which culturable *C. jejuni* became undetectable on day 42 or later (Figure 3.5). The pronounced robustness of *C. jejuni* in UHT milk might be due to the protective effect of proteins in milk. According to a previous study, addition of milk proteins promoted the survival of *E. coli* in milk at low temperature (56). In another study, Rubin concluded that casein micelles contributed to the prolonged survival of pathogens in cheese (57). The strain-specific difference was also observed in the induction process in UHT milk as the reduction in culturable cells was different among four strains. *C. jejuni* strains F38011, NCTC 11168, 81-116, and ATCC 33560^{T} entered into the VBNC state after 56, 56, 56, and 42 days of storage, respectively, indicating the relatively high susceptibility of *C. jejuni* ATCC 33560^{T} towards cold stress. Similarly reported in a study by Feng and colleagues, more *C. jejuni* ATCC 33560^{T} lysed under aerobic and starvation conditions compared with other strains (*e.g.*, *C. jejuni* F38011, NTCC 11168, etc.) (58). In addition, *C. jejuni* ATCC 33560^{T} was observed to be susceptible to various antibiotics, such as amoxicillin, ciprofloxacin, erythromycin, gentamicin and tetracycline (59). According to sequencing analysis, *C. jejuni* ATCC 33560^{T} has less point mutations associated with antibiotic resistance. Moreover, no polymorphism was observed in the

regulatory region of *cmeABC*. CmeABC has been recognized as a multidrug efflux pump contributing to antimicrobial resistance of *C. jejuni*. Transcriptional repressor CmeR can specifically bind to the regulatory region of *cmeABC* to modulate its expression level so as to determine the antimicrobial susceptibility of *C. jejuni* (60). Taken together, these findings demonstrated the vulnerability of *C. jejuni* ATCC 33560^{T} towards physical and chemical stresses. Mechanisms underlying the high susceptibility of this strain in refrigerated milk remain largely unknown and need to be investigated in the future.

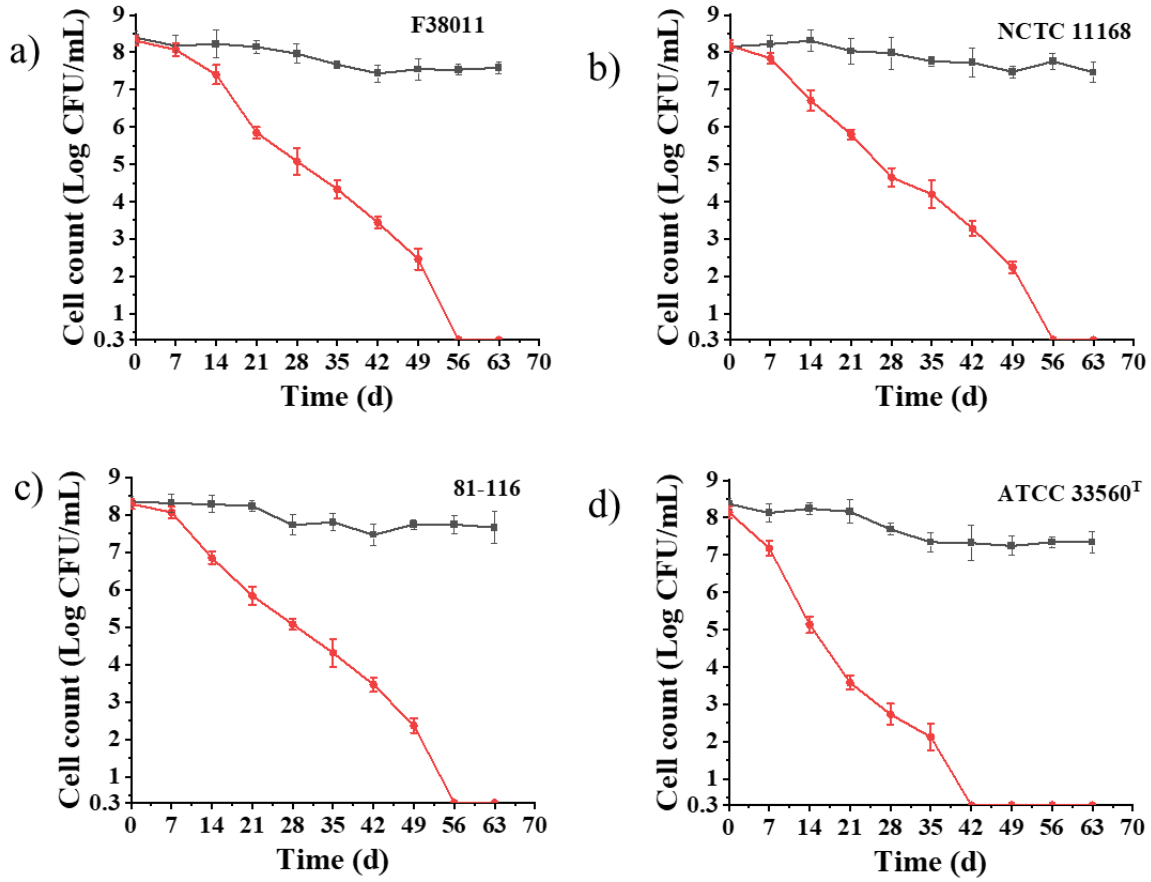

Figure 3.5 Culturable and viable cell counts of *C. jejuni* in UHT milk under low temperature (4°C): a) F38011; b) NCTC 11168; c) 81-116; d) ATCC 33560^{T}. Black squares represent viable cell counts determined by PMA-qPCR and red circles represent culturable cell counts determined by the plating assay. Each data point represents three replicates.

Survival of *C. jejuni* in pasteurized milk is shown in Figure 3.6. Although the number of culturable cells decreased during refrigeration storage, all four strains remained their culturability in the end with the number of culturable cells ranging from 1.5 to 5 log CFU/mL on day 21. Similar to the VBNC induction in UHT milk, *C. jejuni* strains F38011, NCTC 11168, 81-116 showed relatively more robustness while *C. jejuni* ATCC 33560^{T} was susceptible to stressors in pasteurized milk. Inactivation of culturable *C. jejuni* in pasteurized milk was greater than that in UHT milk, suggesting the potential presence of antimicrobials. Pasteurized milk contains lactoperoxidase system (*i.e.*, lactoperoxidase, thiocyanate, and hydrogen peroxide) that can generate antimicrobial intermediate products against a wide range of Gram-negative bacteria including *E. coli*, *L. monocytogenes*, *S. aureus* and *C. jejuni* (61-64). Moreover, the background microflora in pasteurized milk can grow rapidly in late stage of storage and produce metabolites to inhibit *C. jejuni* (54). In contrast, UHT treatment completely inactivates lactoperoxidase and microbes in milk, resulting in less stressors that *C. jejuni* encounters. A previous study reported a slower decline in the culturability of *C. jejuni* in pasteurized milk, in which <1 log CFU/mL reduction was observed over 14 days of storage (65). The difference in the process of VBNC formation of *C. jejuni* in pasteurized milk between the current study and this previous study might be due to the use of different *C. jejuni* strains and different antimicrobials in milk (*e.g.*, lactoperoxidase system, metabolites produced by background microflora, etc.). Although rapid decrease was observed in the culturable population, there was <1 log CFU/mL reduction in viable *C. jejuni* strains during the induction process in the current study. Thus, *C. jejuni* in contaminated milk can be induced to VBNC state, posing a threat to food safety and public health.

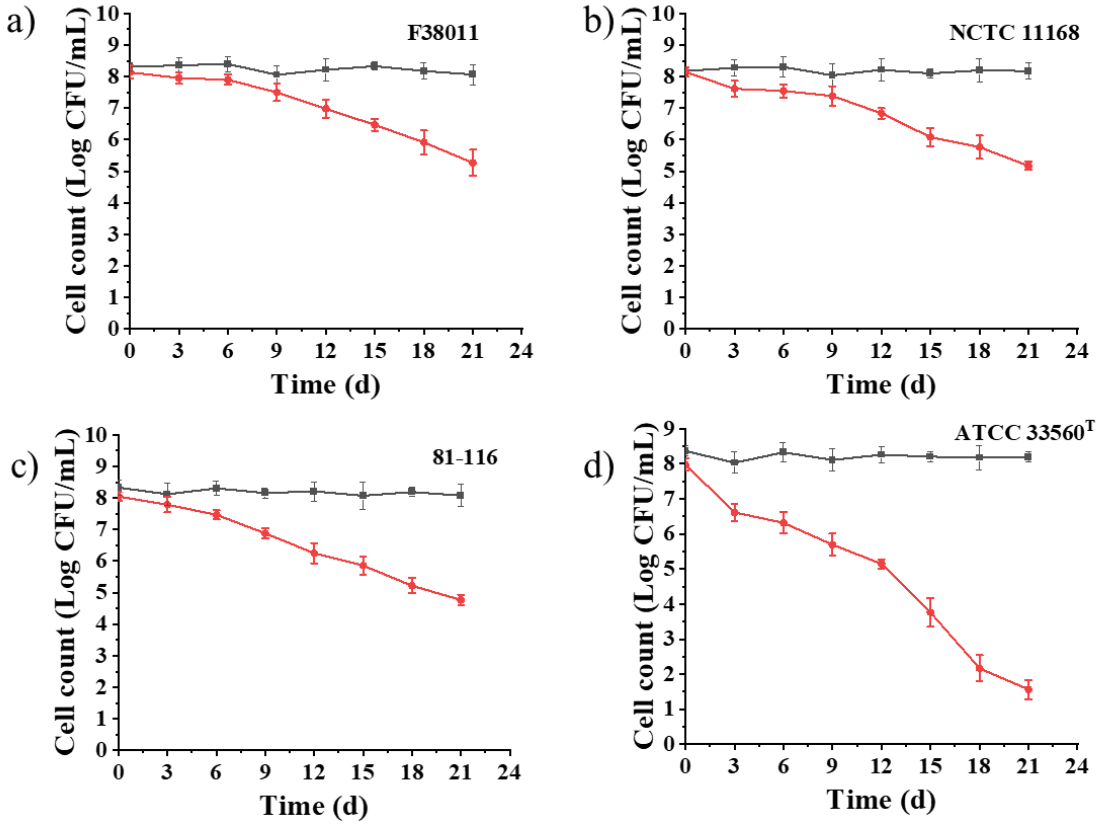

Figure 3.6 Culturable and viable cell counts of *C. jejuni* in pasteurized milk under low temperature (4°C): a) F38011; b) NCTC 11168; c) 81-116; d) ATCC 33560^{T}. Black squares represent viable cell counts determined by PMA-qPCR and red circles represent culturable cell counts determined by the plating assay. Each data point represents three replicates.

3.5. Conclusion

In conclusion, stressors in food processing conditions and food products could trigger the induction of the VBNC state in *C. jejuni*. A certain number of *C. jejuni* transitioned into the VBNC state although they responded differently to various stress conditions. In addition, the heterogeneous behaviors among tested strains under the same stress condition indicated the existence of strain-specific difference. Further study is required to decipher the molecular mechanisms underlying the formation of VBNC state under various stress conditions and the strain-specific difference that existed in different *C. jejuni* strains. Taken together, this study provides insight into the induction and persistence of VBNC *Campylobacter* in the environment and aids in the development of innovative mitigation strategies to reduce the prevalence of this microbe in the agro-ecosystem.

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Connecting text

In the previous chapter, it was demonstrated that the stressors encountered during food processing conditions and within food products have the capacity to induce the VBNC state in *C. jejuni*. Although *C. jejuni* exhibited diverse responses to different stress conditions, a considerable proportion of the bacterial population was transitioned into the VBNC state. Moreover, heterogeneous behaviors were observed among different strains of *C. jejuni* when subjected to the same stress conditions, emphasizing the existence of strain-specific variations in response. The induction of the VBNC state in *C. jejuni* raises concerns regarding the efficacy of current inactivation methods as VBNC bacterial pathogens have exhibited heightened tolerance to antimicrobials. Therefore, development of novel strategies is crucial to effectively control bacterial pathogens persisting in the VBNC state. Additionally, the persistence of VBNC *C. jejuni* in the presence of antimicrobials remains largely unknown and warrants comprehensive investigation.

In Chapter 4, our study aimed to investigate the persistence of VBNC *C. jejuni* in the presence of various antimicrobials, namely carvacrol, diallyl sulfide, and Al_2O_3 NPs. Recognizing the potential advantages of combinational antimicrobial treatments over the use of a single antimicrobial, we also explored the potential synergistic effects of these compounds when used together. By examining the response of VBNC *C. jejuni* to antimicrobials and evaluating their potential synergistic interactions, our research seeks to provide valuable insights into the development of effective mitigation strategies against this persistent pathogen. Understanding the behavior of VBNC *C. jejuni* and exploring the synergistic antimicrobial effects of different compounds can contribute to the development of innovative approaches for combating *C. jejuni* infections and ensuring food safety. The manuscript is under preparation and will be submitted shortly.

Chapter 4. Investigating the synergistic antimicrobial effect of carvacrol, diallyl sulfide and aluminum oxide nanoparticles against viable but non-culturable *Campylobacter jejuni*

4.1. Abstract

Bacteria in the viable but non culturable (VBNC) state demonstrate enhanced persistence towards various environmental stressors including antimicrobials compared to their culturable counterparts. This persistence poses a significant threat to food safety and public health since VBNC pathogenic bacteria resuscitate and initiate diseases under favorable conditions. Therefore, development of effective strategies to combat VBNC bacteria is of great importance. In this study, we investigated the persistence of VBNC *C. jejuni*, a leading cause of bacterial gastroenteritis worldwide, towards various antimicrobials, namely carvacrol, diallyl sulfide, and Al_2O_3 nanoparticles (NPs). Potential synergistic antimicrobial effects of these compounds on VBNC *C. jejuni* were also investigated. VBNC *C. jejuni* displayed a high level of persistence against carvacrol or diallyl sulfide, as single treatment with either compound at low concentrations barely achieved inactivation. In contrast, Al_2O_3 NPs exhibited significant antimicrobial efficacy, suggesting their potential as promising agents for combating VBNC *C. jejuni*. When carvacrol and diallyl sulfide were combined, an additive antimicrobial effect was observed. Synergistic effects were observed when either carvacrol or diallyl sulfide was combined with Al_2O_3 NPs. Notably, the triple combination of carvacrol, diallyl sulfide, and Al_2O_3 NPs generated an enhanced synergistic effect, allowing for decreased concentrations of these antimicrobials. These findings provide an alternative strategy to inactivate VBNC *C. jejuni* and demonstrate the potential applications of these approaches in the food industry for combating other dormant foodborne pathogens.

Keywords

Campylobacter, viable but non-culturable, synergy, plant-based antimicrobials, nanoparticles

4.2. Introduction

Campylobacter jejuni is a prominent cause of gastrointestinal bacterial infection worldwide, which can result in a variety of clinical symptoms ranging from mild, non-invasive conditions (*e.g.*, fever, headache, vomiting, and diarrhea) to more severe long-term complications, including Crohn's disease, Guillain-Barré syndrome, and septicemia (Kaakoush et al., 2015; Kirkpatrick and Tribble, 2011). Despite having fastidious growth requirements (*e.g.*, microaerophilic condition and a temperature range of 32-47°C), *C. jejuni* can survive in atmospheric condition and spread throughout the food-processing and storage environment to infect humans (Farrar et al., 2011). This apparent contradiction may be due to the ability of *C. jejuni* to form biofilms or enter the viable but non-culturable (VBNC) state to evade the environmental stress (Farrar et al., 2011; Svensson et al., 2008). Induction of VBNC state is a specific response of *C. jejuni* to the unfavorable conditions and this has been previously documented (Chaveerach et al., 2003; Lv et al., 2019; Tholozan et al., 1999). Our recent study has also confirmed the induction of VBNC *C. jejuni* in the food processing environment and stored food products (Zhang and Lu, 2023).

Pathogenic bacteria may not be able to cause diseases in the VBNC state, but they can be the potential risk to food safety and public health as they are virulent after resuscitation under suitable condition (Baffone et al., 2006; Cappelier et al., 2007; Fu et al., 2020). Therefore, it is critical to inactivate VBNC pathogenic bacteria before it regains culturability. VBNC bacterial cells have many similarities to their culturable counterparts. However, when bacteria transit from the culturable state to the VBNC state, various physiological changes occur, such as alterations in cellular morphology, metabolism, stress tolerance, gene expression, and potential virulence (Zhao et al., 2017). Due to these changes, VBNC bacteria exhibit a greater resistance to chemical and

physical stresses, as well as antimicrobials, compared to culturable cells (Nowakowska and Oliver, 2013; Ramamurthy et al., 2014; Signoretto et al., 2000). Unlike culturable cells that acquire antibiotic resistance via specific resistance genes, VBNC cells are isogenic to the sensitive bacterial population but exhibit antimicrobial tolerant phenotypes (Ayrapetyan et al., 2018). For instance, VBNC *Escherichia coli* demonstrated a greater tolerance to nine common antibiotics compared to their culturable counterparts (Lin et al., 2017). Increased tolerance to antibiotics, disinfectants and preservatives was also identified in VBNC pathogens, including *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*. VBNC *L. monocytogenes* could survive even when exposed to 1000 mg/L of ampicillin, which is 1000 times higher than the minimum inhibition concentration (MIC) for culturable cells (Robben et al., 2019). Due to the high antimicrobial tolerance of VBNC bacterial pathogens, current inactivation methods might be invalid and novel strategies need to be developed to control bacterial pathogens in the VBNC state. In addition, the persistence of VBNC *C. jejuni* in the presence of antimicrobials remains largely unknown and needs to be investigated.

Combinational antimicrobial treatment offers several advantages over the use of a single antimicrobial, such as a higher effectiveness, lower dose requirement, and reduced chance to develop resistance. Successful antimicrobial combinations are often determined by the synergistic effect, which refers to the potentiated antimicrobial effect that exceeds the sum of individual effects (Chou, 2006). Recent studies identified novel combinations of plant-based antimicrobials and metal oxide nanoparticles (NPs) to exhibit synergistic effects through various mechanisms. For example, the antibacterial activity of zinc oxide NPs and/or copper oxide NPs against *E. coli* and *L. monocytogenes* was enhanced by rosemary or cinnamon extract (Osaili et al., 2019).

Therefore, the synergistic combinations of these antimicrobial agents may facilitate the inactivation of VBNC *C. jejuni.*

Plant-derived volatile compounds known as essential oils (EOs) have been used as natural antimicrobial agents for food preservation (Vergis et al., 2015). One of the most potent chemical groups in EOs is carvacrol, a monoterpenoid phenolic compound that is a major component of several essential oils including oregano, thyme, marjoram, and summer savory (Knowles et al., 2005). Carvacrol exhibits a broad-spectrum antimicrobial effect, and it is particularly effective against major foodborne pathogens such as *B. cereus*, *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *E. coli*, and *L. monocytogenes* (Knowles et al., 2005; Perez-Conesa et al., 2011; Ultee et al., 2000). Diallyl sulfide ($C_6H_{10}S$) is a naturally occurring organosulfur compound that is primarily extracted from *Allium* plant species (Suman and Shukla, 2016). The robust antimicrobial capability of diallyl sulfide has been observed in various studies. For example, diallyl sulfide effectively inhibited both planktonic and sessile cells of *C. jejuni* (Lu et al., 2012). It also demonstrated potent bactericidal effects on various microbes, such as *Klebsiella pneumoniae*, *Helicobacter pylori*, *E. coli*, *L. monocytogenes*, and methicillin-resistant *S. aureus* (Chen et al., 1999; Lu et al., 2011; O'Gara et al., 2000; Tsao et al., 2003).

Metal oxides have been widely used as antibacterial agents, especially in their nano-forms, in various fields including agriculture and environmental protection (Stanić and Tanasković, 2020). Recently, zinc oxide, titanium dioxide, copper oxide, and other metal oxide nanoparticles have been extensively investigated for their antibacterial activities (Mendes et al., 2022; Naseem and Durrani, 2021). Another promising candidate is aluminum oxide NPs, which is known for its

inherent inertness and low cytotoxicity (Radziun et al., 2011; Sliwinska et al., 2015). Additionally, its unique electrical, chemical, and physical properties make it commercially viable and of interest for antimicrobial studies (Mukherjee et al., 2011). In fact, Al_2O_3 NPs was reported to effectively inhibit various bacterial pathogens, such as *C. jejuni*, *E. coli*, and *S. aureus* (Gudkov et al., 2022; Xue et al., 2018).

The aim of this study was to evaluate the antimicrobial effects of plant extracts (*i.e.*, carvacrol and diallyl sulfide) and metal oxide nanoparticles (*i.e.*, Al_2O_3 NPs) alone and in combination against VBNC *C. jejuni*. This study will shed light on how nanoparticles and plant-derived compounds can be used to decrease the presence of VBNC foodborne pathogens such as *Campylobacter* in the agri-food supply chain.

4.3. Materials and methods

4.3.1. Chemical reagents and culture methods

Carvacrol and diallyl sulfide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Al_2O_3 NPs powder with the particle size of 40-50 nm and the surface area of 32-40 m^2/g was purchased from Alfa Aesar™ (Haverhill, MA, USA). Four strains of *C. jejuni* were used in this study, including two isolates from human clinical samples (F38011 and 81-116) and two reference strains (ATCC 33560 originated from bovine and NCTC 11168 originated from human). To cultivate these strains, Mueller-Hinton (MH) agar (BD Difco, Franklin Lakes, NJ, USA) supplemented with 5% (v/v) defibrinated sheep blood (Cedarlane, Burlington, ON, Canada) was used, and microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) at 37°C were maintained. After transferring a single colony of each strain to MH broth (BD Difco), they were subjected to constant shaking at 175 rpm and

incubated under microaerophilic conditions at 37°C. Once in the late-log phase after 16-18 hours of incubation, the bacterial suspension was diluted in MH broth to obtain a final optical density (OD_{600}) of 0.3, roughly equivalent to a bacterial concentration of 9 log CFU/mL.

4.3.2. Induction of VBNC *C. jejuni*

VBNC *C. jejuni* was induced by osmotic pressure according to the protocols described in a previous study conducted in our laboratory (Lv et al., 2019). Four strains of *C. jejuni* were cultivated overnight and combined to form a bacterial cocktail, which was then suspended in a 7% (w/v) NaCl solution to achieve a concentration of 8 log CFU/mL. After incubation at 37°C for 48 hours, both plating assay and monoazide (PMA)-quantitative PCR were used to verify the successful induction of VBNC bacterial cells. The absence of culturable cells was confirmed by transferring 2 mL of bacterial culture onto each MH blood agar plate, with the detection limit (LOD) established at 0.5 CFU/mL. In cases where the concentration of the culturable cells dropped below this threshold, all viable cells measured using PMA-qPCR were regarded as in the VBNC state.

4.3.3. Preparation of antimicrobial working solutions and antimicrobial treatment

To create desired concentrations of carvacrol and diallyl sulfide for antimicrobial treatment, these compounds were separately dissolved in dimethyl sulfoxide (DMSO, Fisher Sci., Hampton, NH, USA) to achieve a concentration of 32 mg/mL. Serial dilutions were then prepared and added to VBNC *C. jejuni* cocktail to create final concentrations of 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, and 1.6 mg/mL. In the meanwhile, serial dilutions of Al_2O_3 NPs were performed in double distilled water (ddH_2O) to yield the final concentrations of 0.05

mg/mL, 0.1 mg/mL, 0.2 mg/mL, and 0.4 mg/mL in VBNC *C. jejuni* cocktail. The control samples included VBNC *C. jejuni* alone and VBNC *C. jejuni* with 5% (v/v) DMSO. All samples, including the controls, were incubated at 37°C with agitation under microaerophilic conditions for 0, 4, 8, 12, and 24 h, and PMA-qPCR was used to quantify the number of viable *C. jejuni* cells.

4.3.4. Synergistic antimicrobial effects of carvacrol, diallyl sulfide, and aluminum oxide NPs against VBNC *C. jejuni*

Synergistic antimicrobial effect of carvacrol, diallyl sulfide, and Al_2O_3 NPs against VBNC *C. jejuni* was investigated using the time-kill assay. VBNC *C. jejuni* was separately challenged with 1) carvacrol (0.1 mg/mL, 0.2 mg/mL) and diallyl sulfide (0.1 mg/mL, 0.2 mg/mL); 2) carvacrol (0.1 mg/mL, 0.2 mg/mL) and Al_2O_3 NPs (0.05 mg/mL, 0.1 mg/mL); 3) diallyl sulfide (0.1 mg/mL, 0.2 mg/mL) and Al_2O_3 NPs (0.05 mg/mL, 0.1 mg/mL); 4) carvacrol (0.1 mg/mL), diallyl sulfide (0.1 mg/mL) and Al_2O_3 NPs (0.05 mg/mL, 0.1 mg/mL). The remaining viable *C. jejuni* cells were quantified at 0, 4, 8, 12, and 24 h using PMA-qPCR.

4.3.5. PMA treatment and DNA extraction

PMA pretreatment coupled with qPCR is one of the promising methods for the quantification of VBNC bacteria. This method relies on the integrity of cell membrane to differentiate between viable and dead cells. PMA selectively penetrates cells with compromised membrane, where it intercalates with DNA and forms a covalent bond (Gao et al., 2021). This modification prevents the extraction and further amplification of the DNA from dead cells.

To determine the number of the remaining viable cells in antimicrobial-treated VBNC *C. jejuni* samples, PMA-qPCR was performed following a protocol adapted from our previous study (Zhang and Lu, 2023). First, 1 mL of each treated bacterial suspension was centrifuged at 15,000 ×*g* for 5 min before being resuspended in an equal volume of phosphate-buffered saline (PBS). To increase the binding of PMA to the DNA of dead cells, 100 μL of a PMA enhancer specially designed for Gram-negative bacteria (Biotium Inc., Hayward, CA, USA) was combined with 395 μL of bacterial suspension. A total of 5 μL of PMA solution (dissolved in ddH_2O, Biotium Inc.) was then added to the cell mixture in a transparent microcentrifuge tube (Froggabio, Concord, ON, Canada) to reach to a final concentration of 15 μM. These samples were incubated in the dark with constant shaking for 10 min. Afterwards, the mixture was placed on ice and exposed to a 600-W halogen lamp (Smith victor, Bartlett, IL, USA) positioned 20 cm above the tubes with constant shaking during the 10 min-exposure to ensure uniform light exposure and cross-linking. After the PMA treatment, *C. jejuni* cells were collected via centrifugation at 15,000 ×*g* for 5 minutes and washed once with ddH_2O to remove any unbound PMA from the samples.

A rapid extraction method using thermal treatment was employed to extract bacterial DNA from PMA-pretreated samples. Briefly, *C. jejuni* cells were subjected to boiling at 100°C for 10 min and then cooled on ice for an additional 10 min. The obtained DNA was subsequently used for qPCR analysis.

4.3.6. qPCR

The primer set was used to amplify a 121-bp segment of *rpoB* from *C. jejuni* and its specificity was previously validated by Lv and coauthors (Lv et al., 2019). The forward and reverse primers

were 5’-GAGTAAGCTTGCTAAGATTAAAG-3’ and 5’-AAGAAGTTTTAGAGTTTCTCC-3’, respectively. The amplification reactions were performed in a 96-well non-skirted polypropylene PCR plate (Agilent, Santa Clara, CA, USA) with optical strip caps using Agilent Mx3005P Real-Time PCR System. A total volume of 20 μL was used for qPCR amplification, consisting of 10 μL of SensiFAST SYBR Lo-ROX Kit, 100 nM each primer, 2 μL of template DNA, and 7.6 μL of sterile ddH_2O. The amplification program was initiated with a cycle at 50°C for 2 min, then a cycle at 95°C for 10 min. This was followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each amplification reaction was carried out in triplicate, and the negative control of DNase-free sterile ddH_2O was included in each experimental run. The cycle threshold (Ct) values of each sample were automatically generated by the MxPro qPCR Software (Agilent, Santa Clara, CA, USA).

4.4. Results

4.4.1. Antimicrobial effect of single agents against VBNC *C. jejuni*

The effect of antimicrobials against VBNC *C. jejuni* was observed in a concentration-dependent manner, as shown in Figure 4.1. All single antimicrobials had mild effect on VBNC *C. jejuni* when used at low concentrations, with <1 log CFU/mL reduction in bacterial cell count throughout the entire antimicrobial treatment. Carvacrol demonstrated a significant reduction of >1 log CFU/mL at concentrations >0.4 mg/mL ($P < 0.05$) (Figure 4.1A). Additionally, these concentrations (0.6 and 0.8 mg/mL) also led to an enhanced antimicrobial effect that increased with longer treatment durations. Notably, upon the addition of 1.6 mg/mL of carvacrol to *C. jejuni* culture, an immediate reduction in bacterial cell count of >2 log CFU/mL was observed, followed by a slight decrease of <0.5 log CFU/mL during the later stage of the experiment. Although the antimicrobial effect of

diallyl sulfide exhibited a similar trend to that of carvacrol, the difference in effectiveness between high and low concentrations was less pronounced compared to carvacrol. As shown in Figure 4.1B, diallyl sulfide at a concentration ≤0.4 mg/mL caused a reduction of <1 log CFU/mL in VBNC *C. jejuni*. Significant reductions in bacterial cell count were observed when the concentration of diallyl sulfide was >0.6 mg/mL ($P < 0.05$). Additionally, the final bacterial count treated with diallyl sulfide at concentrations of 0.8 and 1.6 mg/mL was >5 log CFU/mL, which was higher than that treated with the corresponding concentrations of carvacrol (< 5 log CFU/mL).

In the present study, the time-kill kinetics of individual antimicrobial treatments against VBNC *C. jejuni* indicated that Al_2O_3 NPs generally exhibited a superior antimicrobial effect in comparison to carvacrol and diallyl sulfide (Figure 4.1C). When the same concentrations (*i.e.*, 0.2 and 0.4 mg/mL) of antimicrobials were used, samples treated with Al_2O_3 NPs showed a final bacterial count of ~ 5 log CFU/mL, which was significantly lower than that observed in the samples treated with carvacrol and diallyl sulfide (≥6 log CFU/mL). In this study, 5% (v/v) DMSO was used as a vehicle for carvacrol and diallyl sulfide. Considering the potential cytotoxicity of DMSO towards bacterial cells at high concentrations, samples with DMSO were included in the experiment (Modrzynski et al., 2019). The analysis of bacterial cell count in all DMSO added groups indicated that the viability of VBNC *C. jejuni* remained unaffected, thereby excluding any adverse effects caused by DMSO.

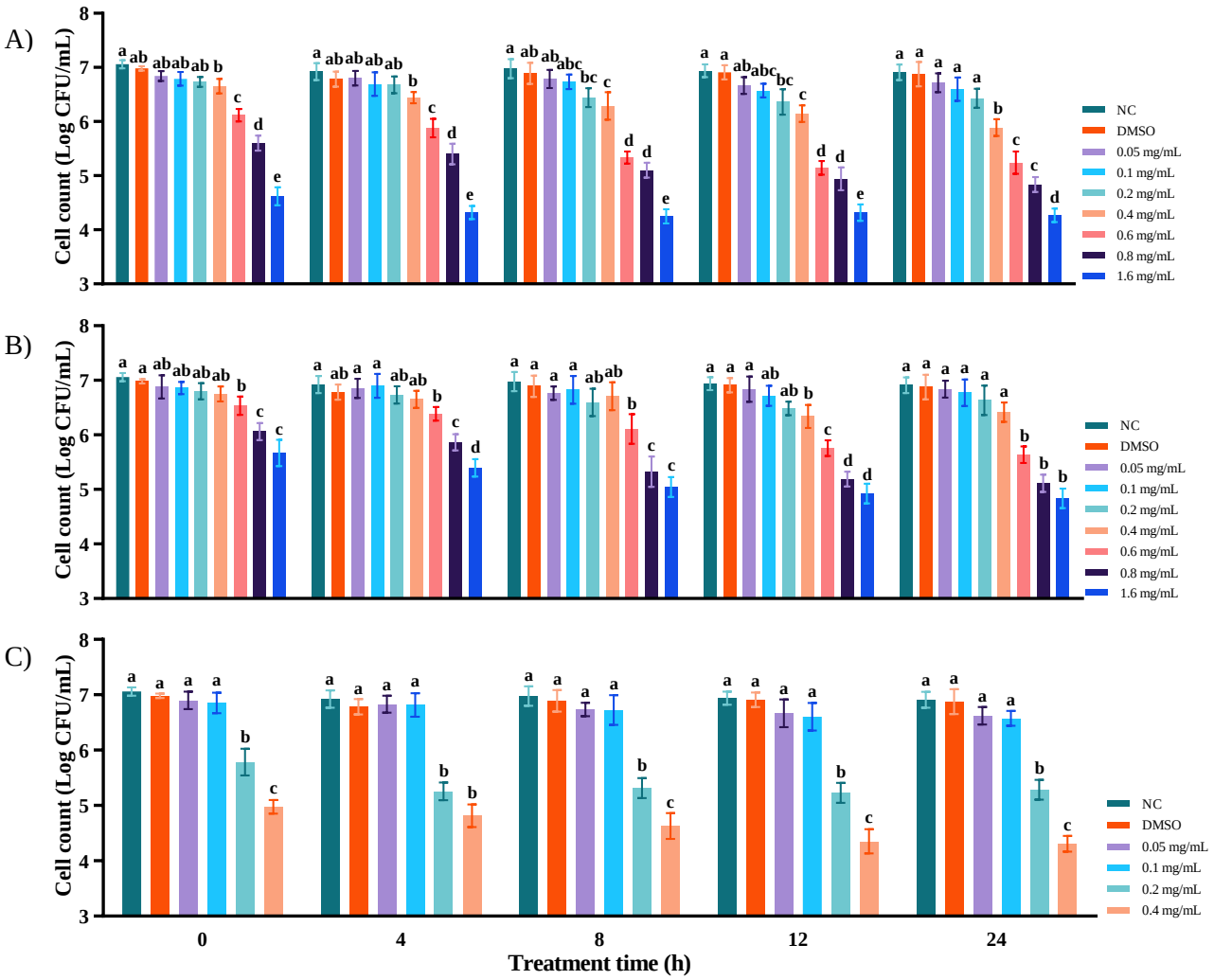

Figure 4.1 Antimicrobial activity of (A) carvacrol, (B) diallyl sulfide, and (C) aluminum oxide nanoparticles (Al_2O_3 NPs) against viable but nonculturable (VBNC) *C. jejuni* cocktail at 37°C in a microaerobic condition. DMSO represents cells treated with 5% DMSO and negative control (NC) represents the untreated cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial counts ($P < 0.05$).

4.4.2. Interactions between carvacrol and diallyl sulfide

To investigate the interactions of carvacrol and diallyl sulfide, four different combinations were used in the present study, namely 1) 0.1 mg/mL carvacrol + 0.1 mg/mL diallyl sulfide, 2) 0.1 mg/mL carvacrol + 0.2 mg/mL diallyl sulfide, 3) 0.2 mg/mL carvacrol + 0.1 mg/mL diallyl sulfide, and 4) 0.2 mg/mL carvacrol + 0.2 mg/mL diallyl sulfide. The bacterial counts at various time points in all experimental groups, including single and dual antimicrobial treatment groups, are shown Figure 4.2A-E. All antimicrobial treated groups exhibited a slight reduction in bacterial population, with the highest reduction observed in samples treated with 0.2 mg/mL of carvacrol and 0.2 mg/mL of diallyl sulfide. Determination of synergistic action in the time-kill assay involves comparing the combination treatment to the most potent single agent and assessing a reduction of ≥2 log CFU/mL in bacterial cell count (Laishram et al., 2017). However, all combination groups exhibited <1 log CFU/mL reduction when compared to the corresponding single antimicrobials at the same time point, indicating the presence of an additive effect rather than a synergistic effect.

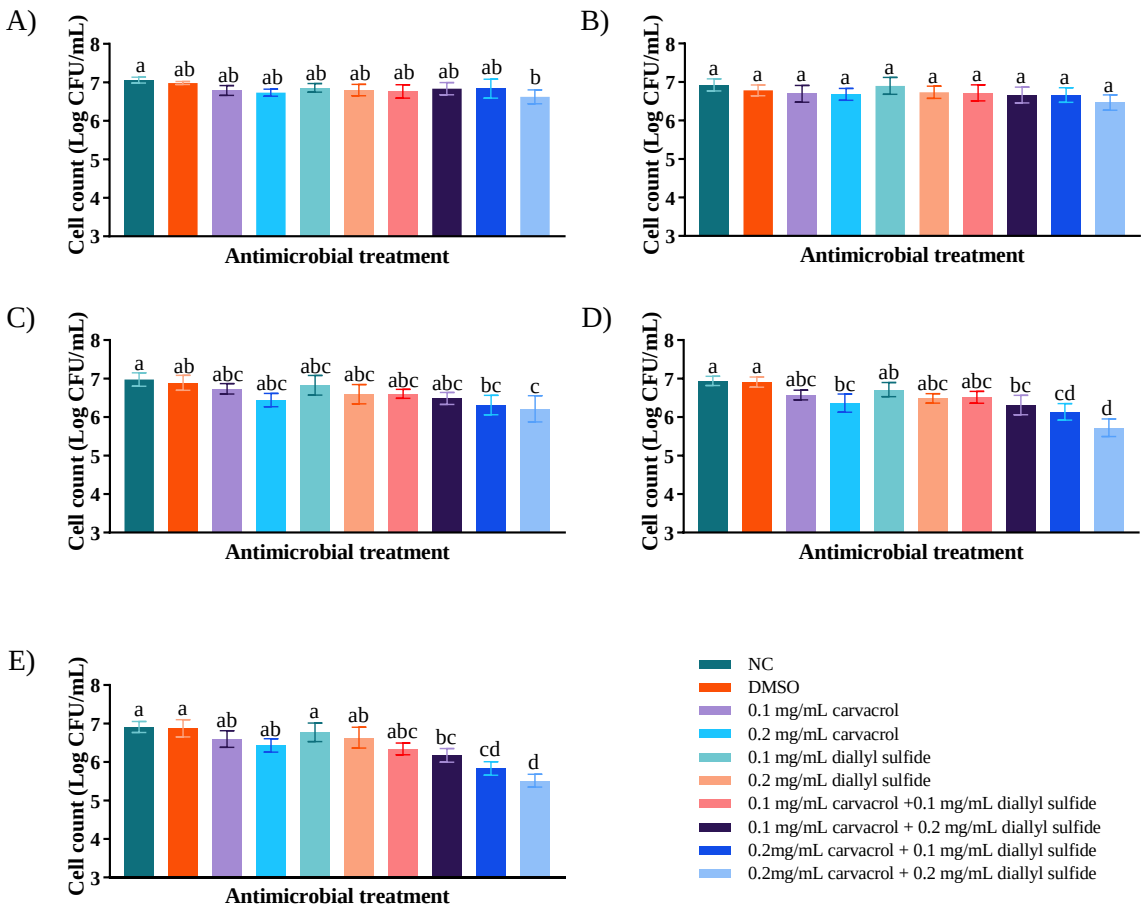

Figure 4.2 Antimicrobial activity of carvacrol (0.1 and 0.2 mg/mL) and diallyl sulfide (0.1 and 0.2 mg/mL) against VBNC *C. jejuni* cocktail at 37°C in a microaerobic condition at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents cells treated with 5% DMSO and negative control (NC) represents the untreated cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial counts ($P < 0.05$).

4.4.3. Interactions between carvacrol and Al_2O_3 NPs

In the present study, four different combinations were generated to investigate the interactions between carvacrol and Al_2O_3 NPs (Figure 4.3). These combinations consisted of two concentrations of carvacrol (0.1 and 0.2 mg/mL) and two concentrations of Al_2O_3 NPs (0.05 and 0.1 mg/mL). These combinations were selected to facilitate the evaluation of antimicrobial efficacy and explore the roles of different antimicrobials in synergistic treatment. All the combinational groups demonstrated superior antimicrobial efficacy compared to the groups treated with a single antimicrobial at 24 h. Notably, when the concentrations of 0.2 mg/mL carvacrol and 0.1 mg/mL Al_2O_3 NPs were employed together, the bacterial count exhibited a reduction of 2 log CFU/mL compared to the single antimicrobial treatment, indicating the existence of a synergistic effect. Increasing the concentration of carvacrol from 0.1 mg/mL to 0.2 mg/mL resulted in an improved antimicrobial effect when combined with 0.05 mg/mL Al_2O_3 NPs. Similar results were observed when carvacrol was combined with 0.1 mg/mL Al_2O_3 NPs. A previous study conducted by Windiasti and colleagues also reported a synergistic effect between carvacrol and ZnO NPs on culturable *C. jejuni*, with the antimicrobial efficacy being dependent on the concentration of carvacrol (Windiasti et al., 2019).

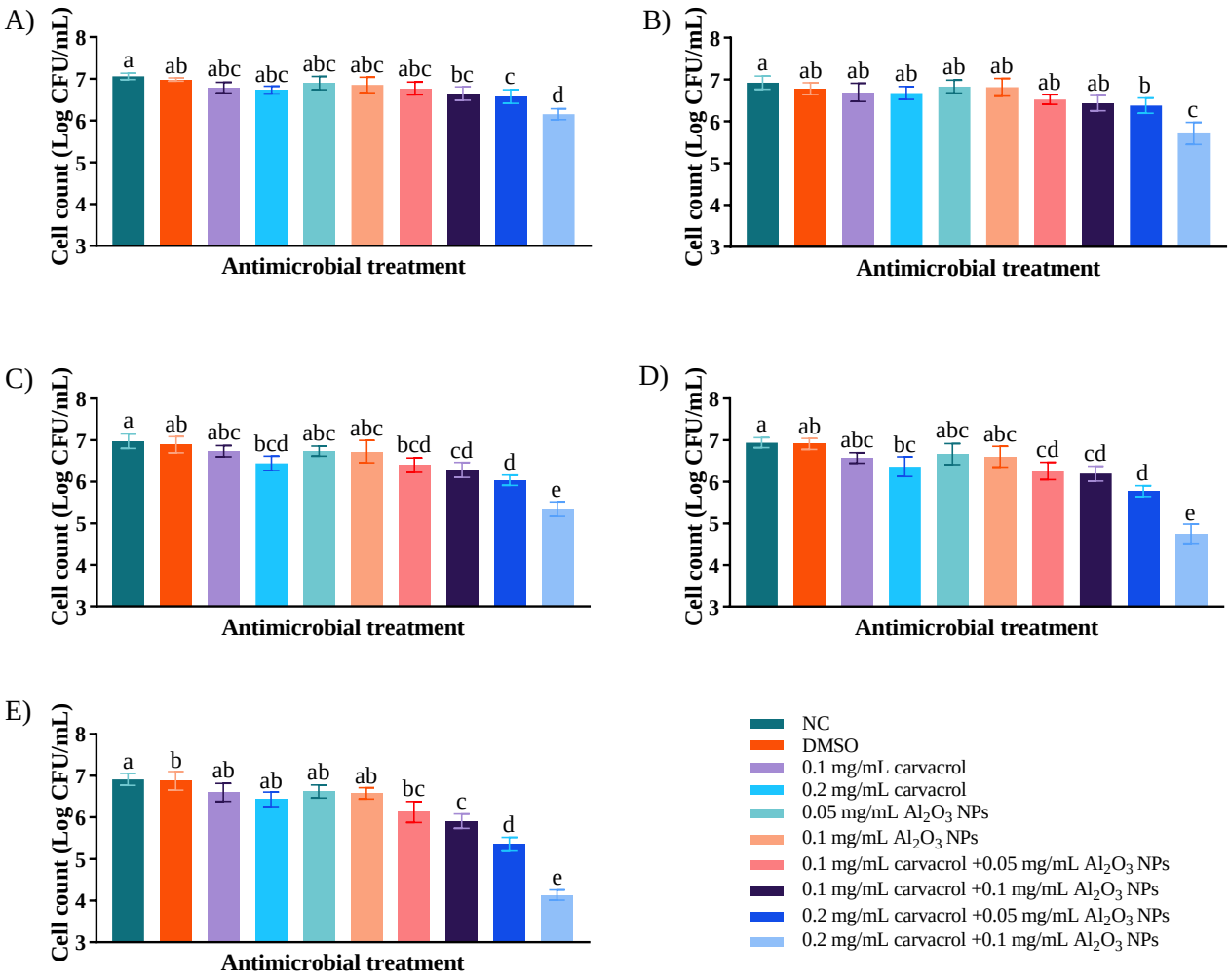

Figure 4.3 Antimicrobial activity of carvacrol (0.1 and 0.2 mg/mL) and Al_2O_3 NPs (0.05 and 0.1 mg/mL) against VBNC *C. jejuni* cocktail at 37°C in a microaerobic condition at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents cells treated with 5% DMSO and negative control (NC) represents the untreated cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial counts ($P < 0.05$).

4.4.4. Interactions between diallyl sulfide and Al_2O_3 NPs

The antimicrobial effects of combinations involving diallyl sulfide (0.1 and 0.2 mg/mL) and Al_2O_3 NPs (0.05 and 0.1 mg/mL) are shown in Figure 4.4. While exhibiting a comparable trend to the combinations of carvacrol and Al_2O_3 NPs, the samples treated with diallyl sulfide and Al_2O_3 NPs demonstrated relatively low antimicrobial activity, as evidenced by higher bacterial cell counts. This could potentially be attributed to the lower antimicrobial potency of diallyl sulfide compared to carvacrol. Nonetheless, a synergistic effect was still observed in the group treated with 0.2 mg/mL diallyl sulfide and 0.1 mg/mL Al_2O_3 NPs. This finding suggests that although carvacrol and diallyl sulfide might act on different targets, similar synergistic effects can still be achieved once combined with Al_2O_3 NPs.

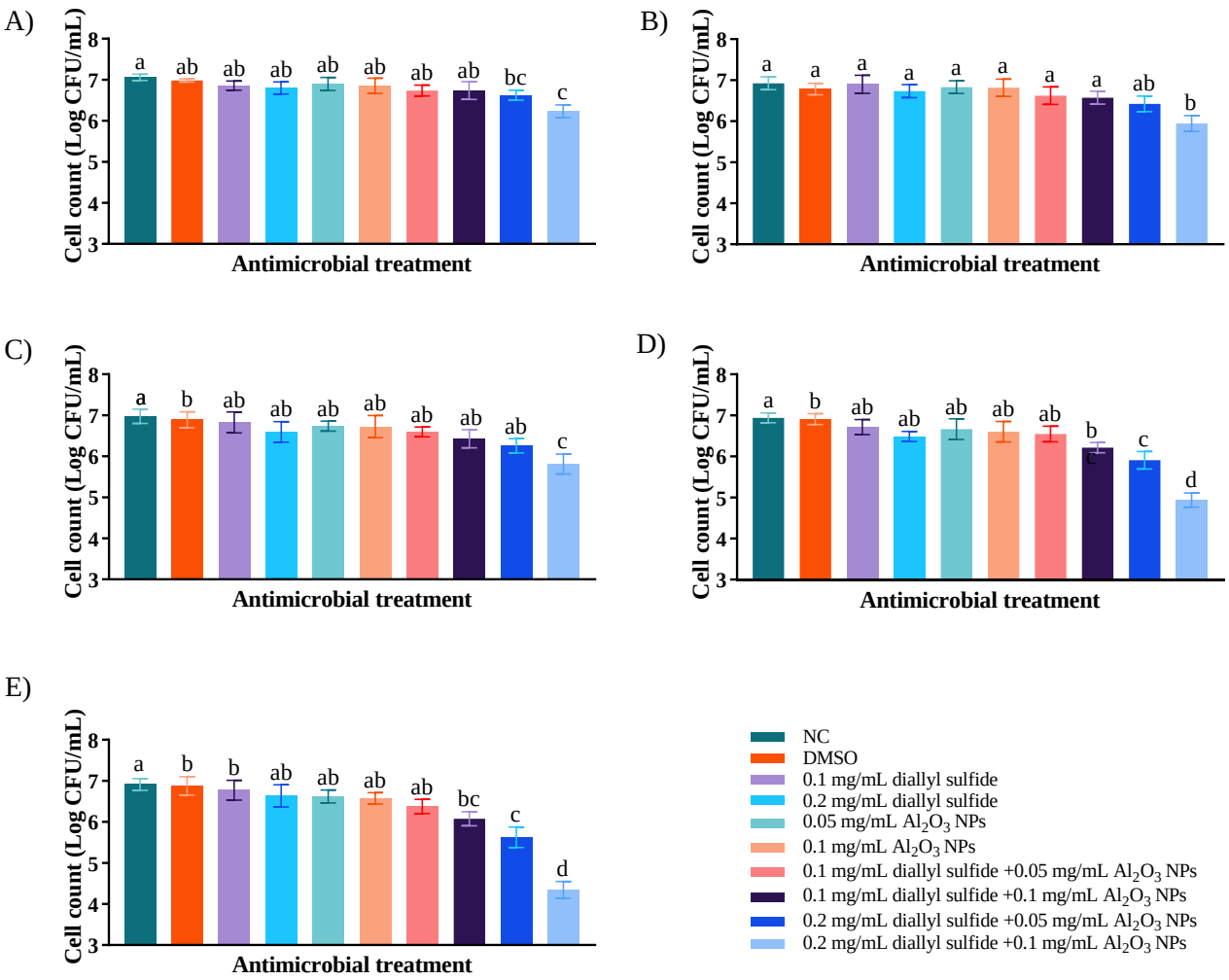

Figure 4.4 Antimicrobial activity of diallyl sulfide (0.1 and 0.2 mg/mL) and Al_2O_3 NPs (0.05 and 0.1 mg/mL) against VBNC *C. jejuni* cocktail at 37°C in a microaerobic condition at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents cells treated with 5% DMSO and negative control (NC) represents the untreated cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial counts ($P < 0.05$).

4.4.5. Interactions among carvacrol, diallyl sulfide and Al_2O_3 NPs

In addition to the dual antimicrobial treatment, the interactions among three antimicrobials were also investigated (Figure 4.5). Two different concentrations of Al_2O_3 NPs (0.05 and 0.1 mg/mL) were combined with carvacrol (0.1 mg/mL) and diallyl sulfide (0.1 mg/mL). Although both combinations exhibited a synergistic effect against VBNC *C. jejuni*, the antimicrobial efficacy of combinatorial treatment was enhanced by increasing the concentration of Al_2O_3 NPs. Specifically, combinatorial treatment with 0.05 mg/mL of Al_2O_3 NPs achieved the synergistic effect within 24 h. In comparison, similar antimicrobial effect was achieved within 12 h when the concentration of Al_2O_3 NPs was increased to 0.1 mg/mL. Notably, the individual concentrations of these antimicrobials did not yield any significant reduction compared to the control group even at the end of treatment period (24 h). Furthermore, pairwise combinations of these three antimicrobials did not generate a synergistic effect, and their remaining bacterial cell counts were significantly higher than that of the triple combinations. These results highlighted the essential contribution of all three antimicrobials to the overall synergistic effect.

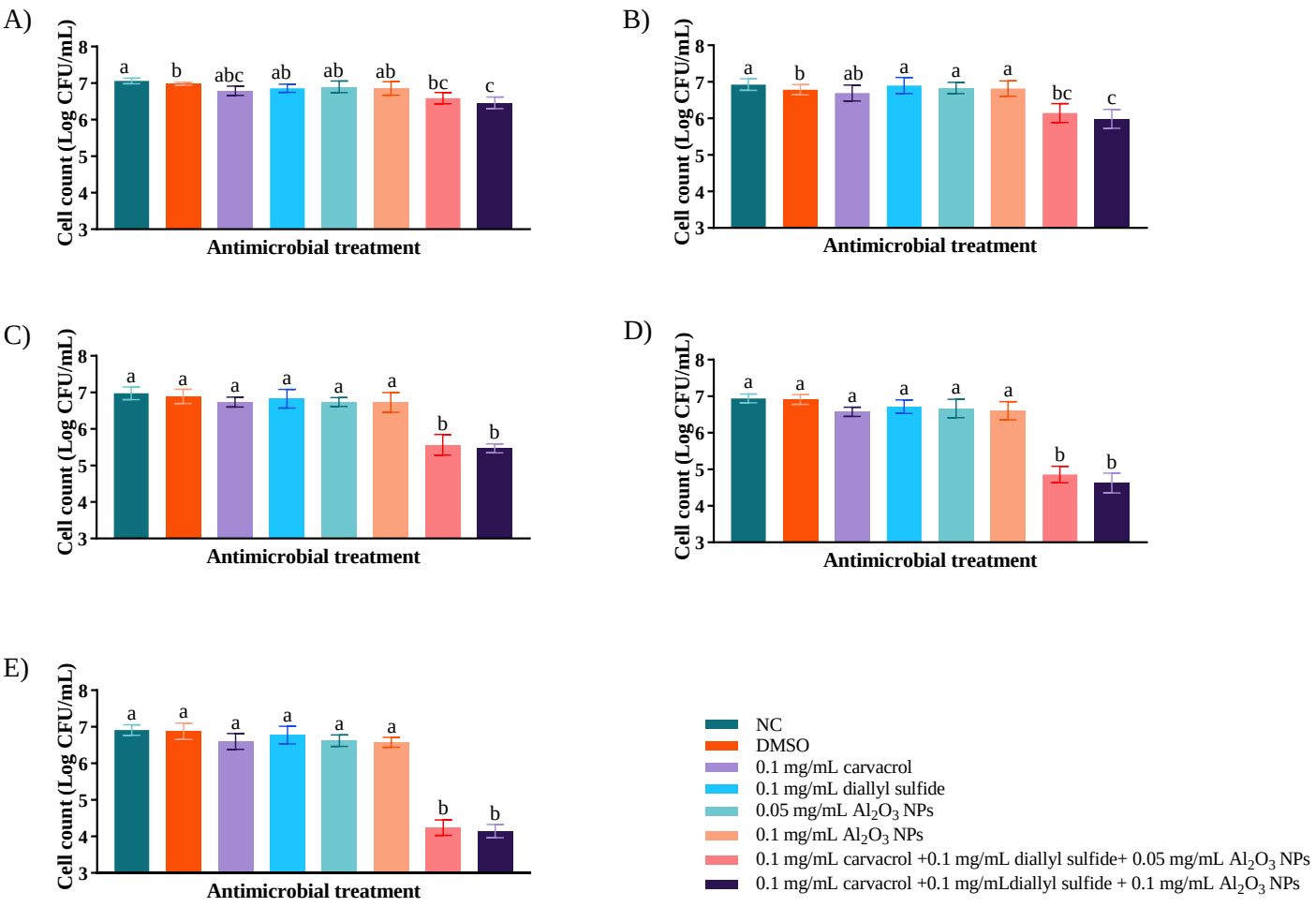

Figure 4.5 Antimicrobial activity of 0.1 mg/mL carvacrol, 0.1 mg/mL diallyl sulfide and Al_2O_3 NPs (0.05 and 0.1 mg/mL) against VBNC *C. jejuni* cocktail at 37°C in a microaerobic condition at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents cells treated with 5% DMSO and negative control (NC) represents the untreated cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial counts ($P < 0.05$).

4.5. Discussion

The antimicrobial activity of carvacrol, diallyl sulfide, and Al_2O_3 NPs against culturable *C. jejuni* has been previously investigated (Hakeem et al., 2019; Lu et al., 2012; Wagle et al., 2019; Windiasti et al., 2019; Xue et al., 2018; Yin and Cheng, 2003). However, the persistence of *C. jejuni* to these antimicrobials when in the VBNC state has not been documented yet. In the present study, higher concentrations of carvacrol and diallyl sulfide were required when they were individually employed to inhibit VBNC *C. jejuni* (Figure 4.1). For example, a previous study conducted by Windiasti and coworkers indicated that carvacrol at the concentration of ~0.02 mg/mL was sufficient to reduce 1 log CFU/mL of culturable *C. jejuni* (Windiasti et al., 2019). However, in the present study, a comparable antimicrobial effect was achieved at a concentration of ~0.4 mg/mL of carvacrol, which was ~20 times higher than that reported in their findings (Figure 4.1A). Furthermore, diallyl sulfide demonstrated a significant antimicrobial effect against *C. jejuni* at a low concentration of 0.04 mg/L as described in a previous study (Lu et al., 2012), whereas a concentration of 0.6 mg/mL of diallyl sulfide was required to achieve a similar antimicrobial effect in the present study (Figure 4.1B). The persistence of VBNC pathogens to these antimicrobials might be due to their reduced metabolic activity, halted growth, and elevated rates of efflux (Ayrapetyan et al., 2018). Al_2O_3 NPs are commonly employed at high concentrations (≥1 mg/mL) to inhibit the growth of bacterial pathogens (Gudkov et al., 2022). For example, Ansari and coauthors determined the MIC of multidrug-resistant strains of *S. aureus* to be 1 mg/mL Al_2O_3 NPs (Ansari et al., 2013). Moreover, a study by Xue and colleagues demonstrated that 16 mM Al_2O_3 NPs (~1.6 mg/mL) resulted in a reduction of ~2 log CFU/mL in culturable *C. jejuni* (Xue et al., 2018). The concentrations of Al_2O_3 NPs employed in the present study remained relatively high, albeit slightly lower compared to the aforementioned findings

(Figure 4.1C). This discrepancy could potentially be attributed to variations in the size of Al_2O_3 NPs used across different studies. In addition, the effectiveness of antimicrobial treatments might be influenced by the altered bacterial membrane fluidity. For instance, the reduced membrane fluidity in *Enterococcus faecalis* hindered the insertion of pediocin into the phospholipid bilayer, leading to an increase in antimicrobial resistance (Kumariya et al., 2015). Upon entry into the VBNC state, the altered fatty acid composition could potentially lead to changes in bacterial membrane fluidity, resulting in distinct membrane characteristics compared to culturable cells (Day and Oliver, 2004). These potential changes hold the plausibility to influence the persistence of VBNC *C. jejuni* against metal oxide nanoparticles. Notably, Al_2O_3 NPs barely induced the transcriptional changes of *C. jejuni* and the antimicrobial mechanism of Al_2O_3 NPs was mainly attributed to the interaction with cell membrane and physical disruption (Xue et al., 2018).

Previous studies have investigated the antimicrobial mechanisms of carvacrol, diallyl sulfide, and Al_2O_3 NPs. The antimicrobial efficacy of carvacrol is primarily attributed to the presence of a free hydroxyl functional group and a system of delocalized electrons (Ben Arfa et al., 2006). These distinct structural characteristics of carvacrol enable it to serve as a proton exchanger, thereby contributing to the reduction of the gradient across the cytoplasmic membrane. Consequently, this disruption leads to collapse of the proton motive force and depletion of the ATP pool, ultimately resulting in cell death. Ultee and colleagues demonstrated that carvacrol induced the efflux of K^+ and influx of H^+ in *B. cereus*, whereas cymene lacking the hydroxyl group did not elicit similar effects (Ultee et al., 2000). Moreover, the hydrophobic nature of carvacrol facilitates its accumulation in the cell membrane, leading to membrane disruption and subsequent leakage of intracellular contents. This mechanism might account for the immediate inactivation of VBNC *C.*

jejuni observed in the present study when treated with a concentration of 1.6 mg/mL carvacrol (Figure 4.1A). A similar phenomenon was observed in a study by Khan and coauthors, in which carvacrol interacted with the lipid bilayer of *E. coli* and induced structural disruption (Khan et al., 2017). Additionally, carvacrol has been proposed to inhibit efflux pumps involved in antibiotic resistance and disturb ATP-mediated cellular activities (Kachur and Suntres, 2020).

Diallyl sulfide exhibits a high reactivity towards the free sulfhydryl groups in proteins, leading to the formation of disulfide bonds. This process induces protein denaturation, enzyme inactivation, and subsequent bacterial death (Bhatwalkar et al., 2021). Furthermore, the interaction between diallyl sulfide and bacterial cell membrane compromises its integrity, resulting in the leakage of cellular contents. In a previous study conducted by Lu and colleagues, diallyl sulfide was reported to effectively eradicate both planktonic cells and sessile cells within biofilms using a concentration (~0.04 mg/mL) that was at least 100 times lower than that required for commonly used antibiotics (*i.e.*, ciprofloxacin or erythromycin) (Lu et al., 2012). In comparison, another study only used ~0.02 mg/mL carvacrol to inactivate culturable *C. jejuni*, indicating the remarkably efficient antimicrobial activity of carvacrol (Windiasti et al., 2019). Similarly, in the present study, carvacrol had a better antimicrobial activity against VBNC *C. jejuni* and resulted in a lower viable bacterial count when used alone at the same concentration (Figure 4.1). It is worth noting that the vapor pressure of diallyl sulfide at 25°C is significantly higher (9.22 mm Hg) than that of carvacrol (0.030 mm Hg) (Information, 2023; Wang et al., 2016). Consequently, a greater amount of diallyl sulfide may evaporate during treatment, leading to its relatively lower antimicrobial activity.

The antimicrobial effect of Al_2O_3 NPs primarily arises from their electrostatic interaction with bacterial outer membrane/cell wall and the aggregation of Al_2O_3 NPs on bacterial cell surface. Furthermore, the release of aluminum cations from Al_2O_3 NPs triggers the generation of reactive oxygen species, resulting in the oxidation of biopolymers and ultimately leading to bacterial cell death. According to a study by Li and coworkers, Al_2O_3 NPs were determined to have a positive surface charge at neutral pH, enabling their electrostatic interaction with the negatively charged surface of *E. coli* cells (Li and Logan, 2004). This observation was further supported by the findings of Pakrashi and colleagues, who demonstrated that Al_2O_3 NPs aggregated at the cell membrane, leading to the disruption of cellular structure in *B. licheniformis* (Pakrashi et al., 2011). Furthermore, Xue and colleagues demonstrated a slight impact of Al_2O_3 NPs on the transcriptional profile of *C. jejuni*, indicating limited induction of gene expression changes. The predominant antimicrobial effect of Al_2O_3 NPs was attributed to their interaction with the cell membrane and subsequent physical disruption (Xue et al., 2018). In the present study, the concentration of Al_2O_3 NPs required to achieve 1 log CFU/mL reduction in VBNC *C. jejuni* was ~0.2 mg/mL, indicating the high efficacy of Al_2O_3 NPs towards VBNC *C. jejuni* (Figure 4.1C).

Considering the requirement for high concentrations of antimicrobials to effectively inactivate VBNC *C. jejuni*, the use of antimicrobial combinations emerges as a promising strategy, especially if synergistic effects can be achieved. Although the antimicrobial activity of most plant-based antimicrobials can be attributed to their ability to disrupt bacterial cells, there exist several distinct mechanisms due to variations in their chemical structures and diverse cellular targets, potentially leading to synergistic interactions among them. Previous studies have reported synergism between different plant-based antimicrobials in their actions against bacterial cells. For instance, a

synergistic inhibition of *C. jejuni* was observed when cinnamon oil and curcumin were combined (Hakeem et al., 2019). Similarly, synergistic effects were observed when carvacrol was combined with (+)-*β*-pinene or when *γ*-terpinene was combined with geranyl acetate against *S. aureus* (van Zyl et al., 2010). Furthermore, combinations of plant-based antimicrobials also exhibited additive effects. For instance, van Zyl and colleagues demonstrated an additive interaction between trans-geraniol and *E*- and *Z*-(±)-nerolidol against *B. cereus* (van Zyl et al., 2010). Importantly, when combining extracts from *Salvia chamelaeagnea* and *Leonotis leonurus*, synergistic actions were observed against Gram-positive bacteria (*i.e.*, *B. cereus* and *S. aureus*), while interactions with Gram-negative bacteria (*i.e.*, *E. coli* and *K. pneumoniae*) varied, including antagonism, synergism, and additive effects, depending on the ratios tested (Kamatou et al., 2006). These studies highlight the involvement of multiple factors in antimicrobial interactions. Consequently, further investigations are warranted to elucidate the underlying mechanisms of the additive effect between carvacrol and diallyl sulfide (Figure 4.2) observed in the present study.

The synergistic interactions between metal oxide nanoparticles and plant-based antimicrobials have been documented in the previous studies. For instance, Babapour and colleagues reported the synergism between ZnO NPs and fennel essential oil, leading to enhanced antimicrobial activity against *S. aureus* and *E. coli* (Babapour et al., 2021). Similarly, Gohargani and coworkers observed synergistic effects when combining *Zataria multiflora* essential oil with TiO_2 NPs (Gohargani et al., 2020). Moreover, the combination of essential oils and metal oxide nanoparticles has shown promising results to control *C. jejuni*. Combination of carvacrol and ZnO NPs demonstrated a synergistic effect, achieving a reduction in *C. jejuni* by > 6 log CFU/mL within 24 h (Windiasti et al., 2019). In another study, Xue and coauthors combined ajoene with Al_2O_3 NPs/TiO_2 NPs and

achieved a remarkable reduction of ≥8 log CFU/mL in *C. jejuni* (Xue et al., 2018). These findings highlight the potential of plant-based antimicrobials and metal oxide nanoparticle combinations as effective antimicrobials against *C. jejuni*. In the current study, a synergistic effect was observed when Al_2O_3 NPs were combined with either carvacrol or diallyl sulfide (Figure 4.3 & Figure 4.4). However, the combination of Al_2O_3 NPs and carvacrol exhibited a higher antimicrobial activity compared to the combination of Al_2O_3 NPs and diallyl sulfide. This difference in activity could be attributed to the relatively lower antimicrobial potency of diallyl sulfide when compared to carvacrol. Furthermore, the combinations of these three antimicrobials also demonstrated a synergistic effect against VBNC *C. jejuni* (Figure 4.5). Similar results were reported by Hakeem and colleagues, who observed a reduction of more than 8 log CFU/mL in *C. jejuni* by combining cinnamon oil, ZnO NPs, and encapsulated curcumin (Hakeem et al., 2019). Overall, the combination treatment exhibited enhanced activity even at lower concentrations compared to individual treatments involving carvacrol, diallyl sulfide, and Al_2O_3 NPs alone.

4.6. Conclusion

In conclusion, VBNC *C. jejuni* demonstrated diverse persistence in response to different antimicrobials, exhibiting tolerance to plant-based antimicrobials while displaying susceptibility to Al_2O_3 NPs. The interactions among different antimicrobials resulted in an additive effect for plant-based antimicrobials and a synergistic effect when combining plant-based antimicrobials with Al_2O_3 NPs. Further investigations are required to elucidate the underlying mechanisms of these antimicrobials when used alone or in combination. This study offers valuable insights into the persistence of VBNC *C. jejuni* against diverse antimicrobials, highlighting the potential

implications of the developed antimicrobial strategies as interventions to mitigate the prevalence of VBNC *C. jejuni* or other dormant foodborne pathogens in the agri-food systems.

4.7 References

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Connecting text

In the previous chapter, the persistence of VBNC *C. jejuni* in response to different antimicrobials was extensively examined, revealing distinct patterns of tolerance and susceptibility. VBNC *C. jejuni* exhibited tolerance to plant-based antimicrobials (*i.e.*, carvacrol and diallyl sulfide), while demonstrating susceptibility to Al_2O_3 NPs. Moreover, investigations into the interactions among these antimicrobials revealed distinct patterns. When plant-based antimicrobials were combined, an additive effect was observed, suggesting that their combined action provided a cumulative inhibitory effect on VBNC *C. jejuni*. In contrast, when plant-based antimicrobials were combined with Al_2O_3 NPs, synergistic effects were observed, suggesting that the combined treatment had a greater antimicrobial efficacy than the sum of their individual effects. These findings not only emphasize the distinct response of VBNC *C. jejuni* to various antimicrobials but also highlight the potential for developing novel antimicrobial combinations to effectively combat this pathogen.

Given that the processing conditions employed in poultry production have the potential to induce *C. jejuni* into the VBNC state, it is crucial to address the associated risks to food safety and public health. In Chapter 5, we focused on assessing the antimicrobial efficacy of the aforementioned antimicrobials and their combinations against VBNC *C. jejuni* under simulated poultry processing conditions. By subjecting the VBNC pathogens to these treatments, we aimed to assess the effectiveness of the antimicrobials, explore their potential synergistic effects, and evaluate their suitability as the control measures in the poultry industry. To assess the synergistic effects of the antimicrobial combinations, both traditional time-kill assay and a mathematical model were employed, allowing us to gain a comprehensive understanding of the synergistic effects of different antimicrobial combinations against VBNC *C. jejuni*.

Overall, the findings from this chapter contribute to the development of effective mitigation strategies to control VBNC *C. jejuni* in the poultry industry. The investigation of antimicrobial interactions and the evaluation of their efficacy under poultry processing conditions provide valuable insights to the design of innovative interventions aimed at reducing the prevalence of VBNC *C. jejuni* and ensuring the safety of poultry products. The manuscript is under preparation and will be submitted shortly.

Chapter 5. A novel mathematical model for studying antimicrobial interactions against viable but non-culturable *Campylobacter jejuni* in the poultry product processing environment

5.1. Abstract

Campylobacter jejuni is a prominent pathogen associated with gastrointestinal illness and is frequently detected in poultry products. The ability of *C. jejuni* to enter the viable but non-culturable (VBNC) state is an adaptive survival strategy triggered by adverse conditions. Consequently, the processing conditions involved in poultry production can potentially induce *C. jejuni* into the VBNC state, posing risks to food safety and public health. This study aimed to evaluate the antimicrobial effectiveness of carvacrol, diallyl sulfide, and Al_2O_3 nanoparticles (NPs) and investigate their synergistic interactions against VBNC *C. jejuni* under conditions relevant to poultry processing. To investigate the synergistic effects of various combinations, both traditional time-kill assay and mathematical model were employed. The time-kill assay demonstrated the exceptional efficacy of Al_2O_3 NPs as well as its combinations with other antimicrobials. However, all combinations exhibited additive effects and their interactions were further examined using the combination index. The mathematical model effectively simulated the antimicrobial effects and interactions across all levels of inhibition. The concentration-effect curves and median-effect equations provided evidence of the potent antimicrobial activity of Al_2O_3 NPs. In addition, an additive effect was observed for the combination of carvacrol and diallyl sulfide, while synergistic interactions were noted for other binary and ternary combinations (*i.e.*, carvacrol/Al_2O_3 NPs, diallyl sulfide/Al_2O_3 NPs, and carvacrol/diallyl sulfide/Al_2O_3 NPs) using the combination index. The mathematical model presents an alternative approach to developing novel antimicrobial strategies.

Keywords: *Campylobacter*, viable but non-culturable, poultry, antimicrobial, combination index

5.2. Introduction

Foodborne illnesses are becoming an increasingly alarming issue for public health worldwide. Among these illnesses, infections resulting from *Campylobacter jejuni* have emerged as a significant global challenge, with it being ranked as the top foodborne gastroenteritis in developed countries (Kirkpatrick and Tribble, 2011). It is estimated that around 1.5 million cases of human campylobacteriosis occur in the United States annually, with the typical symptoms identified as diarrhea, fever, headache, and vomiting (CDC, 2019; Kaakoush et al., 2015). Additionally, *C. jejuni* infection may trigger immune-mediated disorders such as Guillain-Barré syndrome that causes respiratory muscle weakness and even death (Nachamkin et al., 1998). The consumption of *C. jejuni*-contaminated poultry and poultry products is recognized as the primary source of human infection, responsible for 50%-70% of human campylobacteriosis cases (Keener et al., 2004). As a commensal microbe of poultry, *C. jejuni* can colonize the intestine at levels of up to 9 log CFU/g of cecal contents (Sahin et al., 2015). Due to the contamination that frequently occurs during the processing of poultry carcasses, *C. jejuni* is commonly identified in poultry products. In fact, around 70%-90% commercially available raw poultry products in Europe and North America were contaminated with *C. jejuni* (Jorgensen et al., 2002; Willis and Murray, 1997).

Despite its fastidious nutritional requirements and susceptibility to environmental stressors, *C. jejuni* can persist and retain its infectivity from poultry farm to slaughterhouse processing and retail distribution of the final product (Tresse et al., 2017). *C. jejuni* can transit into a viable but non-culturable (VBNC) state under conditions that frequently occur during processing, such as exposure to extreme pH, fluctuations in temperature, osmotic challenges, and nutrient deprivation (Jackson et al., 2009). For example, *C. jejuni* lost its culturability but maintained its viability after

15 days of incubation in microcosm water at 4°C (Cappelier et al., 1999). Similarly, exposure to acid solution induced *C. jejuni* to the VBNC state after 2 h (Chaveerach et al., 2003). Additionally, when inoculated into a NaCl solution, *C. jejuni* became non-culturable but remained viable after 48 h of incubation (Lv et al., 2019). Bacteria in the VBNC state cannot be detected by conventional culture-based methods, but they still pose health risks since they are capable of producing virulence factors (Ayrapetyan and Oliver, 2016). This phenomenon has been observed in various VBNC pathogens, including *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* Serovar Thompson, and *Legionella pneumophila* (Alleron et al., 2013; Highmore et al., 2018; Liu et al., 2010). Moreover, VBNC bacterial pathogens might resuscitate under suitable conditions and initiate diseases (Zhao et al., 2017). In fact, VBNC pathogenic bacteria such as *E. coli* and *S.* Oranienburg were associated with several foodborne outbreaks (Asakura et al., 2002; Aurass et al., 2011; Makino et al., 2000). In the case of *C. jejuni*, the expression of virulence-associated genes, including *flaA*, *flaB*, *cadF*, *ciaB*, *cdtA*, *cdtB*, and *cdtC*, maintained at a low level when it was induced to the VBNC state. In addition, the induced VBNC cells could still invade human intestinal epithelial cells (Chaisowwong et al., 2012). Therefore, the control of VBNC *C. jejuni* in foods, especially in poultry products, is of great importance.

Bacteria were found to have a higher antimicrobial/stress tolerance when in the VBNC state. For example, VBNC *E. coli*, *Enterococcus faecalis*, and *Vibrio vulnificus* exhibited greater resistance to antibiotics compared to their culturable counterparts (Lleo et al., 2007; Nowakowska and Oliver, 2013; Ye et al., 2020). In addition, increased resistance to disinfectants (*i.e.*, ethanol and chlorine) and processing conditions (*i.e.*, thermal and low salinity) were found in VBNC *C. jejuni* and *V. parahaemolyticus* (Rowe et al., 1998; Wong and Wang, 2004), respectively. Moreover, the

intricate composition of chicken matrices, such as lipids and proteins, as well as the presence of indigenous microbiota of chicken could potentially offer protection to *C. jejuni*, subsequently diminishing the effectiveness of antimicrobial agents used (Hakeem et al., 2020). Therefore, novel methods need to be used to control VBNC *C. jejuni* under poultry processing conditions.

In recent years, combination antimicrobial treatment has gained popularity as a strategy to enhance antimicrobial efficacy, mitigate the risk of toxicity, and thwart the emergence of antimicrobial resistance. In this regard, the combination of essential oils and nanoparticles (NPs) may potentially elicit a synergistic antimicrobial effect that contributes to the development of novel strategies to control VBNC bacterial pathogens. Synergy refers to the phenomenon wherein the combined effect of two or more agents exceeds the sum of their individual effects, also known as an additive effect (Chou, 2006). Various methods have been employed to study the synergism between antimicrobials *in vitro*, which include disk diffusion, time-kill assay, checkboard method, and multiple-combination bactericidal antimicrobial testing (MCBT) (Doern, 2014). However, synergism testing methods that rely on growth inhibition, such as disk diffusion, checkboard, and MCBT, are not suitable for testing VBNC bacteria since they are in a dormant state and no longer capable of growing on the agar plates or in the broth. Numerous mathematical models have emerged over the years to facilitate the quantitative evaluation of synergy and enable high-throughput screening of drug combinations. The utilization of the combination index, derived from the Loewe additivity model, offers an advantage in assessing the interaction between drugs. Unlike effect-based methods, this approach considers the dose of each drug required to achieve a comparable quantitative effect, making it particularly valuable when dealing with drugs exhibiting nonlinear dose-effect curves (Duarte and Vale, 2022; Gayvert et al., 2017). In the meanwhile, the

combination index serves as a quantitative metric for evaluating the degree of synergy or antagonism between drugs, independent of the necessity to understand the mechanisms of action associated with each individual drug (Chou, 2006). Although this model has primarily been used in drug combination studies in the pharmaceutical and biomedical fields, there has been limited research on their applications in antimicrobial studies (Liu et al., 2022; Matthews et al., 2017; Zhou et al., 2016).

The current study aimed to assess the synergistic effects of three antimicrobials, namely carvacrol, diallyl sulfide, and Al_2O_3 NPs, on VBNC *C. jejuni* under poultry processing conditions via time-kill assay and mathematical model. This study was the first to use the mathematical model to accurately determine the antimicrobial interactions against VBNC bacterial pathogens under food processing conditions. By using this computational approach, it is possible to identify other combinations that are both synergistic and effective in combating VBNC *C. jejuni* in a high-throughput manner, which could help reduce the incidence of *Campylobacter* infections.

5.3. Materials and methods

5.3.1. Antimicrobials and preparation of antimicrobial stock solutions

Both carvacrol and diallyl sulfide were supplied by Sigma-Aldrich (St. Louis, MO, USA). A powder form of Al_2O_3 NPs (size: 40-50 nm; surface area: 32-40 m^2/g) was purchased from Alfa Aesar™ (Haverhill, MA, USA). The stock solutions for carvacrol and diallyl sulfide were prepared in dimethyl sulfoxide (DMSO, Fisher Sci., Hampton, NH, USA) at a concentration of 32 mg/mL whereas the Al_2O_3 NPs stock solution (20 mg/mL) was prepared by adding Al_2O_3 NPs powder to double distilled water (ddH_2O).

5.3.2. Bacterial cultivation and induction of the VBNC state

5.3.2.1. Bacterial strains and cultivation

Four *C. jejuni* strains were used in this study, including F38011 (clinical isolate), 81-116 (clinical isolate), NCTC11168 (clinical isolate), and ATCC 33560 (bovine isolate). The strains were stored at -80°C in Mueller-Hinton broth (MHB; BD Difco, Franklin Lakes, NJ, USA) containing 20% (v/v) glycerol (Fisher Sci.) and 10% (v/v) defibrinated sheep blood (Cedarlane, Burlington, ON, Canada). Each bacterial glycerol stock (~50 µL) was streaked onto MH agar (BD Difco) containing 5% (v/v) defibrinated sheep blood. The agar plate was then incubated and routinely subcultured at 37°C with microaerobic conditions of 85% N_2, 10% CO_2, and 5% O_2. The overnight culture was prepared by suspending *C. jejuni* colonies in MH broth, followed by incubation at 37°C for 16 h under microaerobic conditions with constant shaking (175 rpm).

5.3.2.2. Induction and validation of the VBNC state

The protocol used to induce VBNC *C. jejuni* by osmotic pressure was previously described by Lv and coauthors (Lv et al., 2019). Briefly, a cocktail of four *C. jejuni* strains was created by combining equal volume of their overnight cultures at an initial concentration of ~9 log CFU/mL. This cocktail mixture was centrifuged at 15,000 ×*g* for 5 min and washed with phosphate-buffered saline (PBS) to eliminate excess nutrients. The cell pellet was added to 7% (w/v) NaCl solution, resulting in a concentration of ~ 8 log CFU/mL for VBNC induction. The bacterial culture was incubated at 37°C under microaerobic conditions with constant shaking for 48 h, during which samples were periodically collected and assessed for culturable and viable cells using the plating assay and propidium monoazide (PMA)-quantitative PCR, respectively. When no colony is formed on the agar plates, it can be inferred that the number of culturable cells in the suspension was too

low to be detected (less than 0.5 CFU/mL) and all viable cells were assumed to be in the VBNC state. The obtained VBNC cells were then centrifuged at 15,000 ×*g* for 10 min and suspended in the simulated chicken juice before antimicrobial treatment.

5.3.3. Preparation of simulated poultry processing environment

A simulated chicken processing environment was prepared according to the frozen-thaw method described by Birk and coauthors with modifications (Birk et al., 2004). In brief, whole chickens without giblets purchased from a local grocery store in Montreal were frozen at -20°C and thawed overnight at room temperature. The collected exudates were centrifuged at 12,000 ×*g* for 10 min to remove large debris. The obtained supernatant was then sterilized using a 0.22-μm polyethersulfone syringe filter (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C as aliquots. Chicken juice was diluted with MH broth at a percentage of 5% (v/v) to simulate the poultry processing environment.

5.3.4. PMA treatment and qPCR analysis

PMA-qPCR was used to determine the number of remaining VBNC *C. jejuni* in antimicrobial treated samples and the protocol was adapted from our previous study (Zhang and Lu, 2023). PMA (Biotium Inc., Hayward, CA, USA) was dissolved in sterile ddH_2O to obtain a stock solution with a concentration of 1.5 mM and stored at -20°C. For PMA treatment, the antimicrobial treated samples (1 mL) were centrifuged at 15,000 ×*g* for 5 min at room temperature, and the resulting pellet was then resuspended in 1 mL of PBS. The PMA (5 μL) and PMA enhancer (100 μL) (5× solution, Biotium Inc.) were mixed with 395 μL of the prepared bacterial suspension, and the mixture was incubated in the dark with constant shaking for 10 min at room temperature.

Afterwards, the samples were exposed to a 600-W halogen light source (Smith victor, Bartlett, IL, USA) 20 cm away while placed on ice with constant shaking for another 10 min. Finally, the mixture was washed once with sterile ddH_2O and boiled at 100°C for 10 min to release the genomic DNA.

The qPCR amplification was carried out using the Mx3005P Real-Time PCR System (Agilent, Santa Clara, CA, USA). The amplification protocol involved an initial denaturation at 50°C for 2 min, followed by another denaturation at 95°C for 10 min, and then 40 cycles of amplification consisting of 15 s at 95°C and 1 min at 60°C. The reaction mixture contained 2 μL of template DNA, 10 μL of SensiFAST SYBR Lo-ROX Kit (Bioline, Memphis, TN, USA), 100 nM each of forward (5'-GAGTAAGCTTGCTAAGATTAAAG-3') and reverse primer (5'-AAGAAGTTTTAGAGTTTCTCC-3') targeting the single-copy gene *rpoB* (Lv et al., 2019), and sterile ddH_2O to a final volume of 20 μL. The experiment was conducted in triplicate, and the negative control (2 μL of sterile ddH_2O) was included in all cases.

5.3.5. Investigation of antimicrobial interactions using the time-kill method

The efficacy of antimicrobial agents against VBNC *C. jejuni* was evaluated using the time-kill method. Various single antimicrobials (Table 5.1) and antimicrobial combinations (Table 5.2) were combined with the VBNC *C. jejuni* cocktail. The survived VBNC *C. jejuni* was quantified at specific time intervals (0, 4, 8, 12, and 24 h) using PMA-qPCR. To investigate the interactions between antimicrobials, the results of individual antimicrobial treatment were compared with those of combined antimicrobial treatment, with the synergistic effect defined as a difference of ≥2 log CFU/mL.

Table 5.1 Working concentrations of single antimicrobials in time-killing method.

Antimicrobials	Concentrations of antimicrobials (mg/mL)
Carvacrol	0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.6
Diallyl sulfide	0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.6
Al_2O_3 NPs	0, 0.05, 0.1, 0.2, 0.3, 0.4

Table 5.2 Working concentrations of combined antimicrobials in time-killing method.

Concentration (mg/mL)		
Carvacrol	Diallyl sulfide	Al_2O_3 NPs
0.1	0.1	0
0.1	0.2	0
0.2	0.1	0
0.2	0.2	0
0.1	0	0.05
0.1	0	0.1
0.2	0	0.05
0.2	0	0.1
0	0.1	0.05
0	0.1	0.1
0	0.2	0.05
0	0.2	0.1
0.1	0.1	0.05
0.1	0.1	0.1

5.3.6. Investigation of antimicrobial interactions using the mathematical model

VBNC *C. jejuni* cocktail was subjected to varying concentrations of carvacrol, diallyl sulfide, and Al_2O_3 NPs or their combinations under simulated poultry processing conditions (Table 5.3). Each antimicrobial/antimicrobial combination was mixed with VBNC *C. jejuni* and incubated at 37°C for 24 h with constant shaking under microaerobic conditions. PMA-qPCR was used to determine the antimicrobial effect based on the logarithmic reduction in bacterial counts.

The median-effect equation based on mass action law was used to assess the combined effect of carvacrol, diallyl sulfide, and Al_2O_3 NPs (Chou and Talalay, 1984). The equation is expressed as $f_a/f_u=(D/D_m)^m$, where f_a represents the fraction of inactivated VBNC *C. jejuni* and f_u represents the fraction of the remaining VBNC *C. jejuni*. D denotes the dose of the antimicrobial administered, D_m represents the median-effect dose, and m indicates the sigmoidicity of the dose-effect curve. The values of 'm' can be quantitatively categorized as hyperbolic, sigmoidal, or flat sigmoidal when m=1, >1, and <1, respectively (Chou, 2006).

Taking the logarithmic form of the median-effect equation gives $\log[f_a/(1-f_a)]=m \log(D)-m \log(D_m)$, which allows for the determination of the slope 'm' and the x-intercept of $\log(D_m)$ by plotting $y=[f_a/(1-f_a)]$ against $x=\log(D)$. Concentration-effect curves and median-effect plots were automatically generated using the CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). The values of D_m and m were then used for the computerized calculation of combination index (CI) values using CompuSyn. A CI of <1 indicates synergy, which means a greater than expected additive effect when two agents are combined. A CI of 1 indicates additive effects, while a CI of >1 indicates antagonism, which means a less than expected additive effect (Chou, 2006).

Table 5.3 Working concentrations of combined antimicrobials in combination index method.

Concentration (mg/mL)			Ratio
Carvacrol	Diallyl sulfide	Al_2O_3 NPs	
0.05	0.05	0	1:1
0.1	0.1	0	1:1
0.2	0.2	0	1:1
0.4	0.4	0	1:1
0.6	0.6	0	1:1
0.8	0.8	0	1:1
0.05	0	0.025	2:1
0.1	0	0.05	2:1
0.2	0	0.1	2:1
0.4	0	0.2	2:1
0.6	0	0.3	2:1
0.8	0	0.4	2:1
0	0.05	0.025	2:1
0	0.1	0.05	2:1
0	0.2	0.1	2:1
0	0.4	0.2	2:1
0	0.6	0.3	2:1
0	0.8	0.4	2:1
0.025	0.025	0.025	1:1:1
0.05	0.05	0.05	1:1:1
0.1	0.1	0.1	1:1:1
0.2	0.2	0.2	1:1:1
0.3	0.3	0.3	1:1:1
0.4	0.4	0.4	1:1:1

5.4. Results and discussion

5.4.1. Antimicrobial effect of single antimicrobial against VBNC *C. jejuni* by time-kill assay

The efficacy of both individual and combined antimicrobial treatments against VBNC *C. jejuni* was investigated using the time-kill assay. The number of VBNC *C. jejuni* remained relatively stable when these antimicrobials were administered at low concentrations (Figure 5.1). Moreover, high concentrations of antimicrobials only led to a reduction in bacterial count ranging from 1-2 log CFU/mL. Specifically, significant bacterial reduction (>1 log CFU/mL) was achieved only in samples treated with carvacrol at concentrations of 0.8 and 1.6 mg/mL, diallyl sulfide at a concentration of 1.6 mg/mL, and Al_2O_3 NPs at concentrations of 0.3 and 0.4 mg/mL. Notably, when comparable antimicrobial effects were observed, higher concentrations of plant-based antimicrobials (carvacrol and diallyl sulfide) were required, indicating the superior antimicrobial activity of Al_2O_3 NPs. Furthermore, when both carvacrol and diallyl sulfide were applied at a concentration of 1.6 mg/mL, the carvacrol-treated group exhibited ~1 log CFU/mL lower bacterial count at 24 h, suggesting that carvacrol exhibited greater antimicrobial activity compared to diallyl sulfide. DMSO was used as a solvent in the present study and there was no significant difference in bacterial viability between the negative control and DMSO group. These findings suggest that the observed antimicrobial effects of carvacrol and diallyl sulfide can be attributed solely to their inherent properties, independent of any influence from DMSO.

The effective concentrations of carvacrol and diallyl sulfide employed in this study were significantly higher compared to the previous investigations. For instance, carvacrol at a lower concentration of 0.02 mg/mL demonstrated the capability to induce a reduction of ~1 log CFU/mL in culturable *C. jejuni* (Windiasti et al., 2019). In a separate investigation by Lu and colleagues,

diallyl sulfide at a concentration of 0.04 mg/mL inactivated both planktonic and sessile cells of *C. jejuni* (Lu et al., 2012). This discrepancy can be attributed to the relatively low metabolic activity exhibited by *C. jejuni* in the VBNC state. VBNC cells exhibit various physiological characteristics distinct from their culturable counterparts. They reduce their metabolic activity and cease growth, enabling them to conserve energy and withstand different forms of stresses (Ayrapetyan et al., 2018). Additionally, the presence of chicken juice in the experimental setup may contribute to higher antimicrobial concentrations. The lipids and proteins present in chicken juice could potentially interact with the added antimicrobials, resulting in reduced antimicrobial availability and increased persistence of VBNC *C. jejuni*. A similar phenomenon was observed in the study conducted by Piskernik and coauthors, where the antimicrobial effect of rosemary extract was four times greater in MHB compared to chicken meat juice for the inactivation of culturable *C. jejuni* (Piskernik et al., 2011). Consequently, the use of antimicrobial combinations becomes imperative to reduce the required dose of antimicrobials, particularly if synergistic effects are present.

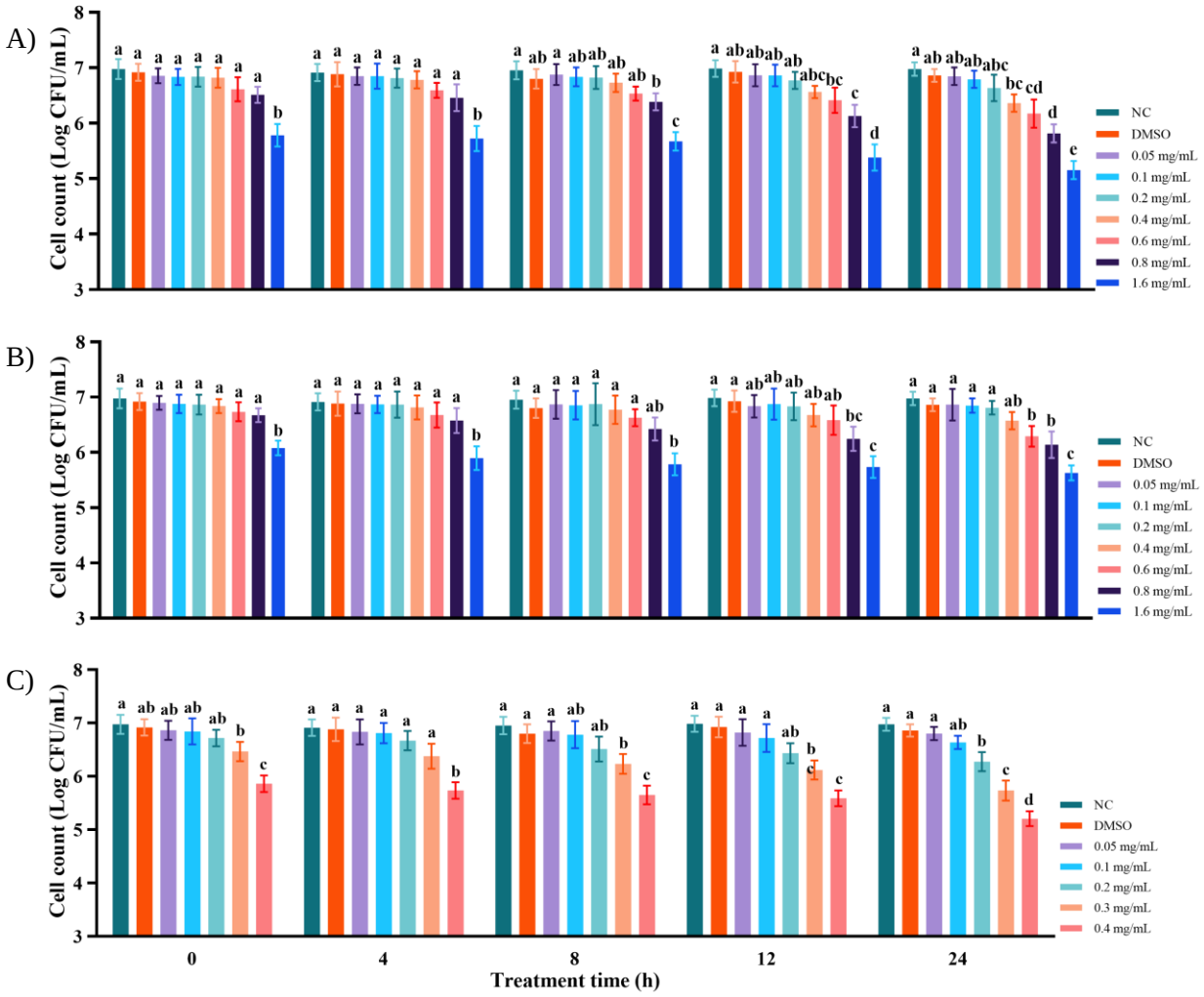

Figure 5.1 Antimicrobial activity of (A) carvacrol, (B) diallyl sulfide, and (C) aluminum oxide nanoparticles (Al_2O_3 NPs) against viable but nonculturable (VBNC) *C. jejuni* cocktail in chicken juice under microaerobic conditions at 37°C. DMSO represents bacterial cells treated with 5% DMSO and negative control (NC) represents the untreated bacterial cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial count ($P < 0.05$).

5.4.2. Investigation of synergistic antimicrobial effect by time-kill assay

The time-kill assay has also been used to investigate antimicrobial interactions involving carvacrol, diallyl sulfide and Al_2O_3 NPs. Synergism is defined as a significant decrease of ≥2 log CFU/mL in bacterial counts observed in the combined group, when compared to the most effective single antimicrobial-treated group. Conversely, antagonism is characterized by an increase of ≥2 log CFU/mL in the combination group, indicating reduced efficacy compared to the most potent single agent. Furthermore, the additive effect was identified when the changes in bacterial count fell within the range of 2 log CFU/mL.

The combination groups comprised both binary and ternary combinations of carvacrol, diallyl sulfide, and Al_2O_3 NPs. The specific concentrations for each combination are provided in Table 5.2. There was no significant difference in bacterial count between the negative control group and all the groups treated with carvacrol and diallyl sulfide throughout the entire experimental period (Figure 5.2). This result indicates the presence of an additive effect between these two antimicrobials when employed for the treatment of VBNC *C. jejuni* in the poultry processing environment.

The bacterial count remained relatively stable when using carvacrol at a concentration of 0.1 mg/mL and Al_2O_3 NPs at a concentration of 0.05 mg/mL, as no significant antimicrobial effect was observed even at the end of the experiment (Figure 5.3). In contrast, other combinations with higher concentrations of antimicrobials resulted in significantly lower bacterial counts compared to the negative control group. The most potent antimicrobial effect was observed when employing carvacrol at a concentration of 0.2 mg/mL and Al_2O_3 NPs at a concentration of 0.1 mg/mL.

However, the bacterial count in this combination was ~1 log CFU/mL lower when compared to groups treated with either 0.2 mg/mL carvacrol or 0.1 mg/mL Al_2O_3 NPs alone, indicating the presence of an additive effect rather than a synergistic effect. Similar trends were observed in the combinations of diallyl sulfide and Al_2O_3 NPs. However, these combinations required longer treatment durations (12 h) to achieve a comparable antimicrobial effect compared to the groups treated with carvacrol and Al_2O_3 NPs (4 h) (Figure 5.4). Furthermore, no synergistic effect was observed in these groups either. Groups treated with the combination of carvacrol, diallyl sulfide, and Al_2O_3 NPs displayed bacterial counts that were comparable to samples treated with dual antimicrobial combinations, indicating an additive interaction among these three antimicrobials (Figure 5.5).

The combinations of carvacrol and Al_2O_3 NPs, diallyl sulfide and Al_2O_3 NPs, as well as the combination of these three antimicrobials all exhibited a synergistic effect against VBNC *C. jejuni* in our unpublished study where the antimicrobial experiment was conducted in PBS. In the present study, although several combinations exhibited antimicrobial activities and achieved reductions in bacterial count of ~1.5 log CFU/mL, only additive effects were observed for these combinations. It is important to note that the determination of synergy is based on the specific criteria of the time-kill assay, which requires a bacterial reduction of ≥2 log CFU/mL for the combination compared to the most effective single antimicrobial treatment. The difference in observed effects could potentially be attributed to the presence of chicken juice, which may reduce the effectiveness of these antimicrobials and offer protection to VBNC *C. jejuni*, resulting in an over- or under-estimation of synergism. Consequently, mathematical modeling was employed to further investigate the interactions between these antimicrobials.

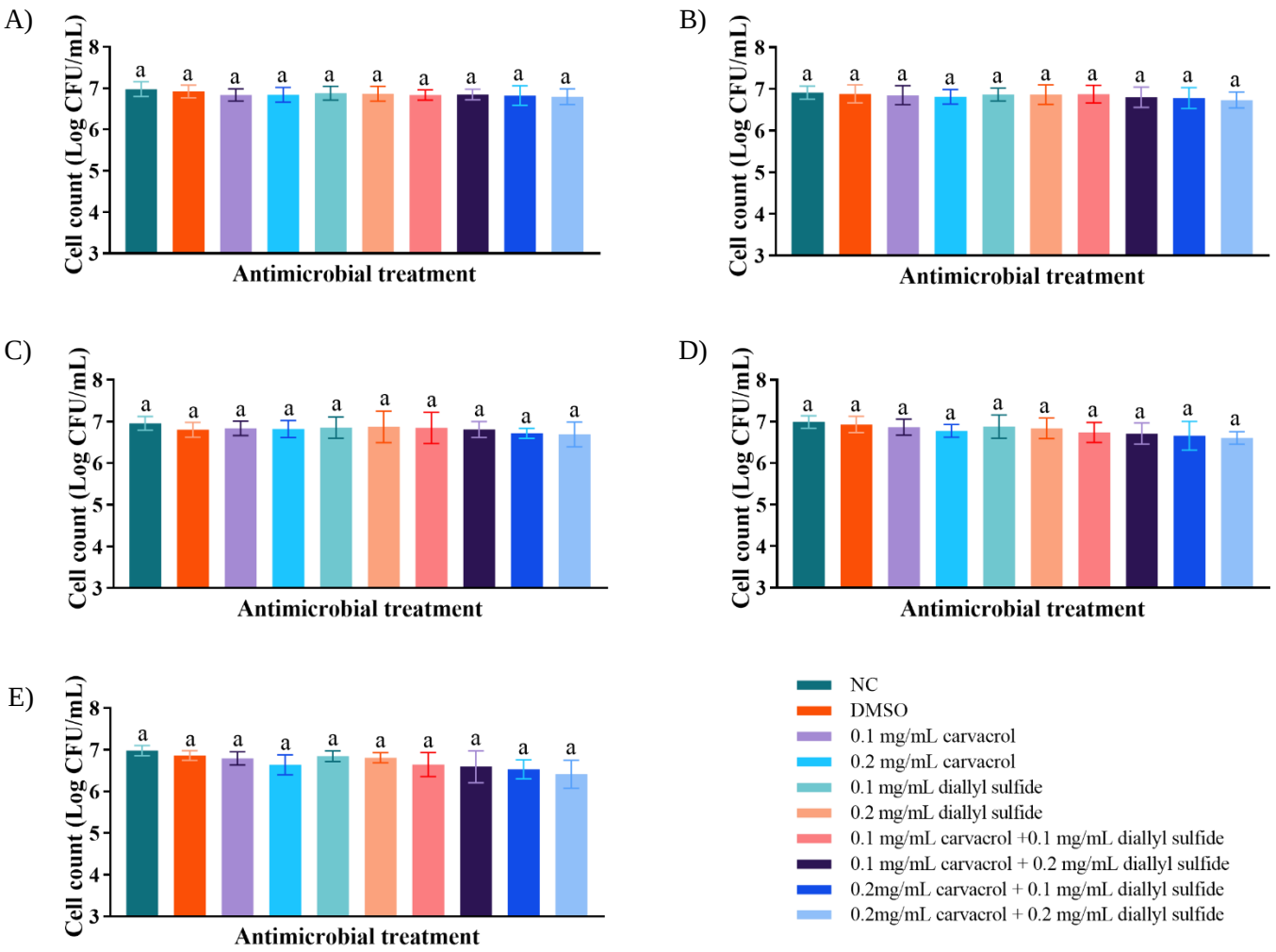

Figure 5.2 Antimicrobial activity of carvacrol (0.1 and 0.2 mg/mL) and diallyl sulfide (0.1 and 0.2 mg/mL) against VBNC *C. jejuni* cocktail in chicken juice under microaerobic conditions at 37°C at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents bacterial cells treated with 5% DMSO and negative control (NC) represents the untreated bacterial cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial count ($P < 0.05$).

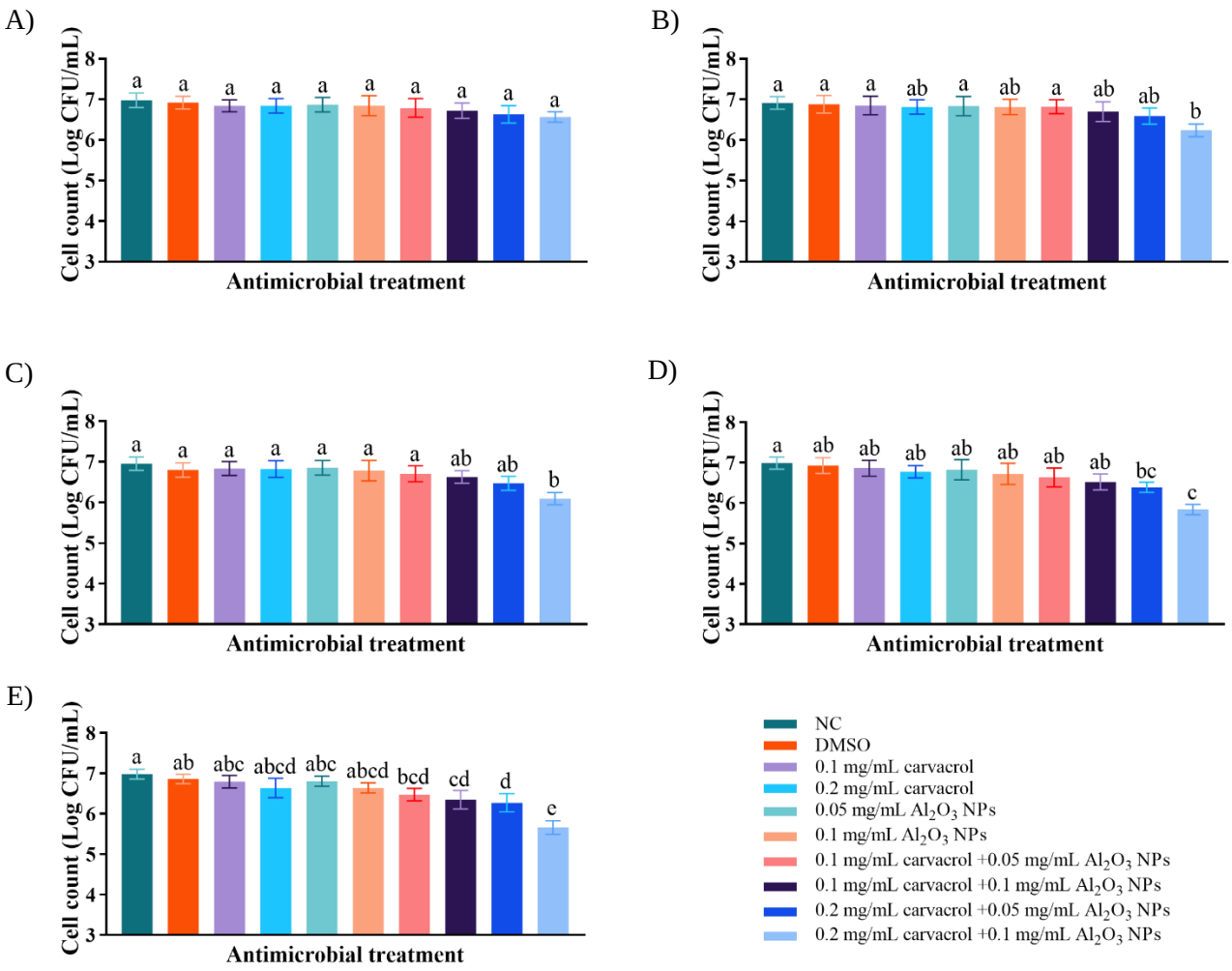

Figure 5.3 Antimicrobial activity of carvacrol (0.1 and 0.2 mg/mL) and Al_2O_3 NPs (0.05 and 0.1 mg/mL) against VBNC *C. jejuni* in chicken juice under microaerobic conditions at 37°C at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents bacterial cells treated with 5% DMSO and negative control (NC) represents the untreated bacterial cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial count ($P < 0.05$).

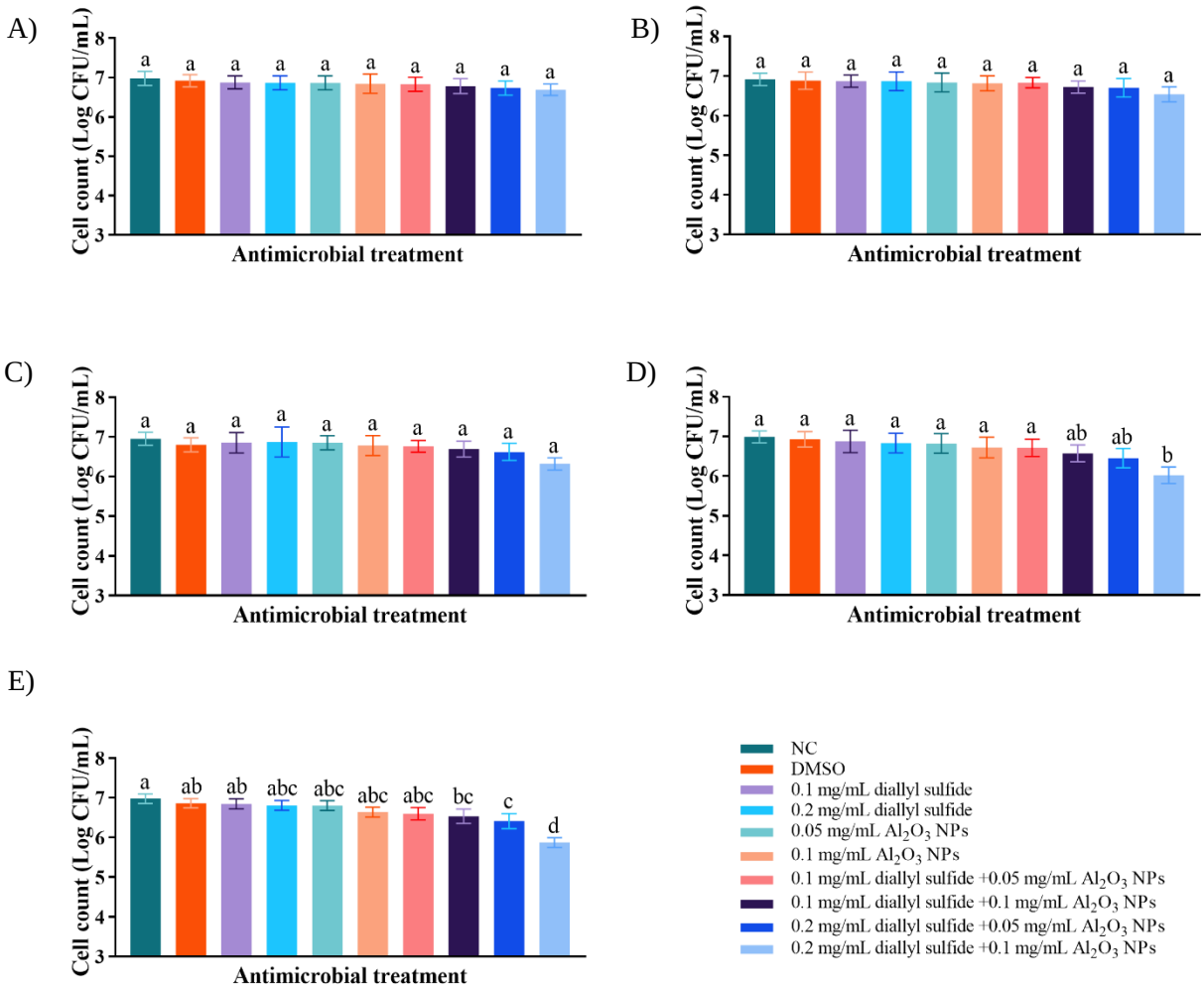

Figure 5.4 Antimicrobial activity of diallyl sulfide (0.1 and 0.2 mg/mL) and Al_2O_3 NPs (0.05 and 0.1 mg/mL) against VBNC *C. jejuni* cocktail in chicken juice under microaerobic conditions at 37°C at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents bacterial cells treated with 5% DMSO and negative control (NC) represents the untreated bacterial cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial count ($P < 0.05$).

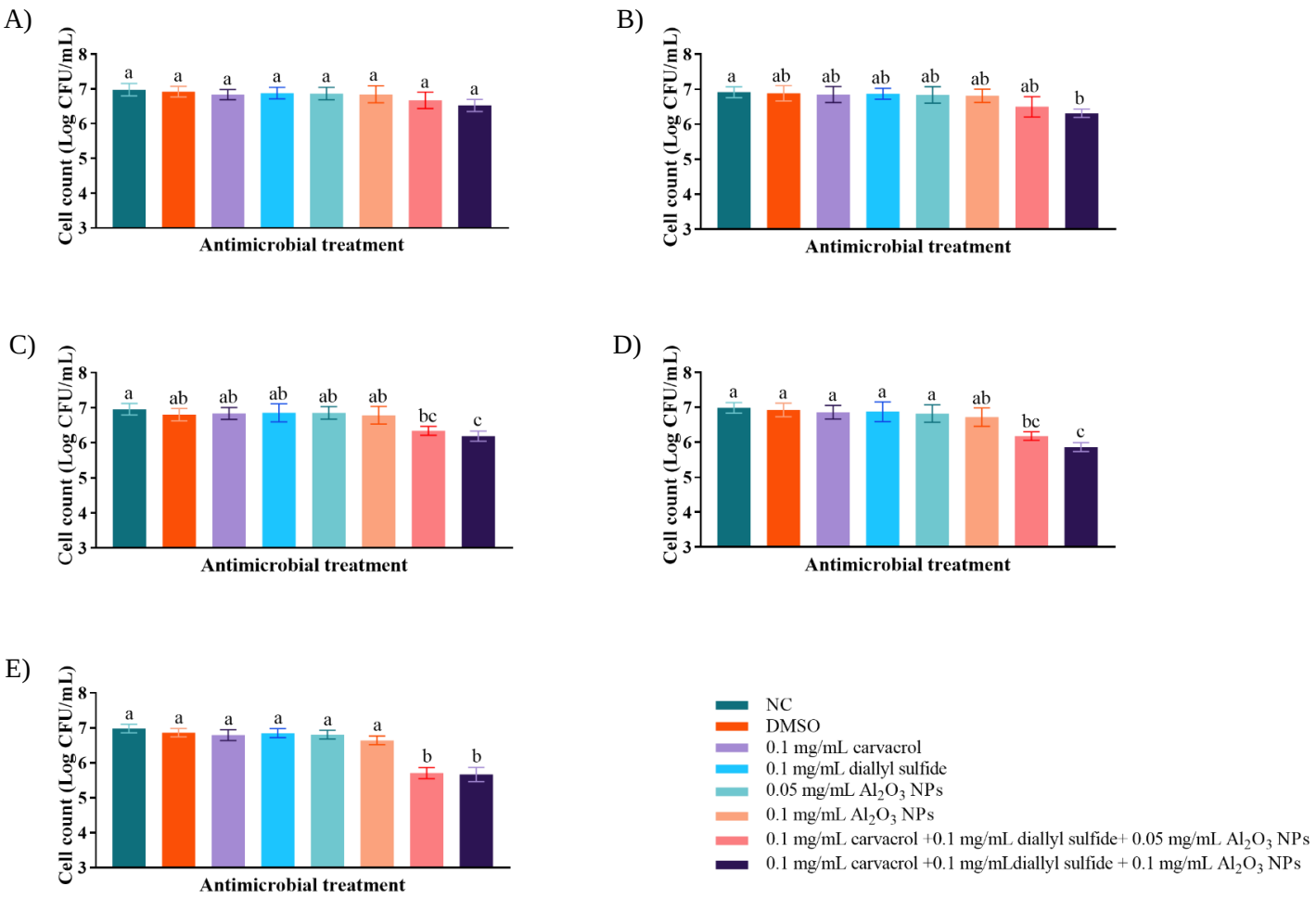

Figure 5.5 Antimicrobial activity of 0.1 mg/mL carvacrol, 0.1 mg/mL diallyl sulfide and Al_2O_3 NPs (0.05 and 0.1 mg/mL) against VBNC *C. jejuni* cocktail in chicken juice under microaerobic conditions at 37°C at different time points: (A) 0 h, (B) 4 h, (C) 8 h, (D) 12 h and (E) 24 h. DMSO represents bacterial cells treated with 5% DMSO and negative control (NC) represents the untreated bacterial cells. Error bars represent the standard deviations (n = 3, duplicates). The results are expressed as the mean ± standard deviation of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Sample groups labeled with different letters indicate that they have significantly different bacterial count ($P < 0.05$).

5.4.3. Combination index used to study the antimicrobial interactions

The combination index based on the median-effect principle is a widely used approach for analyzing drug interactions in pharmaceutical and biomedical studies. However, its application to investigate the interactions among antimicrobials has been limited. A notable example of utilizing this approach was demonstrated by Belardo and coauthors, who identified the synergistic effect between nitazoxanide and neuraminidase on inactivating influenza A viruses using the combination index (Belardo et al., 2015). Similarly, Liu and colleagues conducted a study employing the combination index to evaluate the interactions between combined natural antimicrobials such as thymol, oregano, carvacrol against various poultry-borne pathogens including *E. coli, S.* Pullorum, and *Klebsiella pneumonia* (Liu et al., 2022). These studies highlight the potential of the combination index in investigating antimicrobial interactions, thereby contributing to our understanding of synergistic effects among carvacrol, diallyl sulfide and Al_2O_3 NPs. In the present study, fixed ratios of these antimicrobials were employed. Specifically, ratios of 1:1, 2:1, 2:1 and 1:1:1 were used for carvacrol+ diallyl sulfide, carvacrol+ Al_2O_3 NPs, diallyl sulfide+ Al_2O_3 NPs, and carvacrol+ diallyl sulfide+ Al_2O_3 NPs, respectively (Table 5.3). This approach enables the combination mixtures to exhibit characteristics similar to single antimicrobials, facilitating the computerized simulation of dose-effect curves, median-effect plots, and combination index (CI) plots across the entire range of interactions. By maintaining constant ratios, we gain valuable insights into the effects and interactions of these antimicrobials, allowing for a comprehensive analysis of their combined efficacy.

5.4.3.1. Concentration-effect curves and median-effect equations

The establishment of the potency and slope of the concentration-effect curves for individual antimicrobials (carvacrol, diallyl sulfide, and Al_2O_3 NPs) was necessary prior to determining their potential synergistic activities. Using the CompuSyn software, concentration-effect curves and median-effect plots were generated for each antimicrobial, allowing for the calculation of dose-effect parameters [(D_m), (m), and (r)] for both individual antimicrobials and their combinations. These parameters were further utilized to calculate the combination index (CI).

The concentration-effect curves for all three antimicrobials exhibited similar sigmoidal trends (Figure 5.6). Notably, the curve for Al_2O_3 NPs displayed a sharper slope compared to the others, indicating its high potency. Regarding the combined antimicrobials, the combinations involving Al_2O_3 NPs showed similar shapes in the concentration-effect curves, whereas the combination of carvacrol and diallyl sulfide exhibited a distinct pattern separate from them. These findings highlight the differential effects and interactions among the antimicrobial combinations, emphasizing the unique properties of Al_2O_3 NPs when combined with carvacrol and diallyl sulfide.

The median-effect plot of each antimicrobial provides valuable insights into their potency (Figure 5.7). In this study, the x intercept of the median-effect plot, corresponding to the median-effect dose (D_m, equivalent to IC_{50}), was found to be 0.24 mg/mL for carvacrol, 0.40 mg/mL for diallyl sulfide, and 0.11 mg/mL for Al_2O_3 NPs when used individually. These results indicate the high potency of Al_2O_3 NPs compared to other antimicrobials. When assessing the antimicrobial combinations, the D_m values of carvacrol+ diallyl sulfide, carvacrol+ Al_2O_3 NPs, diallyl sulfide+ Al_2O_3 NPs, and carvacrol+ diallyl sulfide+ Al_2O_3 NPs were determined to be 0.30, 0.15, 0.15 and

0.11 mg/mL, respectively. By analyzing the slopes (m values) of the concentration-effect curves, we can assess how changes in the dose of antimicrobials or their combinations influence the antimicrobial effect. The m values for carvacrol, diallyl sulfide, and Al_2O_3 NPs ranged from 1.63 to 2.83, with the combinations of all three antimicrobials exhibiting the highest slope. This indicates that even a small increase in the concentration of the combination leads to a significant decrease in bacterial viability. Additionally, the regression coefficients (r) for all curves exceed 0.974, demonstrating the excellent fit of the method to the experimental data.

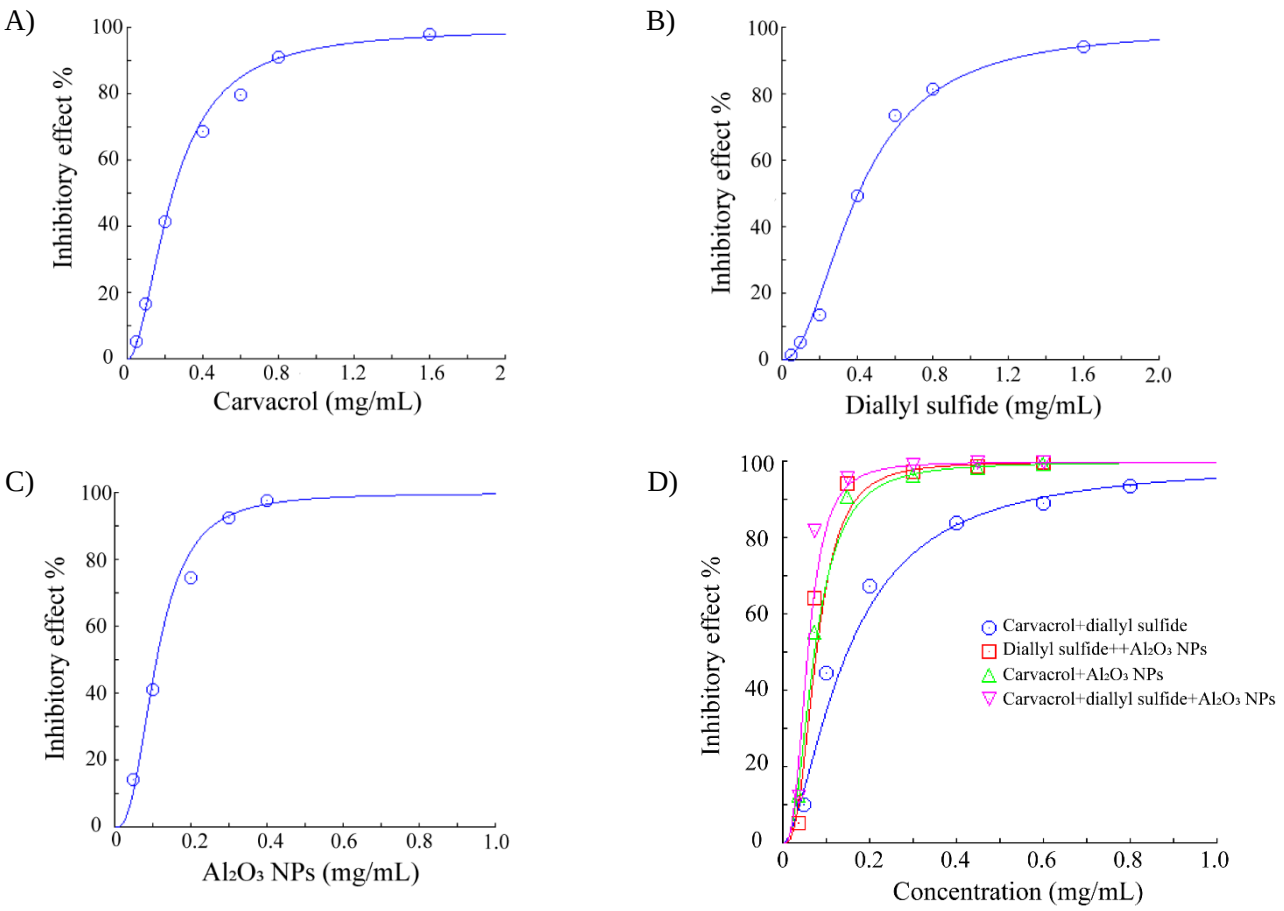

Figure 5.6 Concentration-effect curves for (A) carvacrol, (B) diallyl sulfide, (C) Al_2O_3 NPs and (D) antimicrobial combinations against VBNC *C. jejuni* cocktail in chicken juice after 24 h treatment at 37°C in a microaerobic condition. The inhibitory effect represents the percentage of inactivated bacterial cells.

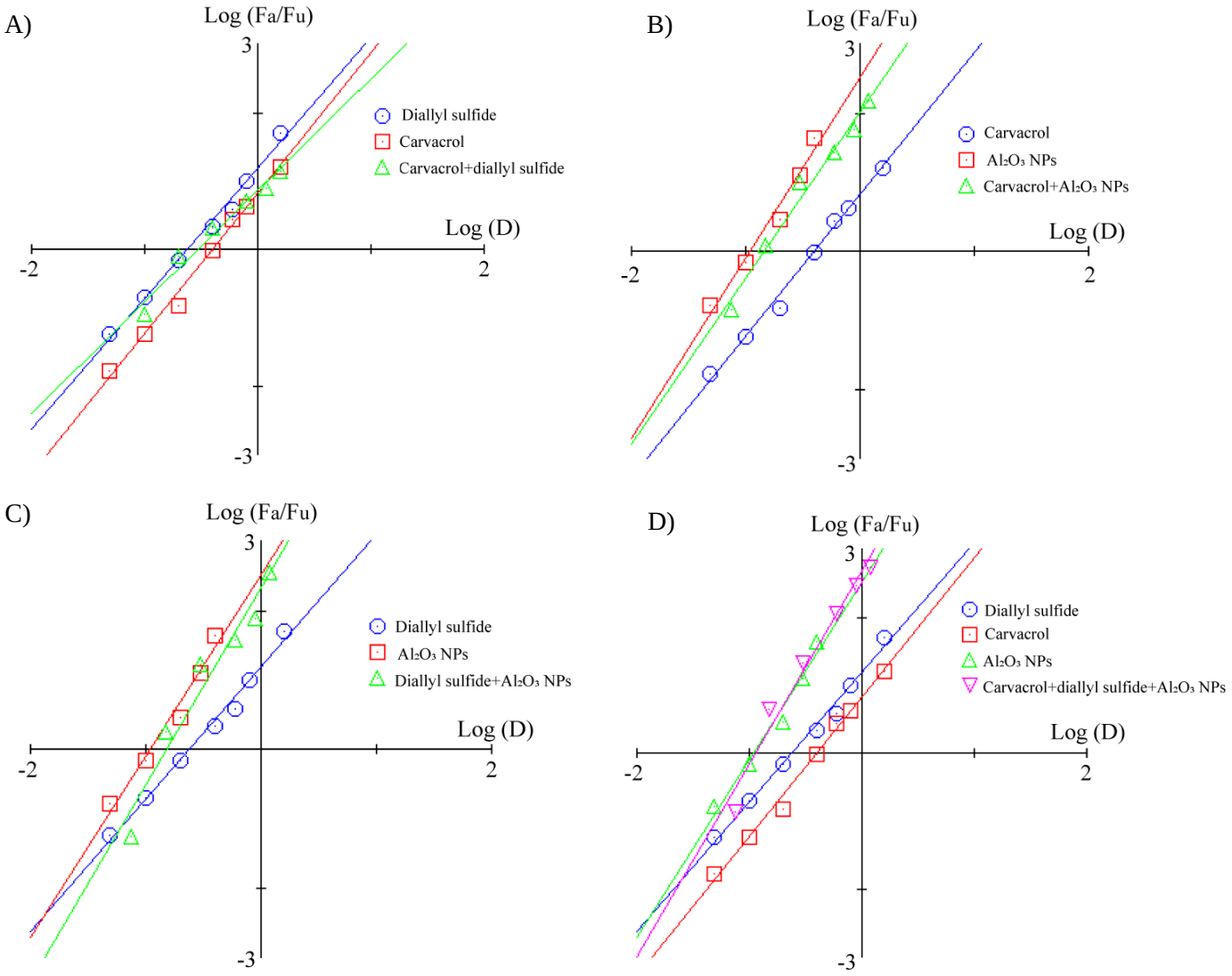

Figure 5.7 Median-effect plots of carvacrol, diallyl sulfide, and Al_2O_3 NPs against VBNC *C. jejuni* cocktail in chicken juice after 24 h treatment at 37°C in a microaerobic condition. Data were plotted for single or dual treatments as (A–C) binary or (D) tertiary combinations. F represents the percentage reduction of bacterial cells. D means the antimicrobial concentration. Each line represents the effect of single or combinational antimicrobial treatment. The "0" on the x-axis represents median-effect and the "0" on the y-axis represents a 50% reduction of viable cells. The line slope represents the potency of the antimicrobial treatment (n = 3, duplicates).

5.4.3.2. Combination index

Antimicrobial interactions were evaluated by determining the combination index (CI) values for various antimicrobial combinations against VBNC *C. jejuni* under simulated poultry processing conditions (Figure 5.8). This analysis involved the application of the median-effect equation and combination index equation, with the CompuSyn software facilitating the automation of the process. Simulated CI values at different f_a levels allowed for the automatic generation of f_a-CI plots for each antimicrobial combination, extending beyond the actual experimental doses and corresponding effect values.

The combination of carvacrol and diallyl sulfide exhibited additive to slight antagonistic behavior across f_a levels ranging from 0.05 to 0.97. In contrast, all binary and ternary combinations involving Al_2O_3 NPs demonstrated synergistic interactions (CI < 1) at f_a values exceeding 0.65. Moreover, the transition of interaction from antagonistic or additive interactions to synergistic was dose-dependent for all combinations. Notably, the combination of carvacrol, diallyl sulfide, and Al_2O_3 NPs displayed promising synergistic antimicrobial interactions, indicating its potential for effectively inactivating VBNC *C. jejuni* under simulated poultry processing conditions. The concentration-effect curves of all synergistic combinations showed a parallel trend with the curve of Al_2O_3 NPs, indicating the significant contribution of Al_2O_3 NPs to the observed synergistic interaction.

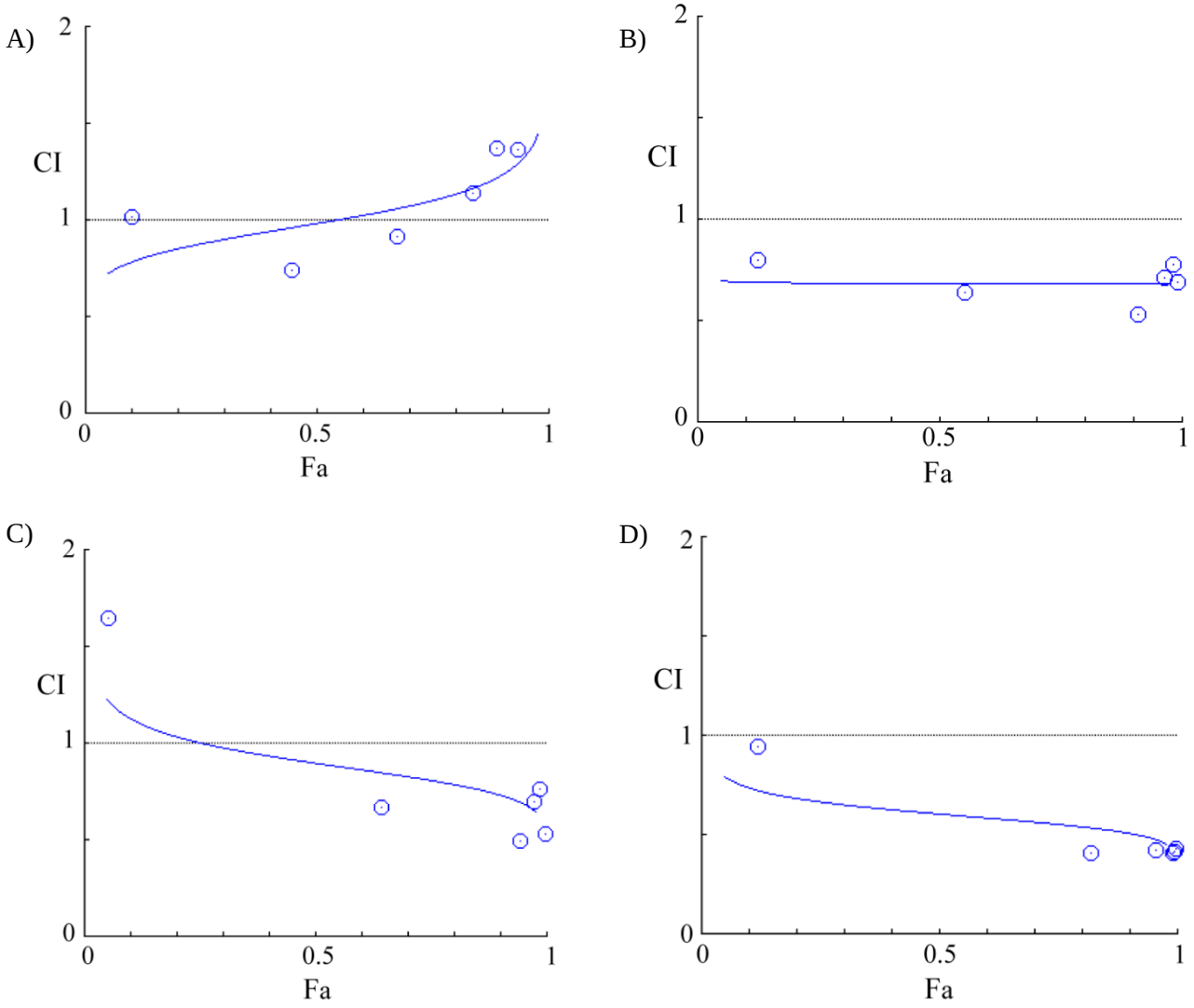

Figure 5.8 Chou-Talalay method F_a-CI plot of carvacrol, diallyl sulfide, and Al_2O_3 NPs against VBNC *C. jejuni* cocktail in chicken juice after 24 h treatment at 37°C in a microaerobic condition. Data were plotted for binary treatments as (A) Carvacrol+ diallyl sulfide, (B) Carvacrol+ Al_2O_3 NPs, and (C) Diallyl sulfide+ Al_2O_3 NPs or (D) tertiary combinations. CI was plotted on the y-axis as a function of effect level (F_a) on the x-axis to evaluate drug synergism. CI < 1, CI = 1 and CI > 1 refers to synergism, additivity and antagonism, respectively.

Although the determination of synergism based on the mass-action law is independent of specific mechanisms, the observed effects of the various antimicrobials employed in this study could potentially be elucidated by their respective modes of action. Carvacrol exhibits its primary antimicrobial function by potentially disrupting the bacterial cell wall and cell membrane through the presence of a free hydroxyl functional group and a system of delocalized electrons (Ben Arfa et al., 2006). On the other hand, diallyl sulfide displays high reactivity towards free sulfhydryl groups in proteins, resulting in protein denaturation, cell wall disruption, and leakage of cellular contents (Bhatwalkar et al., 2021). The antimicrobial action of Al_2O_3 NPs involves interactions with negatively charged bacterial cells and their aggregation on the cell surface, leading to the disruption of bacterial cellular structure. Furthermore, the generation of reactive oxygen species by Al_2O_3 NPs can oxidize biopolymers, contributing to bacterial cell death (Gudkov et al., 2022).

Plant-based antimicrobials have been found to exhibit both synergistic and additive interactions. For example, a study by Hakeem and colleagues demonstrated synergistic inhibition of *C. jejuni* when cinnamon oil and curcumin were combined (Hakeem et al., 2019). Additionally, Lambert and coworkers observed an additive effect when mixtures of carvacrol and thymol were used against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Lambert et al., 2001). Previous studies have documented the synergistic interaction between metal oxide NPs and plant-based antimicrobials. In a study by Babapour and colleagues, it was demonstrated that the combination of ZnO NPs and fennel essential oil exhibited synergistic effects, resulting in enhanced antimicrobial activity against *S. aureus* and *E. coli* (Babapour et al., 2021). Furthermore, the combination of ajoene with Al_2O_3 NPs /TiO_2 NPs exhibited a notable reduction in *C. jejuni* count (Xue et al., 2018). These findings highlight the complex nature of antimicrobial interactions and

underscore the importance of evaluating the combined effects of different antimicrobials to optimize their efficacy against specific microbial pathogens. Therefore, comprehensive investigations are required to unravel the specific molecular mechanisms involved in the enhanced antimicrobial activity observed.

5.5. Conclusion

VBNC *C. jejuni* exhibited high persistence to plant-based antimicrobials (*i.e.*, carvacrol and diallyl sulfide) under conditions relevant to poultry processing. In contrast, Al_2O_3 NPs demonstrated relatively high potency as evidenced by both the time-kill assay and mathematical model. The interactions among antimicrobials were determined to be additive in all the combinations using the time-kill assay. However, when assessed using the mathematical model, synergistic effects were observed in both binary and ternary combinations of plant-based antimicrobials and Al_2O_3 NPs, while additive effects were observed in the combinations of plant-based antimicrobials. Further investigations are necessary to elucidate the underlying mechanisms of these antimicrobials when employed individually or in combination under poultry processing conditions. This study provides valuable insights into antimicrobial interactions, and the application of the mathematical model can facilitate the development of innovative mitigation strategies aimed at reducing the prevalence of VBNC *C. jejuni* in the agri-food system.

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Chapter 6. Contribution to knowledge and recommendations for future research

6.1. General conclusions

The primary objective of this thesis is to gain a better understanding of the induction and persistence of VBNC *C. jejuni* within the agri-food system. The literature review indicated that despite its seemingly vulnerable nature, *C. jejuni* employs specific survival strategies such as entering the VBNC state that could pose substantial risks to both food safety and public health. Additionally, the review presented a comprehensive analysis of diverse mitigation strategies aimed at reducing the prevalence of *Campylobacter* in both environment and agri-foods (Chapter 2). In light of these findings, our investigation focused on elucidating the formation of VBNC *C. jejuni* in the food processing environment and its presence in food products, thereby providing valuable insights for risk assessment (Chapter 3). Furthermore, we evaluated the efficacy of antimicrobial combinations and their potential synergistic interactions in inactivating VBNC *C. jejuni*, with a specific focus on the poultry processing environment. The developed strategies hold potential as the promising candidates for mitigating *C. jejuni* (Chapters 4-5).

In Chapter 3, a comprehensive investigation was conducted to examine the impact of stressors encountered in food processing conditions and food products on the induction of VBNC *C. jejuni*. Our study revealed that a significant proportion of *C. jejuni* entered the VBNC state in response to these stress conditions (*i.e.*, chlorine, aerobic stress, and low temperature). Interestingly, distinct responses were observed among *C. jejuni* populations when subjected to different stressors, highlighting variations in their ability to withstand environmental pressures. Additionally, our findings provided evidence of heterogeneous behaviors exhibited by different *C. jejuni* strains under the same stress condition, suggesting the presence of strain-specific differences in their stress response mechanisms.

In Chapter 4, the efficacy of various antimicrobials and their combinations in inactivating VBNC *C. jejuni* was investigated. The findings revealed distinct patterns of persistence in VBNC *C. jejuni* when exposed to different antimicrobials, indicating their varied responses to treatment. Notably, VBNC *C. jejuni* exhibited tolerance towards plant-based antimicrobials (*i.e.*, carvacrol and diallyl sulfide), while demonstrating susceptibility to Al_2O_3 NPs. Moreover, we investigated the interactions among these antimicrobials to explore potential synergistic effects. The results demonstrated an additive effect when plant-based antimicrobials were combined, while the combination of plant-based antimicrobials with Al_2O_3 NPs elicited a synergistic effect, enhancing the overall antimicrobial activity against VBNC *C. jejuni*.

In Chapter 5, we focused on investigating the effects of the aforementioned antimicrobials and their potential synergistic interactions against VBNC *C. jejuni* in a simulated poultry processing environment. The effectiveness of each antimicrobial showed a similar trend as observed in the previous study. However, the presence of chicken juice posed additional challenges as it interacted with the antimicrobials, thereby affecting their overall effectiveness. To assess the interactions among these antimicrobials, we initially employed the time-kill assay, which demonstrated additive effects in all tested combinations. Nevertheless, to achieve a high throughput and more precise evaluation of these interactions, we developed a customized mathematical model. The application of this computational approach revealed notable synergistic effects in both binary and ternary combinations of plant-based antimicrobials and Al_2O_3 NPs. These combinations demonstrated enhanced antimicrobial activity against VBNC *C. jejuni* in the poultry processing environment. In contrast, the combinations of plant-based antimicrobials demonstrated additive effects, highlighting their distinct mode of interaction.

Overall, this study not only sheds light on the interactions between *C. jejuni* and the food processing environment but also presents effective antimicrobial combinations for inactivating VBNC *C. jejuni*. Moreover, the utilization of a mathematical model expands the scope of antimicrobial evaluation, enabling comprehensive assessments of other potential antimicrobial agents. These findings contribute to the advancement of knowledge in the field and provide a basis for further research and the development of improved strategies to ensure food safety and mitigate the risks associated with foodborne pathogenic bacteria.

6.2. Scientific contributions

The work presented in this thesis contributes to several novel research findings, as outlined below:

- The study examined the induction of VBNC *C. jejuni* in the food processing environment and food products, thereby enhancing our understanding of the interactions between *C. jejuni* and the agri-food system.
- *C. jejuni* demonstrated significant adaptability to thrive in the food processing environment through a diverse range of strategies, thereby necessitating comprehensive risk assessment and development of novel mitigation methods.
- A highly effective method for inactivating VBNC *C. jejuni* was developed, providing promising mitigation strategies to combat VBNC pathogenic bacteria.
- This study represents the pioneering attempt to investigate the inactivation of VBNC pathogenic bacteria in a simulated food processing environment.
- A novel mathematical model was developed to examine antimicrobial interactions in VBNC bacterial pathogens in a high-throughput manner, thereby providing a screening method for identifying potential antimicrobial combinations.

6.3. Recommendation for future research

Based on the insightful findings presented in this thesis, several critical recommendations for future research have been identified:

- Additional studies are needed to uncover the molecular mechanisms involved in the formation of the VBNC state in *C. jejuni* under different stress conditions, as well as the strain-specific variations observed among different *C. jejuni* strains. This investigation will provide insights into the genetic and physiological factors influencing the transition to the VBNC state.
- Further investigations are necessary to elucidate the mechanisms underlying the efficacy of these antimicrobials when employed individually or in combination. Such understanding will aid in optimizing antimicrobial combinations and designing more effective treatment strategies against VBNC *C. jejuni*.
- It is necessary to conduct further investigations to understand the underlying mechanisms of action of these antimicrobials when used alone or in combination within poultry processing conditions. This knowledge will contribute to the development of targeted and efficient approaches for reducing *Campylobacter* contamination in poultry products, thereby improving food safety.

General Reference List

Note: In accordance with the Guidelines for Manuscript Style Thesis Preparation, each manuscript chapter (Chapters 3-5) includes its own reference list. Therefore, the reference list below corresponds to the remaining chapters of the thesis (Chapters 1 and 2).

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