Characterisation and potential impacts of *Clostridium* spp. in agroecosystems

JCJ Fourie

orcid.org/ 0000-0002-8559-6485

Thesis accepted in fulfilment of the requirements for the degree *Doctor of Philosophy in Science with Microbiology* at the North-West University

Promoter:	Prof CC Bezuidenhout
Co-promoter:	Prof RA Adeleke
Co-promoter:	Prof CMS Mienie

Graduation: July 2025

PREFACE

As I was writing this thesis, which has been years in the making,

I had to stop and take a breath, because I found myself thinking…

Of a world without oxygen, silent yet alive,

Where *Clostridium*'s resilience allows it to thrive.

No breezes to carry, no skies to embrace,

Yet life stirs softly in this breathless space.

Decay turns to promise, in shadows it grows,

A quiet persistence no daylight knows.

Personifying strength in the absence of air,

It whispers a truth: *survival is rare*.

Even in stillness, life finds its way -

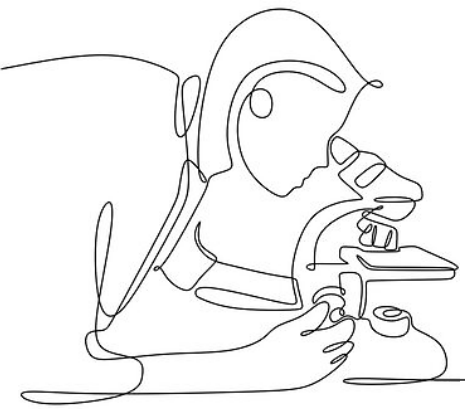
A spark in the dark, defying decay.

So I paused, reflecting, amidst my own fight,

That even in struggle, there's beauty in might.

A lesson from spores, unseen and small -

That life, against odds, can flourish through all.



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ACKNOWLEDGEMENTS

This thesis is the result of a challenging journey and like most successes in life, this one would not have been possible on my own. Many people have contributed and given their support throughout the duration of this study, and I would like to take this opportunity to acknowledge them:

My supervisor, Prof Carlos Bezuidenhout. Thank you for your guidance, patience, and valuable input in making this study possible. I'm truly grateful for all the opportunities, from undergrad all the way to this PhD.

My co-supervisor, Prof Rasheed Adeleke. I have learned so much from Prof over these years, and I can't thank Prof enough. It was a great honour to be part of Prof's team. I am extremely proud to have completed this program under Prof's unwavering guidance.

My other co-supervisor, Prof Charlotte Mienie. Prof's support, time and encouragement have given me the motivation in times when I needed it most. It was a true privilege to work with Prof. Thank you.

The National Research Foundation (NRF), Agricultural Research Council and the North-West University postgraduate bursary for their financial assistance.

A special thanks to Abram Mahlatsi, Lee Chenhaka, Dr Clara-Lee Van Wyk, Dr Deidre Van Wyk, Nolwandle Khumalo and Raeesa Bhikhoo for always lending an ear, support and all their help in and around the lab. I am proud to call you friends.

Thank you to the data scientists from Fathom Data, Ms Leani Bothma and Dr Kalonji Tshisekedi, for sharing their knowledge and amazing skillsets in the RNA-seq analysis. It was a very informative experience.

The Environmental Microbiology and Agricultural Biotechnology (EMAB) Research Group and Microbiology department at the North-West University, Potchefstroom Campus (My seconded home these last couple of years).

My best friends, Trohandi de Klerk, Gerhard Crous, and Shannan de Klerk. Thank you for all the support, laughs and motivational pep talks. *#LapaCorp*

The biggest thanks of all must go to my parents, Johan and Sonja Fourie (whose endless love and support never fails their children) and the best sister, Carmen Van Wyngaard. You all mean the world to me, and I'm beyond blessed to have you in my life.

ABSTRACT

This study investigated the behaviour of *Clostridium* species, with a focus on *Clostridium perfringens*, in agroecosystems, examining their genetic features, adaptability under environmental pressures, and potential dissemination through agricultural practices. Using a next-generation technologies, whole-genome sequencing of surface water-derived *C. perfringens* isolates revealed 11 antibiotic resistance genes (ARGs) and 35 virulence factors, with 79% associated with *C. perfringens*-specific pathogenic traits. This highlights their adaptability and potential risks to human and environmental health. Comparative analyses demonstrated strong genetic similarity to clinical isolates, emphasising the role of agroecosystems as reservoirs and amplifiers of antibiotic resistance and virulence traits. Transcriptome analysis using RNA-Seq revealed 166 significantly differentially expressed genes mapped to 26 KEGG pathways, including 73 up-regulated and 93 down-regulated genes under sublethal antibiotic exposure. Several resistance and virulence-associated genes were up-regulated, enhancing survival and adaptability under stress conditions. Notably, exposure to cefoxitin resulted in nine differentially expressed ARGs, the highest among the tested antibiotic. Concurrently, genes involved in energy-intensive metabolic pathways, such as lipid and carbohydrate metabolism, were notably down-regulated, indicating a shift in bacterial physiology toward a survival-oriented state. These findings emphasise the ecological risks posed by residual antibiotics in agricultural environments, which foster the persistence and pathogenicity of resistant bacterial populations. The study also explored the survival and transfer of *C. sporogenes*, a surrogate for pathogenic *Clostridium* species, in irrigation systems. Contaminated irrigation water facilitated the persistence of bacteria in both soil and the phyllosphere of lettuce, with surface irrigation primarily contaminating the soil and spray irrigation targeting the plant phyllosphere. Surface irrigation led to a peak concentration of 5.59 log copy numbers/g in non-rhizosphere soil at day 22, while spray irrigation resulted in an initial phyllosphere concentration of 9.09 log copy numbers/g leaves, which declined to 0.019 by day 42. Although bacterial concentrations decreased over time, trace amounts remained detectable, presenting potential food safety risks. This research highlights the interconnectedness of environmental reservoirs, antibiotic resistance, and agricultural practices. It also highlights the need for integrated management strategies, including enhanced wastewater treatment, rigorous water quality monitoring, and the adoption of sustainable irrigation practices, to mitigate the spread of antibiotic-resistant and pathogenic bacteria in agroecosystems and ensure food safety.

Keywords: Agroecosystems, *Clostridium perfringens*, Antibiotic Resistance, Pathogenicity, Irrigation Contamination, Lettuce

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Problem statement

Agroecosystems are essential for the continued survival of humanity on Earth. They play a critical role in ensuring food security, preserving biodiversity, and supporting livelihoods (Banerjee *et al.*, 2021). However, in the current era of climate change and ecosystem degradation, these agroecosystems are under significant stress, threatening their capacity to fulfil these essential functions (Toju *et al.*, 2018). Agroecosystems consist of environmental compartments, ranging from animals and plants to soil and water. All these compartments are interlinked and have their own functioning microbiome. These microorganisms play a pivotal role in maintaining the health of agricultural environments and promoting sustainable production systems (Liu *et al.*, 2022). Among these microorganisms, *Clostridium* species are particularly noteworthy due to their ubiquitousness and resilience. While many *Clostridium* species are beneficial, aiding in processes such as nitrogen fixation and organic matter breakdown, they are foremost known for their pathogenic nature (Palmer *et al.*, 2019). Therefore, the presence of *Clostridium* pathogens in these compartments is problematic. Studies have shown *Clostridium* pathogens are introduced into surface water systems by agricultural runoff (Rieke *et al.*, 2018; Thurston-Enriquez *et al.*, 2005). This is of concern since these water systems are used to irrigate agricultural fields and could jeopardise food safety and put consumers at risk.

Agroecosystems are also a hub for the development and spread of antibiotic resistance bacteria (ARB) and genes (ARG) (Franklin *et al.*, 2016). This is due to the overuse and misuse of antibiotics in various settings, such as agriculture, animal husbandry, and human medication, and subsequently being introduced into our soil and water environments (Franklin *et al.*, 2016; Manyi-Loh *et al.*, 2018). Although the concentrations of these active antibiotic compounds are relatively low, it has been shown to affect the functions and behaviour of microorganisms that are present (Larsson & Flach, 2022). Additionally, it was reported that exposure to antibiotic residue resulted in increased pathogenicity of several pathogenic microorganisms, such as *Bacillus cereus* and *Escherichia coli* O157:H7 (Liu *et al.*, 2020; Villegas *et al.*,2015). *Clostridium* is not excluded from this narrative. Previous studies demonstrated that the presence of antibiotic contaminants (ARG, ARB and antibiotic residue) affects *Clostridium* species, with studies reporting the phenotypic resistance to several antibiotic classes used to treat clostridial-related infections (Archambault & Rubin, 2020;

Symochko *et al.*, 2021). However, the genetic adaptations in antibiotic-resistant *Clostridium* pathogens and their possible role in the spread of antibiotic resistance in agricultural environments have not been properly investigated.

1.2 Study justification

The vast majority of living organisms require water, nourishment, and, above all, oxygen to survive. However, the *Clostridium* genus personifies life without oxygen. This anaerobic genus encompasses over 200 different species, most of which are benign. It is noteworthy that several *Clostridium* species have gained clinical recognition and have been extensively studied due to their pathogenicity (Mainil, 2006; Sanchez Ramos & Rodloff, 2018). Even though these anaerobic pathogens are naturally present in various environments, including the gastrointestinal tract of mammals and other sources, such as soil, water, and aquatic sediment, their representation has been neglected in the context of environmental research (Samanta & Bandyopadhyay, 2020; Pahalagedara *et al.*, 2020). Acknowledging the presence of *Clostridium* species and pathogens in the various compartments of agroecosystems and the threats they currently face, specifically the development of antibiotic resistance, the question arises as to how these environmental compartments affect the pathogenicity of *Clostridium* species and, in turn, how they may impact agroecosystems. Thanks to advances in technology such as Next-generation Sequencing, *Clostridium* species from these environments can be better studied (Motlagh & Yang, 2019). By investigating these pathogens at a genomic level, coupled with the use of application-based approaches, this study will provide the much-needed insight into the genetic characteristics of environmentally obtained *Clostridium* species, specifically *C. perfringens*, and the potential impacts they may have on agroecosystems.

1.3 Aim and objectives

1.3.1 Aim of study

This study aimed to investigate the genetic characteristics of environmentally obtained *Clostridium* pathogens, as well as investigating the survival of *Clostridium* species in the agricultural environment, and the potential associated health risks to humans and animals (Figure 1.1).

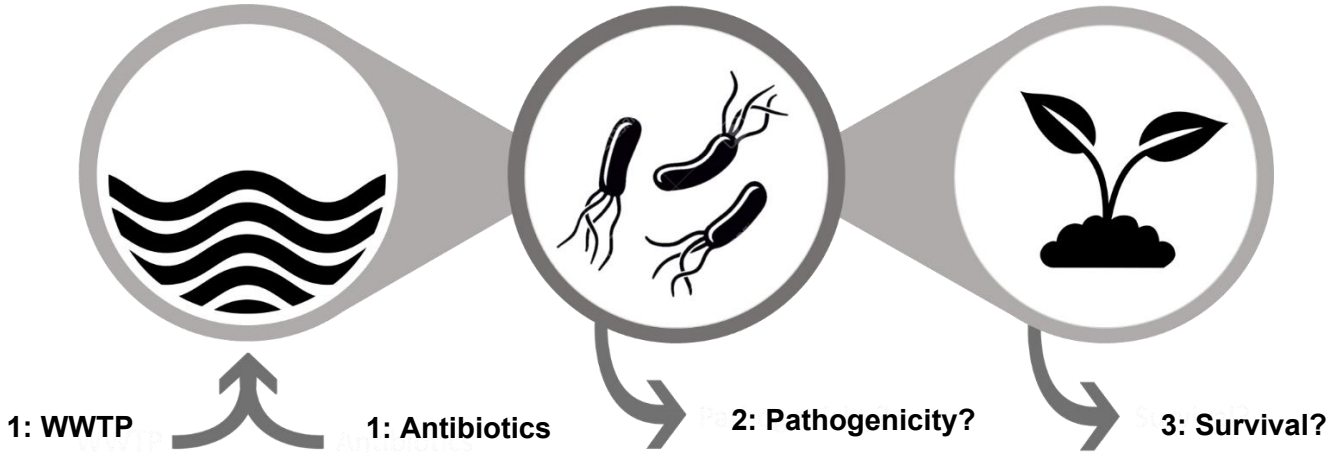


Figure 1.1: Schematic illustration of study aim. 1: Surface water systems used for irrigation are contaminated with poorly treated wastewater from wastewater treatment plants (WWTP) and antibiotics from agricultural runoff. 2: Pathogenic *Clostridium* isolated from these water systems can be characterized via next generation sequencing. 3: Furthermore, using a non-pathogenic surrogate strain of *Clostridium sporogenes*, the effect of irrigation on the survival of *Clostridium* on fresh produce and in the surrounding soil environment can be investigated.

1.3.2 Specific objectives

I. To investigate antibiotic resistance, virulence factors, and other genomic features in environmentally isolated *Clostridium perfringens* using whole-genome sequencing.

II. To determine the transcriptomic response of multidrug-resistant *Clostridium perfringens* to sublethal concentrations of various antibiotics using RNA-Seq.

III. To investigate the survival of *Clostridium sporogenes* in ready-to-eat produce using an experimental greenhouse pot study.

1.4 Thesis layout

In order to address the aforementioned objectives, this thesis comprises of 6 chapters and is in line with the format prescribed by the North-West University's requirements for the fulfilment of the degree Doctor of Philosophy (PhD) in Microbiology.

Chapter 1 is the current chapter. It includes the problem statement, aim and objectives, thesis outline and research publications. **Chapter 2** provides an overview of *Clostridium* species present in natural resources that are utilised in agriculture and related produce. Additionally, Chapters 3 and 4 investigate multidrug-resistant *Clostridium perfringens* strains obtained from surface water that are utilised for various purposes, such as irrigation. **Chapter 3** provides the genetic features of these strains with a specific focus on antibiotic resistance, virulence factors and mobile genetic elements, whereas **Chapter 4** reports on the gene expression of *C. perfringens* during exposure to sublethal concentrations of several classes of antibiotics. **Chapter 5** reports on a greenhouse experiment attempting to determine the survival of *C. sporogenes*, a non-pathogenic surrogate for *Clostridium* pathogens, in the phyllosphere, rhizosphere and non-rhizosphere soil of lettuce (*Lactuca sativa*). Lastly, **Chapter 6** includes a general summary of all chapters and the relevant conclusions to address the aim and objectives set out in this study, followed by possible recommendations for future avenues of research in this field.

1.5 Research publications from this study

i) Title: Draft genome sequences of potentially pathogenic *Clostridium perfringens* strains from environmental surface water in the North West Province of South Africa

Authors: Johannes Cornelius Jacobus Fourie, Tomasz J Sanko, Cornelius Carlos Bezuidenhout, Charlotte Mienie, Rasheed Adegbola Adeleke

Journal: Microbiology Resource Announcements (Published in 2019, Volume 8, Issue 32, Page 10)

Digital Object Identifier (DOI): https://doi.org/10.1128/mra.00407-19

ii) Title: Inside environmental *Clostridium perfringens* genomes: antibiotic resistance genes, virulence factors and genomic features

Authors: Johannes Cornelius Jacobus Fourie, Cornelius Carlos Bezuidenhout, Tomasz Janusz Sanko, Charlotte Mienie, Rasheed Adeleke

Journal: Journal of Water and Health (Published in 2020, Volume 18, Issue 4, Page 477-493)

Digital Object Identifier (DOI): https://doi.org/10.2166/wh.2020.029

iii) Title: Transcriptomic study on environmentally obtained *Clostridium perfringens* under sub-MIC concentrations of antibiotics

Authors: Johannes Cornelius Jacobus Fourie, Cornelius Carlos Bezuidenhout, Charlotte Mienie, Rasheed Adeleke

Target journal: Anaerobes

iv) Title: The effects of irrigation on the survival of *Clostridium sporogenes* in the phyllosphere and soil environments of lettuce

Authors: Johannes Cornelius Jacobus Fourie, Cornelius Carlos Bezuidenhout, Deidre van Wyk, Charlotte Mienie, Rasheed Adeleke

Journal: 3Biotech (Published in 2024, Volume 14, Issue 10, Page 1-9)

Digital Object Identifier (DOI): https://doi.org/10.1007/s13205-024-04069-5

CHAPTER 2: LITERATURE REVIEW

2.1. Background on *Clostridium* genus

The term 'anaerobic' was first used in the 19th century by Louis Pasteur to describe life without oxygen. This was owed to his discovery of a bacterium, *Vibrion butyrique*, that could exist and grow in the absence of oxygen. However, *V. butyrique* was renamed two decades later as *Clostridium butyricum* by Adam Prażmowski (Dürre, 2001; Popoff & Legout, 2023). Since then, the anaerobic genus has grown to include approximately 235 species and subspecies and is seen as one of the most important genera within the Firmicutes phylum (Brasca *et al.*, 2022; Figueiredo *et al.*, 2020).

The genus *Clostridium* exhibits an array of morphological characteristics that contribute to its identity and adaptability to various environments. When stained, they present as Gram-positive bacilli; however, some strains present as Gram-variable or even Gram-negative (Brook, 2014; Fader, 2015; Hashmi *et al.*, 2020). Most species are motile due to peritrichous flagella, but some *Clostridium* species rely on other mechanisms for movement, if at all (Samanta & Bandyopadhyay, 2020; Varga *et al.*, 2006). One example of such a mechanism can be observed in *Clostridium perfringens,* strain SM101. It has shown migration across surfaces by forming filaments of cells that line up in an end-to-end conformation, resulting in a gliding motility (Liu *et al.*, 2014). The genus also comprises heterogeneous phenotypes, which include psychrophiles, thermophiles and acidophiles, synthesising quinones, and cytochromes (Hashmi *et al.*, 2020; Wiegel *et al.*, 2006).

One of the most distinguished features of *Clostridium* species is their ability to form endospores (up to five endospores per cell) (Duda *et al.*, 1987; Samanta & Bandyopadhyay, 2020). These spores can be spherical or oval and are highly resistant to heat, chemicals, and desiccation. This allows them to survive in adverse environments for extended periods of time, after which then can germinate and resume growth under favourable conditions (Brasca *et al.*, 2022). Subsequently, this genus is widely distributed in the natural environment, such as soil, water, sediment, and other ecological niches (Samanta & Bandyopadhyay, 2020). They are also present in the gastrointestinal tract (GIT) of mammals, where they form up to 40% of the microbiota in adult humans (Nagano *et al.*, 2012; Samanta & Bandyopadhyay, 2020). Furthermore, some species have been found to be present in clinical settings, making *Clostridium* truly ubiquitous (Moore & Lacey, 2019; Wells & Wilkins, 1996).

2.2. Taxonomy of *Clostridium* genus

The *Clostridium* genus is known to be described as a highly heterogeneous group that is phylogenetically large (Schaumann *et al.*, 2018). Originally, to be classified as part of this genus, a bacteria had to meet four criteria: (i) Gram-positive strain; (ii) the ability to produce endospores; (iii) being restricted to an anaerobic metabolism; and (iv) the absence of dissimilatory reduction of sulphate. This led to an influx of species being assigned to this genus. However, classifying a bacterium as a *Clostridium* species based on these phenotypic characteristics has become problematic since some species consistently present as Gram-negative (such as *C. clostridiforme* and *C. ramosum)* or only form endospores under specific conditions (such as *C. ramosum*), while others have shown tolerance to atmospheric oxygen (such as *C. histolyticum* and *C. tertium*). These discrepancies resulted in complications in the taxonomic structure of the genus (Cruz-Morales *et al.*, 2019; Stackebrandt & Hippe, 2001).

The (re)classification of species based on the 16S rRNA gene sequences has been the gold standard for molecular taxonomic research for decades (Mizrahi-Man *et al.*, 2013; Yutin & Galperin, 2013). From this, a new taxonomic criterion based on phylogenetic analyses was used to revise the inconsistencies in the *Clostridium* genus. Using the first pure culture obtained, namely *C. butyricum*, which now represents the type species of the genus, 19 distinct clusters and five new genera were described (Collins *et al.*, 1994). Most previous members of *Clostridium* were assigned to *Clostridium* cluster I (*Clostridium sensu stricto*) and are considered to be the true genus of *Clostridium*, while many of the formally recognized members were assigned to new or established genera (Lawson & Rainey, 2016; Stackebrandt & Hippe, 2001). However, even with this revision, the phylogeny of the 19 clusters in the genus *Clostridium* remains diverse. This created more questions regarding the monophyly and the prediction of functions and traits in the genus (Cruz-Morales *et al.*, 2019). Recent studies have questioned whether the current 16S rRNA-based classification should be retained or simplified using genomic analyses (Chun *et al.*, 2018; Cruz-Morales *et al.*, 2019). Nonetheless, the reclassification of members from the genus *Clostridium* continues, and further study is needed to improve its taxonomic classification.

2.3. Pathogenicity within the *Clostridium* genus

Clostridia is known to produce the largest number of life-threatening toxins, including the two most potent toxins known to date, of any bacterial class (Baldassi, 2005; Rossetto & Montecucco, 2019). Although most *Clostridium* species are harmless, the genus is of great

clinical importance, as some species may cause rapid and lethal infections in both humans and animals (Mainil, 2006; Sanchez Ramos & Rodloff, 2018). The genus encompasses 40–50 species that are associated with clinical conditions, where 30 are described as minor pathogens and 15 as major pathogens that often result in species-specific diseases (Mainil, 2006). Based on their pathogenicity traits, these major pathogens can be grouped into (i) neurotoxic clostridia, (ii) enterotoxic clostridia, and (iii) histotoxic clostridia (Figure 2.1) (Dierick *et al.*, 2022; Num & Useh, 2014).

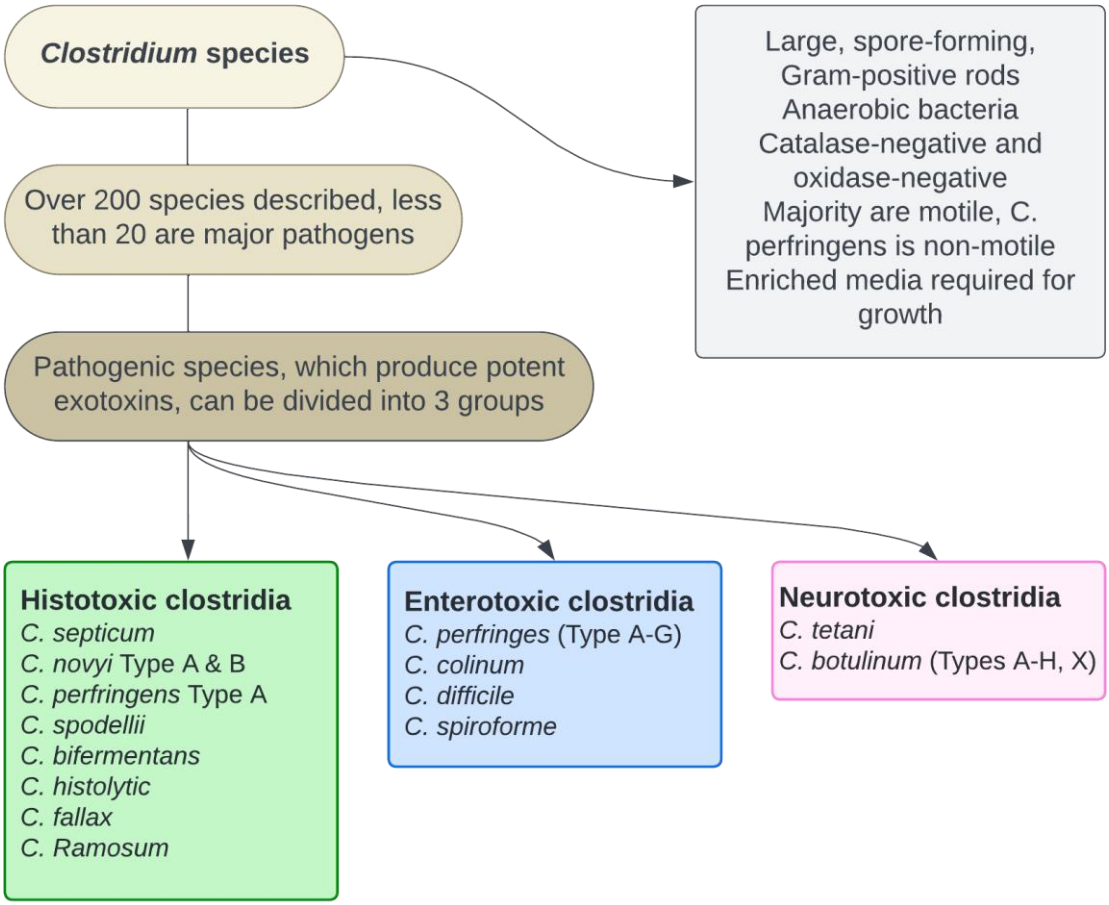


Figure 2.1: The categorisation of pathogenic *Clostridium* species into three major groups (neurotoxic clostridia, enterotoxic clostridia, and histotoxic clostridia) based on their toxin activity (Dierick *et al.*, 2022; Num & Useh, 2014).

2.3.1. Histotoxic clostridia

Histotoxic clostridia (Figure 2.1) are responsible for various human and animal diseases, including gas gangrene or myonecrosis (Stevens & Bryant, 2002). *Clostridium perfringens* Type A is most often the causal agent (80% - 95% of cases), followed by *C. novyi* (10% - 40%

of cases) and *C. septicum* (5% to 20% of cases). However, other *Clostridium* species, such as *C. bifermentans*, *C. histolyticum*, *C. fallax*, *C. ramosum*, and *C. sordellii* have been implicated on rare occasions (Leiblein *et al.*, 2020; Pasternack & Swartz. 2015). These species target various cells, such as muscle, epithelial, erythrocytes, and lymphocytes and then damage their intercellular junctions, actin cytoskeleton, and cell membranes (Popoff, 2016). In the case of *Clostridium perfringens*, necrosis usually begins at the site of infection, where the bacteria then migrate deep into the host tissue presumably using the previously described gliding motility trait (Section 2.1.) (Valeriani *et al.*, 2020). The production of *C. perfringens* alpha toxin (*plc*/*cpa*) and perfringolysin O (*pfo*), along with the secretion of hydrolytic enzymes, leads to the degradation and death of soft tissue. The infection then spreads quickly and, within several hours, results in sepsis and death (Janik *et al.*, 2019; Popoff, 2016; Valeriani *et al.*, 2020).

2.3.2. Enterotoxic clostridia

The GIT of mammals offers an ideal environment for *Clostridium* species to colonise, providing ample nutrients and anaerobic niches (Shrestha *et al.*, 2018). Species such as *Clostridium perfringens* and *Clostridium difficile* (*Clostridioides difficile*) are particularly problematic when in great quantity (Shrestha *et al.*, 2018; Uzal *et al.*, 2018). These species replicate in the GIT and produce elaborate toxins that result in both localised and generalised tissue damage (Uzal *et al.*, 2018). *Clostridium difficile* is a major nosocomial pathogen in humans and causes antibiotic-associated diarrheal disease and pseudomembranous colitis (McClane & Rood, 2001; Moore & Lacey, 2019). It produces two toxins, namely toxin A and B. Both are enterotoxins, whereas toxin B has additional cytotoxic effects (Guo *et al.*, 2020). *Clostridium perfringens* can cause a variety of intestinal diseases, as well as histotoxic infections (Section 2.3.3.) (Moore & Lacey, 2019). The pathogenicity of *C. perfringens* is reflected in its toxin producing proficiency. This species can produce at least 20 distinct exotoxins, many of which have GIT activity (Dierick *et al.*, 2022). Because the toxin-production patterns differ amongst strains, Table 2.1 shows how *C. perfringens* is classified into seven types (A-G) according to the combination of six typing toxins (alpha: *plc*, beta: *cpd*, epsilon: *etx*, iota: *iap/ibp*, CPE: *cpe,* and NetB: *netB*) and five non-typing toxins (Moore & Lacey, 2019).

Table 2.1: The toxin typing scheme of *Clostridium perfringens* (Moore & Lacey, 2019).

Toxinotype	Typing Toxins						Non-typing toxins				
	plc/cpa	*cpb*	*etx*	*iap/ibp*	*cpe*	*netB*	*cpb2*	*pfo*	*netF*	*cpd*	*tpeL*
A	+	-	-	-	-	-	+/-	+	-	-	-
B	+	+	+	-	-	-	+/-	+	-	+/-	-
C	+	+	-	-	+/-	-	+/-	+	-	+/-	+/-
D	+	-	+	-	+/-	-	+/-	+	-	-	-
E	+	-	-	+	+/-	-	+/-	+	-	-	-
F*	+	-	-	-	+	-	+/-	+	+	-	-
G*	+	-	-	-	-	+	+/-	+	-	-	+/-

+: Present, -: Absent, +/-: Present in some isolates, absent in others.
*Type F and G strains were formally categorised as Type A until reclassification in 2018.

This toxinotyping scheme assists with differentiating isolates associated with particular diseases. *Clostridium perfringens* enterotoxin (CPE) is one of the main causes of GI diseases. The CPE-encoding gene (*cpe*) can be chromosomal or plasmid-borne and is present in about 2-5% of the global *C. perfringens* population (McClane & Rood, 2001; Miyamoto *et al.*, 2011). *Clostridium perfringens* Type A and F strains are the causative agents of *C. perfringens* food poisoning, which is the 2nd most common bacterial food-borne illness, whereas Type B, C, D and E strains, are associated with enteritis, enterocolitis and/or enterotoxemia in livestock (Miyamoto *et al.*, 2011; Shrestha *et al.*, 2018).

2.3.3. Neurotoxic clostridia

Tetanus and botulism are two severe neurological diseases resulting from toxins produced by *Clostridium tetani* and *Clostridium botulinum*, respectively (Peck & Duchesnes, 2006). Both are seen as environmental bacteria, with great clinical significance (Popoff, 2022). The neurotoxin produced by *C. tetani*, namely tetanus neurotoxin (TeNT), is characterised by hyperactivity of voluntary muscles leading to rigidity and tetanic spasms in humans and other animals (Popoff, 2022; Rossetto & Montecucco, 2019). The TeNT toxin is produced when *C. tetani* replicates locally in damaged or infected tissue, exerting its effect on the synaptic junctions (Rings, 2004). Although there are effective vaccines available for tetanus, there are still several hundred thousand human deaths from tetanus each year (Moore & Lacey, 2019). The botulinum neurotoxin (BoNT) is the deadliest toxin currently known and expressed by at least four species of *Clostridium* (*C. botulinum*, *C. baratii*, *C. butyricum,* and *C. argentinense*) (Peck & Duchesnes, 2006; Popoff, 2022). *Clostridium botulinum* is known to replicate and produce BoNT in organic matter or in contaminated canned foods. When ingested, the toxin is absorbed into the bloodstream from the GIT and affects the functioning of neuromuscular

junctions. This results in flaccid paralysis with limb weakness and respiratory distress (Rings, 2004). These *Clostridium* species are classified into six groups (I–VI) based on the nine BoNT toxinotypes (A-H, X) they produce (Popoff, 2022; Rossetto & Montecucco, 2019). Despite the contrast in how TeNT and BoNT present clinically, they share a common mechanism of action (Revitt-Mills *et al.*, 2019).

2.4. Impact of *Clostridium* genus in agroecosystems

Microorganisms are essential in the entangled webs of interactions that form part of agroecosystems. Members of the *Clostridium* genus partake in these interactions, both beneficial and potentially harmful, depending on the specific species and conditions (Toju *et al.*, 2018). Even though *Clostridium* species are usually seen as the 'villain' in clinical environments, their role as plant pathogens has also been found to impact agroecosystems. *Clostridium puniceum* and other pectolytic *Clostridium* species are described as important plant pathogens that cause slimy rot in root vegetables, specifically potatoes (da Silva *et al.*, 2019; Shabuer *et al.*, 2015). Other species such as *C. bifermentans* and *C. subterminale* have also been associated with kiwifruit vine decline (Spigaglia *et al.*, 2020).

On the other hand, this genus has also shown to be a beneficial member in agroecology. They form part of the consortium of microorganisms involved in the nutrient cycle, breaking down complex organic material, cellulose, and lignin, thereby improving soil fertility (Palmer *et al.*, 2019). Some *Clostridium* species have been found to contribute to plant growth through their nitrogen-fixing abilities, production of gibberellins-like substances that promote root growth, and their capacity to effectively colonise the rhizosphere, as well as endophytic colonisation (Doni *et al.*, 2014; Oleńska *et al.*, 2020; Polyanskaya *et al.*, 2002; Zeiller *et al.*, 2015). Certain species reportedly alleviate plant damage and enhance the salinity tolerance of the host plant (Doni *et al.*, 2014; Gamalero & Glick, 2011; Ye *et al.* 2005; Zeiller *et al.*, 2015). Their ability to produce various compounds, including volatile fatty acids, that suppress pathogens makes them possible candidates for biocontrol (Mowlick *et al.*, 2012). Strains of *C. beijerinckii* (strain H110 and TB8) have been shown to be effective against *Fusarium oxysporum* f. sp. *Spinaciae* (causal agent of spinach wilt disease) by degrading the major fungal cell wall components due to antifungal activities (Ueki *et al.*, 2017). Furthermore, *C. beijerinckii* strain Sneb518 demonstrated significant inhibition against the root-knot nematode, *Meloidogyne incognita*, and a biomass enhancer in tomato plants (Lian *et al.*, 2022). However, a better understanding of the survival and behaviour of *Clostridium* species in agroecosystems is required to mitigate disease outbreaks or their possible use in beneficial agricultural applications.

2.4.1. Prevalence of *Clostridium* species in soil environments

Soil is believed to serve as the primary reservoir for numerous pathogenic species, representing a crucial route for transmitting diseases to food products and foraging animals (Samadda *et al.*, 2021). Previous works have investigated the prevalence of individual pathogenic species within the *Clostridium* genus, with relevant soil studies outlined in Table 2.2. According to a survey utilising 16S rDNA gene libraries, approximately 0.59% of the soil bacteria community can be attributed to Clostridia (Janssen, 2006). Additionally, the diversity of *Clostridium* species and toxinotypes can exhibit significant variations across different locations, ranging from broad continental and regional differences (del Mar Gamboa *et al.*, 2005; Hang'ombe *et al.*, 2000; Kumar *et al.*, 2017; Lúquez *et al.*, 2005), to microscale disparities within the same soil sample (Kirk *et al.*, 2004).

A study by Kim *et al.* (2004) investigated the distribution of *Clostridium* species in Korean soil, isolating 16 different species from 152 soil specimens taken from five locations. *Clostridium perfringens* was the only species present across all soil samples. However, other *Clostridium* pathogens, such as *C. difficile*, *C. novyi*, *C. chauvoei* and *C. septicum* were only identified in specific locations. A similar study done by del Mar Gamboa *et al.* (2005) reported 54 different species from 117 soil specimens collected across Costa Rica, of which *C. perfringens* and *C. sordellii* were the most prevalent pathogenic species present. Additionally, other pathogenic species, such as *C. tetani*, *C. botulinum*, *C. difficile, C. septicum*, *C. baratii* and *C. novyi*, were also isolated.

Clostridium species have also shown diverse abundance in arctic soil environments, such as Antarctica. A total of 155 strains were categorised into 11 species, which included *C. perfringens*, *C. bifermentans*, *C. sordellii*, C. *sporogenes*, *C. plagarum*, *C. paraperfringens*, *C. septicum*, *C. tertium*, *C. cadaveris*, *C. butyricum*, and *C. felsineum*. However, 38 strains remained unidentified (Miwa, 1975). Notably, *C. perfringens* and *C. sordellii* were frequently isolated, reinforcing their omnipresence as pathogens in various soil and agricultural environments.

The geographical distribution of *C. botulinum* in soil has been extensively investigated, with a particular focus on European and Asian countries (Creti *et al.*,1990; Huss, 1980; Yamakawa & Nakamura, 1992; Serikawa *et al.*, 1977; Smith & Milligan, 1979; Smith & Young, 1980; Sonnabend *et al.*, 1987; Yamakawa *et al.*, 1988). However, limited studies are focusing on botulism outbreaks in natural settings within subtropical and tropical climate zones (Espelund & Klaveness, 2014). In Africa, the presence of *C. botulinum* in Kenyan soil has been confirmed, including the identification of serotypes A to D (Yamakawa *et al.*, 1990). Furthermore, 2 009 soil samples from five different geographical regions of Argentina were

analysed for *C. botulinum* resulting in a 23.5% prevalence. Notably, there was a non-uniform distribution among the regions and a diverse range of serological types was observed, with a higher prevalence detected in nonvirgin soil compared to virgin soil (Lúquez *et al.*,2005). Another neurotoxic *Clostridium* pathogen, namely *C. tetani*, has also been identified in soil specimens from around the globe. The prevalence of *C. tetani* ranged from 25% to 60% in four different studies (total of 697 soil samples) (Béland & Rossier, 1971; Bukar *et al.*, 2008; Sanada & Nishida, 1965; Wilkins *et al.*, 1988). Whereas *C. difficile*, which is more known for its nosocomial infections, was isolated in 60.4% of 159 soil samples in Western Australia (Perumalsamy *et al.*, 2019), in 36.7% of 79 soil samples collected in eastern parts of Slovenia (Janezic *et al.*, 2016), in 37% of 147 soil samples from rural Zimbabwean homesteads (Simango, 2006) and 21% of 104 Welsh soil samples (Al Saif & Brazier, 1996).

These studies highlight the widespread presence of *Clostridium* species, specifically pathogens, across various soil environments and why a more comprehensive understanding of their behaviour in these environments would be of global importance. Despite the number of studies focusing on the prevalence of *Clostridium* pathogens, the major factors that influence their existence and abundance in soil remain unexplored. Additionally, the use of different microbiological methods and techniques may impede the potential for result integration.

Table 2.2: Prevalence of *Clostridium* species in soil.

Species	Sampling area	Samples (n)	Isolates (n)	ID Method	Authors
C. perfringens	Japan	30	288	PCR	Hashimoto *et al.*, 2023
	Punjab province, Pakistan	970	69	Multiplex PCR	Naureen *et al.*, 2022
	South Korea	45	16	PCR	Park *et al.*, 2019
	Egypt	30	12	PCR	Hamza *et al.*, 2018
	Egypt	100	41	Multiplex PCR	Nayel *et al.*, 2013
	Greece	750	376	Biochemical	Voidarou *et al.*, 2011
	USA	14	33	Biochemical, PCR	Soge *et al.*, 2009
	Pittsburgh, USA	502	343	Multiplex PCR	Li *et al.*, 2007
	USA	129	6	PCR	Kuske *et al.*, 2006
	Japan	15	13	Biochemical	Oka *et al.*, 1989
C. difficile	Western Australia	159	96	PCR	Perumalsamy *et al.*, 2019
	Slovenia	79	29	PCR	Janezic *et al.*, 2016
	Sweden	598	16	Biochemical, Gas-liquid chromatography	Båverud *et al.*, 2010
	Zimbabwe	146	54	Biochemical	Simango, 2006
	South Wales, UK	104	22	Biochemical, ELISA	Al Saif & Brazier, 1996
C. botulinum	Argentina	2 009	472	Morphology	Lúquez *et al.*,2005
	Japan	113	128	Biochemical	Yamakawa & Nakamura, 1992
	Rome	520	7	Biochemical, Gas-liquid chromatography	Creti *et al.*,1990
	Paraguay	17	4	Biochemical	Yamakawa *et al.*, 1990
	Kenya	12	3	Biochemical	Yamakawa *et al.*, 1990
	Japan and Shinkiang Province, China	266	286	Biochemical	Yamakawa *et al.*, 1988
	Switzerland	41	18	Biochemical, ELISA	Sonnabend *et al.*, 1987
	Denmark, Faroe Islands and Iceland	684	195	Biochemical	Huss, 1980
	Great Britain	174	10	Trypsinization of culture filtrates	Smith & Young, 1980

Table 2.2: Prevalence of *Clostridium* species in soil (*continue*).

Species	Sampling area	Samples (n)	Isolates (n)	ID Method	Authors
C. botulinum	London	18	16	Trypsinization of culture filtrates	Smith & Milligan, 1979
	Japan	230	129	Biochemical	Serikawa *et al.*, 1977
C. tetani	Nigeria	5	24	Biochemical	Bukar *et al.*, 2008
	South Africa	60	15	Biochemical, Gas-liquid chromatography	Wilkins *et al.*, 1988
	Quebec, Canada	328	214	Biochemical	Béland & Rossier, 1971
	Japan	304	87	Biochemical	Sanada & Nishida, 1965
Multiple	Taiwan	21	76	PCR	Huang *et al.*, 2013
	South India	115	27	DNA restriction digestion analysis, SDS-PAGE	Sathish & Swaminathan, 2009
	Costa Rica	117	1 945	Biochemical, Gas-liquid chromatography	del Mar Gamboa *et al.*, 2005
	South Korea	152	421	Biochemical, Immuno-fluorescent assay	Kim *et al.*, 2004
	Zambia	46	107	Immuno-fluorescent assay	Hang'ombe *et al.*, 2000
	Antarctica	31	193	Biochemical	Miwa, 1975

2.4.2. Prevalence of *Clostridium* species in surface water systems

The interconnected function of water within agroecosystems, and its growing demand across various sectors is widely acknowledged (Fleiner *et al.*, 2013). Globally, agriculture has the highest demand for water of all human activities (Hoover *et al.*, 2023). However, despite water's crucial role in this system, it is a finite resource, with the amount of fresh and readily available water being less than 1% (Mishra, 2023). This natural resource is also facing several threats, be it scarcity, accessibility or degradation, but also climate change, a growing population, and changing consumption patterns (Mishra, 2023). Freshwater systems, such as rivers, lakes, streams and aquifers, are habitats for a large portion of the world's biodiversity (Dudgeon *et al.*, 2006). Additionally, the *Clostridium* genus forms part of the indigenous microorganisms present in these water systems, as evidenced by the studies listed in Table 2.3, and has significant impacts on soil health and agriculture processes (Saber *et al.*, 2021).

A study done by Manafi *et al.* (2013) collected 139 surface water samples across 5 locations in Vienna, Austria. Of all the samples, 131 showed the presence of several *Clostridium* species, with *C. perfringens* being the most prevalent (96.3%). Eighteen strains, which included other species such as *C. sordellii*, *C. bifermentans*, *C. tetani, C. fallax*, *C. botulinum*, and *C. tertium,* were also isolated and identified. Additionally, a study done by Sathish & Swaminathan (2009) investigated the genetic diversity among toxigenic *Clostridium* species in 59 water samples collected from South India. A total of 71 Clostridial strains were isolated, of which 3 strains were toxigenic and identified as *C. bifermentans* and *C. chauvoei*. Several other studies have also investigated the prevalence of the *Clostridium* genus in surface water systems across the African continent but did not identify strains at species level (Olalemi *et al.*, 2020; Onifadé *et al.*, 2017; Saber *et al.*, 2021).

Table 2.3: Prevalence of *Clostridium* species in surface water.

Species	Sampling area	Samples (n)	Isolates (n)	ID Method	Authors
C. perfringens	Poland	19	847	Biochemical	Matuszewska & Mąka, 2023
	Kashmir, India	45	682	PCR	Hafeez *et al.*, 2020
	South Africa	3	835	Selective media	Potgieter *et al.*, 2020
	Egypt	60	19	PCR	Hamza *et al.*, 2018
	Tanzania	30	33	NA	Mushi, 2018
	South Africa	558	77 400	qPCR	Abia *et al.*, 2015
	Wisconsin, USA	24	153	Biochemical, PCR	Mueller-Spitz *et al.*, 2010
	Ontario, Canada	1600	301	Selective media	Wilkes *et al.*, 2009
	USA	14	68	Biochemical, PCR	Soge *et al.*, 2009
	Finland	139	44	Selective media	Horman *et al.*, 2004
	USA	179	690	Selective media	Francy *et al.*, 2000
	Greece	223	1 700	Selective media	Bezirtzoglou *et al.*, 1994
	Japan	5	114	Biochemical	Saito, 1990
	Japan	17	41	Biochemical	Oka *et al.*, 1989
	South Africa	16	849	Biochemical	Sartory, 1986
C. difficile	Czech Republic	16	15	MALDI-TOF MS	Cizek *et al.*, 2022
	Western Australia	277	70	PCR, Whole-genome sequencing	Lim *et al.*, 2018
	Taiwan	29	7	Multiplex PCR	Tsai *et al.*, 2022
	Slovenia	104	15	PCR	Janezic *et al.*, 2016
	Slovenia	94	154	qPCR	Zidaric *et al.*, 2010
	Zimbabwe	234	14	Biochemical	Simango, 2006
	South Wales	16	40	Enzyme immunoassay	Al Saif & Brazier, 1996

Table 2.3: Prevalence of *Clostridium* species in surface water (*continue*).

Species	Sampling area	Samples (n)	Isolates (n)	ID Method	Authors
C. botulinum	Finland	110	34	PCR	Hielm *et al.*, 1998
	Great Britain & Ireland	554	194	Mouse bioassay	Smith *et al.*, 1978
	USA	131	4	Mouse bioassay	Sayler et al,. 1976
	London	69	50	Mouse bioassay	Smith & Moryson, 1975
Multiple	Egypt	5	46 000 000	Selective media	Saber *et al.*, 2021
	Nigeria	24	20 892	Selective media	Olalemi *et al.*, 2020
	Bénin	48	49 300	Selective media	Onifadé *et al.*, 2017
	Vienna	139	483	Biochemical	Manafi *et al.*, 2013
	Malaysia	70	24 404	Biochemical	Lee *et al.*, 2012
	South India	59	71	DNA restriction digestion analysis, SDS-PAGE	Sathish & Swaminathan, 2009

The distribution of *C. difficile* in surface water systems has been studied in various countries (Table 2.3), most of which were situated in Europe and Asia. In recent years, hypervirulent strains of *C. difficile* RT078 were identified in a river system in southern Taiwan, where four isolates harboured multiple toxin-producing genes, namely *tcdA*, *tcdB*, *cdtA* and *cdtB* (Tsai *et al.*, 2022). A similar study done by Zidaric *et al.* (2010) reported a high diversity of *C. difficile* genotypes in 25 river systems across Slovenia, with 38.1 % of the isolates being distributed into 34 different ribotypes, of which RT014 was predominant. These findings suggest that *C. difficile* may be more widely distributed in aquatic environments than previously thought and act as potential reservoirs of genetically diverse strains (Al Saif & Brazier, 1996; Janezic *et al.*, 2016). Additionally, when the core genomes of several *C. difficile* RT014 strains from various environmental water sources were compared to its clinical counterpart, 19.2% (5/26) of the human-derived strains were closely related to one or more water strains (Lim *et al.*, 2018). This supports the growing hypothesis of an environmental transmission route for *C. difficile* infections (CDI) (Lim *et al.*, 2018; Warriner *et al.*, 2017).

In the case of *C. botulinum*, it is important to note that the mechanisms responsible for a botulism outbreak in the aquatic environment remain poorly understood (Espelund & Klaveness, 2014). However, there are environmental characteristics that influence a botulinum outbreak in nature. Low water levels, temperatures above 20°C and a pH between 7.5 and 9 have been linked to larger outbreaks (Espelund & Klaveness, 2014; Rocke *et al.*, 1999). A study done by Sayler *et al.* (1976) investigated its incidence in environmental water and the adjoining sediment. The findings showed *C. botulinum* to be present in 12.3% of sediment samples, however, no *C. botulinum* strains were detected in the water samples. Its ubiquitous presence in aquatic sediment has also been the focus of several studies. In the United Kingdom, several botulinum toxin serotypes, including types B, C, D and E, were identified (Smith *et al.*, 1978; Smith & Moryson, 1975). A study conducted by Hielm *et al.* (1997) on aquatic environments in Finland reported the presence of serotypes A, B, E and F, with serotype E being predominant in 61% of all freshwater sediment samples. Additionally, spores of *C. botulinum* in sediment may be mobilised by surface water and spread throughout water systems (Espelund & Klaveness, 2014).

Of all the *Clostridium* pathogens, the prevalence of *C. perfringens* in aquatic environments is the most well-documented. *Clostridium perfringens* and its spores can survive in various aquatic environments (Manafi *et al.*, 2013). These spores are very resilient to various physicochemical effects and other stressors, such as chlorination, radiation and heating (Bezirtzoglou *et al.*, 1994). A study by Oka *et al.* (1989) investigated the distribution of enterotoxigenic *C. perfringens* in 17 water samples from three different surface water systems. Water samples were initially heat treated at 75°C for 20 minutes and 100°C for 60 minutes

before bacterial culturing. A total of 32 and nine *C. perfringens* strains were isolated following the 75°C and 100°C treatment, respectively. Therefore, *C. perfringens* and especially its spores, could serve as a useful indicator in aquatic ecosystems with stress factors (Bezirtzoglou *et al.*, 1994).

Sulphite-reducing clostridia (SRC), which include *C. perfringens*, has also shown to be a valuable indicator organism for both point and non-point faecal pollution, as well as monitoring water quality to control waterborne diseases, especially in developing countries (Abia *et al.*, 2015; Hafeez *et al.*, 2020; Hamza *et al.*, 2018; Lee *et al.*, 2012; Mushi, 2018; Potgieter *et al.*, 2020; Sartory, 1986). A study done by Potgieter *et al.* (2020) assessed the water quality of 8 river systems in South Africa during the winter and summer season. The levels of *C. perfringens* present in the water samples reached a high of 35 colony forming units (CFU)/100 ml during the summer and >500 CFU/100 ml in the winter, indicating intermittent faecal contamination in the various rivers. Water systems, such as rivers, lakes, estuaries, and lagoons, are often utilized as receiving environments for wastes from residential areas, manufacturing industry and wastewater treatment plants, which dump their untreated or inadequately treated wastewater (Onifadé *et al.*, 2017). The spores of *C. perfringens* present in wastewater can survive longer in water systems than that of other vegetative faecal indicator bacteria, such as *Escherichia coli* (Abia *et al.*, 2015; Manafi *et al.*, 2013). These spores have also been used to monitor protozoan pathogens, such as cysts of *Giardia* sp., and oocysts of *Cryptosporidium* sp. in surface waters associated with agricultural landscapes (Horman *et al.*, 2004; Wilkes *et al.*, 2009). Ferguson *et al.* (1996) investigated the relationship between indicator organisms, environmentally stable pathogens and enteric parasites in an estuarine environment. The results showed that when compared to faecal coliforms and faecal streptococci, *C. perfringens* spores were the most useful indicator of faecal pollution. Furthermore, *C. perfringens* was also the only indicator that showed significant correlation between pathogenic *Giardia* and the opportunistic *Aeromonas* sp.

All these studies highlight that surface water systems are extremely vulnerable to microbiological contamination, particularly with *Clostridium* pathogens. Although certain *Clostridium* species can assist with identifying faecal pollution in water systems, the potential transmission of pathogenic species via water is problematic. These pathogens have multiple means of transmission throughout agroecosystems and are not always prevented by improving water quality alone.

2.4.3. Prevalence of *Clostridium* species in fresh produce

There is a global increase in the consumption of fresh vegetables, with production increasing from 682 million tons to 1.15 billion tons over the last few years, showing an approximate 69% increase (FAO, 2024). However, alongside this, there is also an uprising of etiological agents that cause foodborne outbreaks from these production environments, posing a major health risk to consumers and the world at large (Balali *et al.*, 2020). Several *Clostridium* species have been associated with foodborne disease, with the most well-known being *C. perfringens*, responsible for food poisoning, and *C. botulinum*, which causes the rare but highly fatal food-borne botulism. However, the prevalence of clinically relevant *C. difficile* in fresh produce is on the rise across the world (Han *et al.*, 2018; Lim *et al.*, 2018; Usui *et al.*, 2020). Several studies have shown strong associations between *Clostridium* pathogens and fresh produce that is consumed raw (Table 2.4), particularly in countries such as Iran, South Korea, Nigeria, Thailand, Slovenia, Australia and USA (Azimirad *et al.*, 2021; Chukwu *et al.*, 2016; Han *et al.*, 2018; Lim *et al.*, 2018; Tango *et al.*, 2018; Tassanaudom *et al.*, 2017; Tkalec *et al.*, 2019). Furthermore, countries such as Nigeria, the USA, Mexico and Egypt are also among the world's leading producers of fresh vegetables, cultivating 74.62 million tons collectively in 2022 alone (FAO, 2024).

Fresh produce can be contaminated with disease-causing bacteria at any point during the production chain, during preharvest or postharvest processes (Figure 2.2) (Gil *et al.*, 2015). Regarding the preharvest sources of contaminations, the direct contact between produce and the soil they are cultivated in has shown to be a major transmission route for pathogens, especially in root vegetables. This is evident in a study by Hashimoto *et al.* (2023), where 93% of soil samples attached to the surface of 30 potatoes tested positive for *C. perfringens.* Additionally, of the 613 *C. perfringens* isolates obtained, the majority were classified as type A (73%), followed by type F (20%), type C (6%) and type E (0.003%). In a different study, Tkalec *et al.* (2019) investigated the contamination rates of *C. difficile* in potatoes and leaf vegetables grown in Slovenian soil. Of all the collected vegetables, potatoes exhibited a higher positivity rate than the leafy vegetables, with *C. difficile* being detected in 60% of potato samples, as opposed to 14.3% in lettuce samples. Another source of preharvest contamination of produce includes contaminated water that is used for irrigation and pesticides to be applied to crops (Balali *et al.*, 2020). Alegbeleye & Sant'Ana (2023) reported on the microbiological quality of different water sources (surface water, groundwater, and municipal water) that are utilised to irrigate 69 vegetable farms in Sao Paulo, Brazil. The results showed elevated levels of enteric pathogens, including *C. perfringens*, across all irrigation sources.

Table 2.4: Prevalence of *Clostridium* species in fresh produce.

Species	Sampling area	Samples (n)	Isolates (n)	ID Method	Source	Authors
C. perfringens	Iran	366	66	Biochemical, PCR	Raw and RTE leafy vegetables	Azimirad *et al.*, 2021
	Egypt	10	4	PCR	Vegetables	Hamza *et al.*, 2018
	South Korea	360	48	qPCR	Lettuce, Spinach, Sprouts	Tango *et al.*, 2018
	Nigeria	50	28	Biochemical, PCR	Water leaf, Green peppers, Pumpkin leaf, Cabbage, Carrot	Chukwu *et al.*, 2016
	Thailand	100	160	Biochemical, Multiplex PCR	Dried peppers	Tassanaudom *et al.*, 2017
	Mexico	300	6	qPCR	Parsley	Gómez-Govea *et al.*, 2012
	Seoul, Korea	345	21	Biochemical	Salad, Sprouts, Lettuce	Seo *et al.*, 2010
	South Wales	300	7	Enzyme immunoassay	Potato, Onion, Mushroom, Carrot, Radish, Cucumber	Al Saif & Brazier, 1996
C. difficile	Ireland	100	5	PCR	Spinach, Lettuce, Rocket, Coleslaw	Marcos *et al.*, 2021
	Japan	242	8	PCR	Root vegetables	Usui *et al.*, 2020
	USA	297	41	qPCR	Lettuce	Han *et al.*, 2018
	Slovenia	154	28	PCR, MALDI-TOF	Leafy vegetables, Ginger, Potatoes	Tkalec *et al.*, 2019
	Western Australia	300	30	Biochemical, PCR	Root vegetables	Lim *et al.*, 2018
	Iran	70	3	PCR	Salad	Rahimi *et al.*, 2015
	USA	125	3	Biochemical, PCR	Lettuce, Green pepper, Eggplant	Rodriguez-Palacios *et al.*, 2014
	Iran	106	6	Multiplex PCR	Salad	Yamoudy *et al.*, 2015
	France	104	4	Biochemical, PCR	Salad	Eckert *et al.*, 2013
	Canada	111	5	PCR	Carrot, Ginger	Metcalf *et al.*, 2010
	Scotland	40	3	PCR	Salad	Bakri *et al.*, 2009
C. botulinum	Florida, USA	1118	4	Enzyme immunoassay	Cabbage, Green pepper, Salad	Lilly *et al.*, 1996

When these pathogens are introduced onto fresh produce and the adjacent soil environment via irrigation, they may internalize into the edible parts of vegetables or persist on or within vegetables until harvest (Alegbeleye *et al.*, 2018). Hamza *et al.* (2018) aimed to demonstrate this by investigating the possible link between *C. perfringens* strains from livestock and contaminated produce through water and soil transmission. The results reported a significant correlation made between the prevalence of *C. perfringens* in livestock and these environmental samples ($P < 0.05$), with 55.81% prevalence of *C. perfringens* in livestock, 40% in soil samples, 31.7% in irrigation water samples and 40% prevalence in all produce samples.

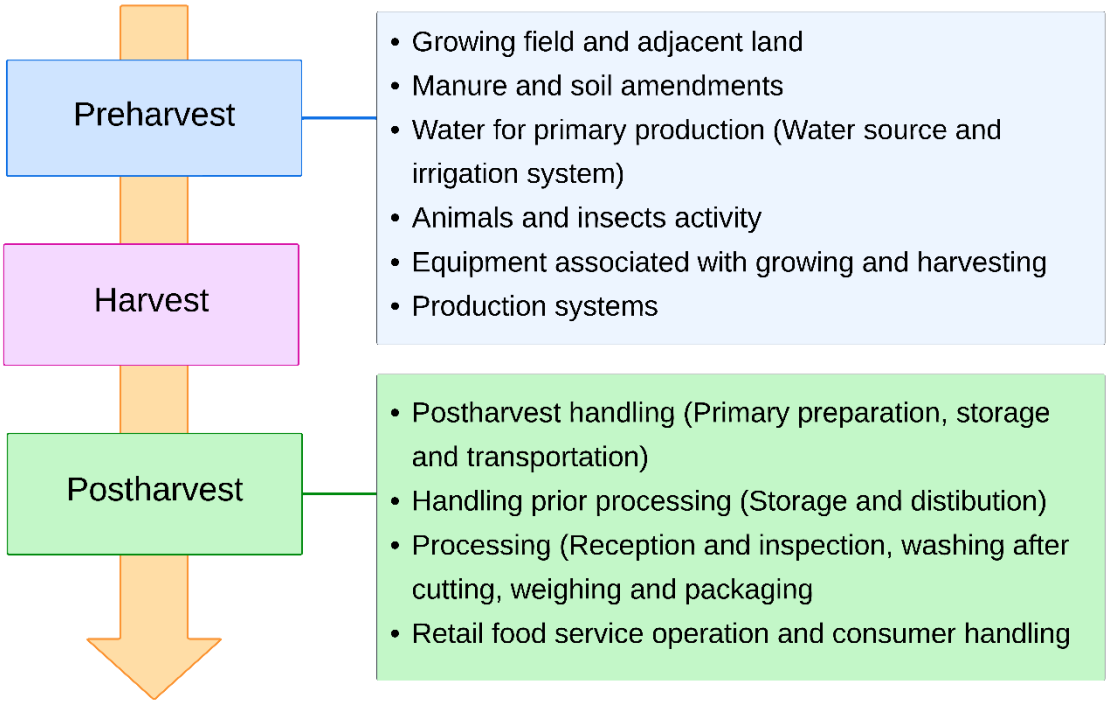


Figure 2.2: Microbial contamination of fresh vegetables in the farm-to-table cycle. This graphic depicts probable contamination occurrences at the various stages of the process. Contamination can occur before, during, and after harvesting, resulting in a disease outbreak (Gil *et al.*, 2015).

Postharvest contamination of fresh produce can occur during field harvesting and field packing, as well as during processing practices, storage, distribution, sale or consumer handling. Although the washing of fresh produce, especially produce that is seen as 'ready-to-eat' (RTE), is crucial to remove unwanted contaminants before being prepared for consumption (Gil *et al.*, 2015), it is not always successful in eliminating pathogens such as *Clostridium*. Several studies have detected *Clostridium* pathogens in retail produce and RTE raw salads, with *C. perfringens* and *C. difficile* being the most frequently isolated from leafy

vegetables sold at market (Azimirad *et al.*, 2021; Han *et al.*, 2018; Rodriguez-Palacios *et al.*, 2014; Tango *et al.*, 2018). Marcos *et al.* (2021) tested a variety of vegetables sold at retail and detected *C. difficile* in five out of the 100 samples. This included spinach (2 samples), lettuce, rocket, and coleslaw (1 sample each). Furthermore, direct counts were obtained for spinach (5.8 $\log_{10}$ CFU/g) and coleslaw (4.3 $\log_{10}$ CFU/g). According to a study conducted in South Wales, 71% of *C. difficile* strains isolated from vegetables were toxigenic (Al Saif & Brazier, 1996). Toxigenic *C. difficile* was also present in RTE salads sold in Iran, Scotland and France, with the ribotypes 078 and 014 being most prevalent in Iran (Rahimi *et al.*, 2015; Yamoudy *et al.*, 2015), RT017 and RT001 in Scotland (Bakri *et al.*, 2009), and RT001, RT014/020/077 and RT015 in France (Eckert *et al.*, 2013). *Clostridium perfringens*, along with *Escherichia coli*, were the most frequently identified pathogens among minimally processed vegetables in Seoul, Korea (Seo *et al.*, 2010). It was present in 21 of the 345 samples, which included lettuce (3.3%), mixed salads (3.9%) and sprouts (12.5%). A study done in Tehran, Iran, assessed the microbiological safety of raw and RTE leafy vegetables purchased from different markets (Azimirad *et al.*, 2021). Of the 21 foodborne pathogens tested for, *C. perfringens* was the third most prevalent pathogen (66/366), after *Staphylococcus aureus* (134/366) and *Escherichia coli* (85/366).

Foodborne botulism, brought on by botulinum neurotoxin (BoNT) produced by *C. botulinum*, has been widely acknowledged as rare but severe paralytic disease (Fleck-Derderian *et al.*, 2017). Although botulism is mostly associated with the consumption of cooked canned vegetables, there have been reports of outbreaks implicating fresh produce as transmission source (Beuchat, 1996; Solomon *et al.*, 1990). An outbreak of botulism in Florida, USA, was linked to RTE coleslaw which was prepared from raw shredded cabbage (Solomon *et al.*, 1990). A study done by Lilly *et al.* (1996) investigated the prevalence of *C. botulinum* spores in commercially packaged vegetables across Florida, USA. The results showed, however, a low prevalence (0.36%) of *C. botulinum*, with only 4 of the 1118 samples tested positive. These 4 strains were isolated from shredded cabbage, green pepper and 2 salad mixes. Additionally, all strains were identified as serotype A, except for one salad mix, which was type A and B.

The prevalence of *Clostridium* pathogens from the existing data indicates that they can contaminate cultivated fresh produce, be it from pre- or postharvest practices. The presence of these pathogens is concerning, since possible exposure to or consumption of these produce may put humans at risk for *Clostridium* infections. There is also an overall need for more efficient decontamination processes during the production chain. Furthermore, additional information regarding the water quality of irrigation sources, as well as the hygiene procedures used throughout processing, storage, and marketing, is warranted to identify crucial contamination points better and define appropriate intervention measures.

2.5. Antibiotics in agroecosystem and effects on *Clostridium*

The World Health Organisation has declared antibiotic resistance a global threat to humans and animals, as well as the natural environment (WHO, 2023). Modern agroecosystems have been shown to harbour and spread pathogenic bacteria, including but not limited to *Clostridium* species, which present with antibiotic resistance (Franklin *et al.*, 2016; Williams-Nguyen *et al.*, 2016). To help illustrate the pathways through which exposure to antibiotics (AB), antibiotic-resistant bacteria (ARB), and antibiotic-resistance genes (ARGs) may occur and their associations to expected effects, a cause model was put forth for agroecosystems (Figure 2.3) (Williams-Nguyen *et al.*, 2016). Embracing a One Health perspective, the causal model delineates crucial interactions among antibiotics, ARB, and ARGs, along with their subsequent effects within agroecosystems and focuses on three distinct endpoints: (i) human health, (ii) ecosystem function, and (iii) agricultural system productivity. This phenomenon results from the overuse and misuse of antibiotics in both health and agricultural sectors (Manyi-Loh *et al.*, 2018). Antibiotics are used to treat serious bacterial infections in humans and animals, as well as prevent stress-related diseases and help promote faster growth in livestock (Franklin *et al.*, 2016). However, these antibiotics are only partly metabolised and then excreted, leaving as much as 90% of them unchanged, i.e., still active (Kumar *et al.*, 2005). These antibiotics are then introduced into the natural environment through various means (Samreen *et al.*, 2021). Studies have found that wastewater treatment facilities are unable to remove antibiotic residue successfully and are subsequently released into surrounding surface water systems (Baquero *et al.*, 2008; Kraemer *et al.*, 2019). Antibiotics are also readily found in agricultural soil. This is largely due to manure and sewage sludge application as fertiliser (Symochko *et al.*, 2023). The exposure of these antibiotics to the indigenous microbes has resulted in the resurgence of antibiotic-resistant bacteria and genes in these environments (Kunhikannan *et al.*, 2021). This encourages genetic mutations and exchange between non-target species of bacteria, whether they perform critical ecosystem functions, are pathogenic or have undefined roles in the ecosystem (Symochko *et al.*, 2021).

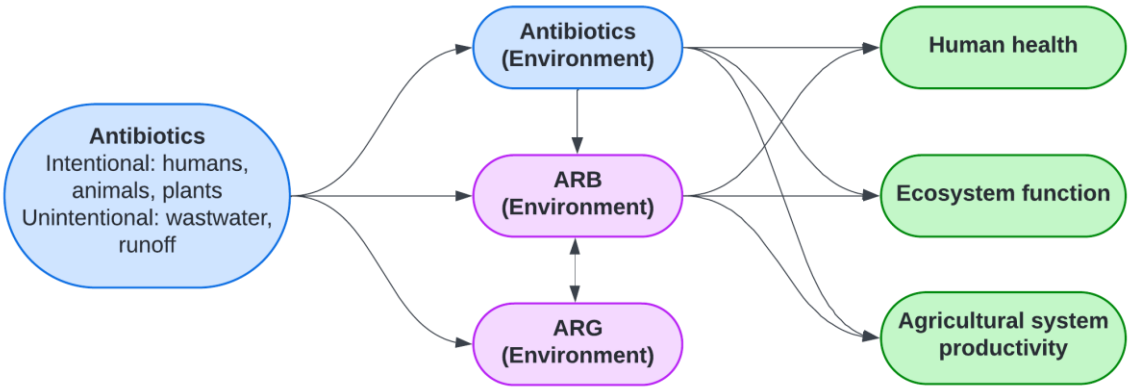


Figure 2.3: Proposed causal model depicting effects of antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic-resistance genes (ARGs) in agroecosystems and the environment. The use of antibiotics results in increased levels of antibiotics, ARB, and ARGs in agroecosystems. Residues of antibiotics cause an enrichment of ARB in the environment. ARB and ARGs, though distinct, are intrinsically linked with unknown or mixed causal directions. Antibiotics and ARB in agroecosystems affect human health, ecosystem function, and agricultural system productivity. Blue represents active antibiotic compounds. Purple represents resistance elements. Green represents the outcomes of interest (Williams-Nguyen *et al.*, 2016).

Considering the above-mentioned information, antibiotic resistance among *Clostridium* species is on the rise (Khademi & Sahebkar, 2019). Several studies have identified antibiotic-resistant *Clostridium* species in agricultural environments, such as soil, surface water and livestock (Fourie, 2017; Khan *et al.*, 2021; Symochko *et al.*, 2023). Resistance to several antibiotics, such as clindamycin, erythromycin and tetracycline, have been found in major *Clostridium* pathogens globally (Archambault & Rubin, 2020). A study done by Symochko *et al.* (2021) isolated *C. perfringens* from an agroecosystem in Ukraine, that was treated with organic fertiliser continuously for 3 years, which showed multidrug resistance against 6 different antibiotics (amoxicillin erythromycin, rifampicin, clindamycin, tetracycline, and metronidazole). In the Czech Republic, high levels of antibiotic resistance (>256 mg/l) to clindamycin and erythromycin have also been reported in 22 *C. difficile* strains isolated from wastewater and receiving surface water systems, of which 62.6% carried the macrolide resistance gene, *erm*B (Cizek *et al.*, 2022). Additionally, *C. perfringens* and *C. difficile* have even shown resistance to last resort antibiotics, specifically vancomycin (Eubank *et al.*, 2022; Osama *et al.*, 2015; Tansuphasiri *et al.*, 2005). Several *Clostridium* pathogens have also been shown to contribute to the environmental spread of antibiotic resistance via conjugal transfer of tetracycline (*tet*M) and macrolide (*mef*A) resistance genes (Soge *et al.*, 2009), as well as

plasmid mediated resistance for metronidazole (Cizek *et al.*, 2022). Treating infections caused by these pathogens is, therefore, more challenging, as it requires the use of more noxious and expensive drugs (Serwecińska, 2020). Followingly, the Centre for Disease Control and Prevention classified *C. difficile* under 'Urgent Threats' that urgently require new antibiotics to treat (CDC, 2019). However, the spread of antibiotic-resistance bacteria and genes is currently outpacing the discovery and development of novel antibiotics, which could suggest that the golden era of antibiotics ending (Shim, 2023). Therefore, it is crucial to monitor susceptibility data of *Clostridium* species to maintain effective treatment against infections.

2.6. Anaerobic culturing and characterisation approaches for *Clostridium* species

It is important to remember that the experiments conducted today are rooted in history. In the late 1950s, Dr Horace Barker successfully used a small flask filled to the brim with specific substrates and nutrients and capped with a ground-glass stopper to cultivate anaerobic bacteria. This method of cultivation became widely used to grow anaerobes (Barker, 1956; Wolfe, 1999). However, cultivating and characterising anaerobic bacteria, such as *Clostridium*, in a laboratory setting today, still comes with some challenges.

2.6.1. Cultivation methods

Although culture-based methods have been essential in increasing our understanding of specific bacteria, they can only provide information on the 1% of bacteria that are readily cultivated (Nocker *et al.*, 2007). Certain *Clostridium* species can be identified and enumerated from different type of samples using culture assays (Palmer *et al.*, 2019) The most routinely used methods include direct plate count and most probable number (MPN) (Sonnabend *et al.*, 1987; Vijayavel & Kashian, 2014). However, they require specific growth conditions, such as anaerobiosis (stable anaerobic environment) and nutrition-rich media (Gajdács *et al.*, 2017). Non-selective growth mediums such as reinforced clostridial agar, blood agar and brain heart infusion agar are used to cultivate a wide variety of fastidious *Clostridium* species before being used for other diagnostic purposes (Byrne *et al.*, 2008). However, the unusual colony morphology on plates may contribute to the misidentifying *Clostridium* species. The choices of selective and differential media currently available for cultivating *Clostridium* species are limited, making it challenging to differentiate closely related species in samples containing multiple strains (Palmer *et al.*, 2019). However, for the selective growth of sulphite-reducing

clostridia, which includes *C. perfringens*, agars such as tryptose sulphite cycloserine (TSC) or Shahidi-Ferguson Perfringens (SFP) is recommended (Barrios *et al.*, 2013; Byrne *et al.*, 2008). These types of media rely on the principle that the bacteria reduce sulphites to sulphides, resulting in the formation of black colonies that are easily recognized (Barrios *et al.*, 2013).

An anaerobic environment can be created by several means, such as using anaerobic jars or chambers (glove boxes) and gassing stations. Although, these types of equipment are not always readily available in laboratories. This is due to the associated cost, maintenance, and tedious efforts in operating them (Hanišáková *et al.*, 2022). However, there have been several approaches to overcome these challenges of anaerobic cultivation. One such example is the Fung double tube (FDT) method. Developed by Fung in the 1980s, this method only requires two glass test tubes (one screw cap and the other being small enough to fit inside the aforementioned) to create the desired anoxic environment (Fung & Lee, 1981). Several studies have also reported that the FDT method resulted in more effective anaerobic enumeration than traditional anaerobic incubation using plates (Ali & Fung, 1990; Barrios *et al.*, 2013). In short (Figure 2.4), liquified agar (e.g. TSC), along with the sample, are mixed and form a thin layer of agar between the two test tubes (creating anaerobiosis) for *Clostridium* to grow. This method was found to be extremely efficient and cost effective in enumerating *Clostridium* species from various sources, including food and water samples, delivering results within 6 hours with no additional equipment to achieve anaerobiosis (Fourie, 2017; Riley *et al.*, 1999).

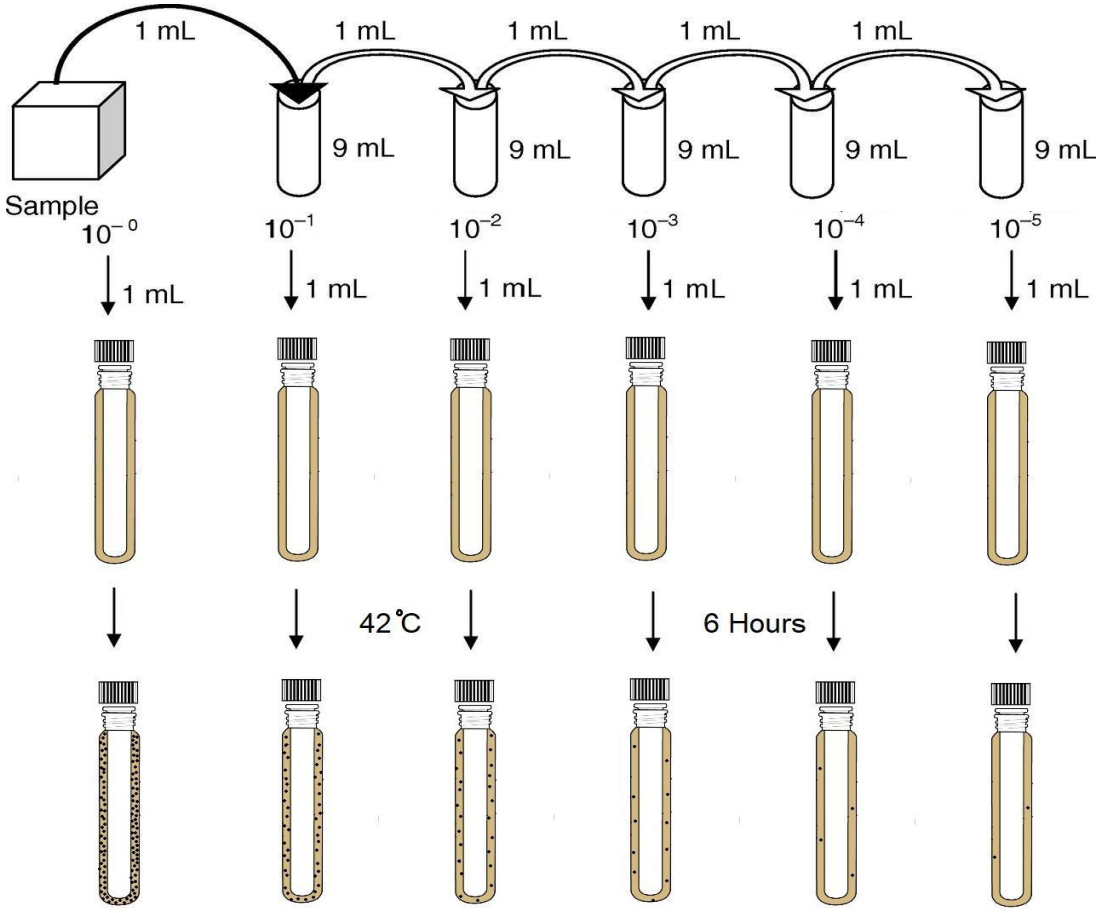


Figure 2.4: Schematic of isolating *Clostridium* species from environmental samples using the Fung double tube (FDT) method. A 10-fold serial dilution is made up of the samples, following which it is mixed with liquefied agar. Sulphite-reducing clostridia, such as *C. perfringens,* will appear as black colonies after incubation at 42°C for 6 hours when using tryptose sulphite cycloserine (TSC) agar.

2.6.2. Biochemical based analysis

In microbiology, biochemical or metabolic tests have been used to isolate, identify, and characterise diseases causing microorganisms for almost 150 years (Altheide, 2019). Over the years, the enzymatic versatility of bacteria was harnessed and refined to where differential, culture-based, biochemical testing became an important and effective methodology in any diagnostic laboratory (Altheide, 2019). With the use of a multistep methodology, using diverse growth substrates in both solid and liquid media, a distinct metabolic profile of an unknown bacteria could be established, offering a reproducible method for identification (Franco-Duarte *et al.*, 2019; Palmer, 2019). Based on the British standards for microbiology investigations,

identification of *Clostridium* species from Public Health England (2014), clinically relevant *Clostridium* pathogens can be preliminary identified based on their growth characteristics, colony morphology and biochemical characteristics, such as lecithinase, indole, lipase, and urease activity (Figure 2.5). Several studies have identified *Clostridium* species from environmental samples, solely based on their biochemical profiles and gas-chromatographic analysis of fermentation products (Båverud *et al.*, 2010; Creti *et al.*,1990; del Mar Gamboa *et al.*, 2005; Wilkins *et al.*, 1988). However, the accurate identification of *Clostridium* species based on these methods is laborious and time-consuming, posing challenges for inexperienced users. Additionally, strictly standardized media, growth conditions and pure cultures are required to achieve consistent results. There are several *Clostridium* species that are characterized by only a single strain which could lead to misidentification due to inherent variation among intraspecies or interspecies phenotypes (Grosse-Herrenthey *et al.*, 2008). Another limitation of these culture-based methods is the inability to identify non-culturable bacteria (Franco-Duarte *et al.*, 2019). A study done by Bagge *et al.* (2009), reported the misidentification of *C. chauvoei* in 42.9% of samples when utilising biochemical tests, as opposed to molecular identification methods, such as polymerase chain reaction (PCR). Commercially available kits, such as RapID™ ANA II and API® systems, have been developed to identify *Clostridium* species (Palmer, 2019). Although these kits are faster and more convenient, they lack both sensitivity and specificity to correctly identify some *Clostridium* pathogens, such as *C. difficile* and *C. botulinum* (Lindström *et al.*, 1999; Head & Ratnam, 1988). Furthermore, distinguishing between *C. bifermentans* and *C. sordellii* proves challenging with traditional methods as their biochemical profiles are almost identical (Sanchez Ramos & Radloff, 2018).

The use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a reference standard for the identification of bacteria (Palmer, 2019). MALDI-TOF MS has been described as an analytical chemistry identification method, rather than a traditional biochemical technique (Altheide, 2019). It is a true departure from other phenotypic approaches, since organisms are not cultivated with various substrates and media to assess metabolic utilisation, and the detection of specific enzymes is not conducted. MALDI-TOF MS rather establishes the protein "fingerprint" of an organism by subjecting a single colony to a high-energy laser. This process generates individual peptide fragments of varying sizes, resulting in a distinctive chemical spectrum (Altheide, 2019). Studies have emphasised the advantages of MALDI-TOF MS over traditional identification methods, which include rapidity, reproducibility, sensitivity, associated costs, and low sample volume requirements, making it an ideal method for routine and high-throughput use (Al-Mogbel, 2015; Chean *et al.*, 2014). However, the high initial investment and maintenance costs of MALDI-

TOF MS may prove too expensive for some laboratories (Palmer, 2019). Although only a few studies have used MALDI-TOF MS to investigate anaerobic bacteria, it has shown great potential for typing applications in *Clostridium* species, as well as the development of fully automated workflows (Al-Mogbel, 2015; Grosse-Herrenthey *et al.*, 2008; Sanchez Ramos & Radloff, 2018).

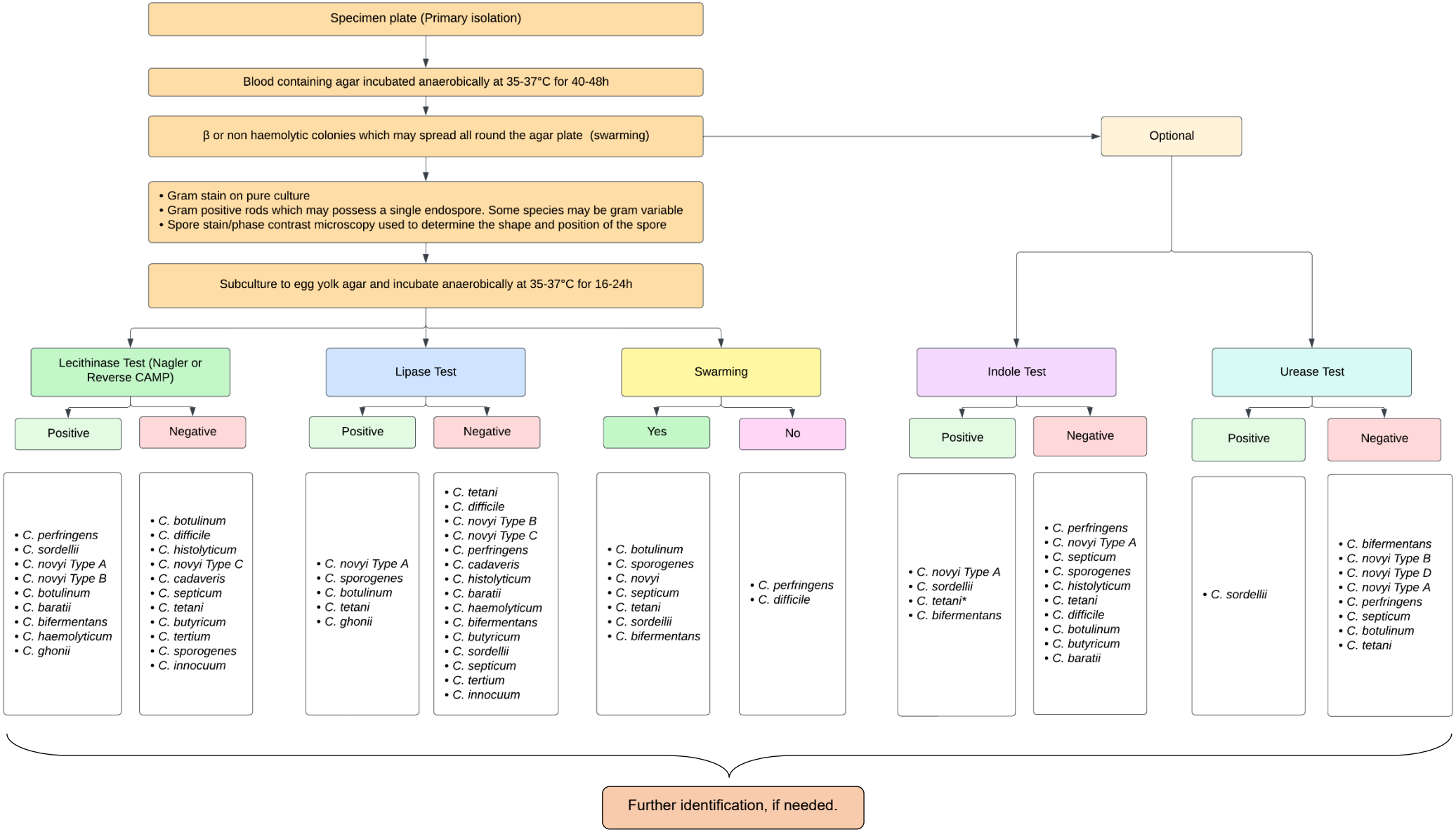


Figure 2.5: Identification of *Clostridium* species based on their phenotypical and biochemical characteristics (Public Health England, 2014).

2.6.3. Antibiotic susceptibility testing

Methods used for antibiotic susceptibility testing (AST) in anaerobic bacteria provide information on the expected bacterial response to antibiotics in the form of minimum inhibitory concentration (MIC). These standards and practices for AST are governed by organisations such as the Clinical Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (Sood *et al.*, 2022). Testing methods such as disk diffusion (Kirby-Bauer Method), broth microdilution and gradient strips are routinely used in practice (Gajdács *et al.*, 2017). However, agar dilution is considered the 'golden standard' for AST in anaerobic bacteria. The current problem facing AST in anaerobes is the lack of literature, with the main issue being that many existing studies do not follow the guidelines set out by the CLSI or EUCAST. This results in methodology and interpretive breakpoint differences, which adds to the variation in resistance data available (Sood *et al.*, 2022).

Although international attempts to monitor antibiotic resistance are focused on culture-based methods, there are molecular diagnostics approaches to detect antibiotic resistance, however, these methods have their limitations and are currently not standardised (Banerjee & Patel, 2023; Franklin *et al.*, 2016). While both polymerase chain reaction (PCR)-based methods and metagenomic approaches have a lot of potential for understanding the diversity and amount of antibiotic resistance genes (ARGs) in complex environments, they are not able to confirm the functionality of the resistance mechanisms that have been identified, or to link specific ARGs to certain bacterial groups (Franklin *et al.*, 2016). Studies have compared the effectiveness of traditional culture-based methods and characterization methods with newer techniques that focus on specific antibiotic resistance genes, rather than whole bacteria. However, there has not yet been a single method or combination of methods proven to be the most accurate (Campbell *et al.*, 2011; Nordmann *et al.*, 2012). To accurately evaluate antibiotic resistance in the environment in the future, standardized methods that use both culture-based and molecular techniques will be necessary (Franklin *et al.*, 2016).

2.6.4. Molecular approaches

2.6.4.1. Polymerase chain reaction (PCR)-based techniques

Polymerase chain reaction (PCR) is an enzyme-dependent procedure based on in vitro replication by using heat-stable DNA polymerase and highly precise oligonucleotide primers to identify and amplify sections of the target gene. If present, this method can generate large quantities of the targeted gene from extracted DNA (Kadri, 2019). This is achieved by a reaction that consists of template DNA, gene-specific primers, Taq polymerase and the four deoxyribonucleoside triphosphates (dNTPs) in a balanced buffer solution which is then

subjected to repetitive temperature cycles of denaturation, annealing and extension (Figure 2.6) (Kadri, 2019; van Pelt-Verkuil & te Witt, 2019, Wilson, 2002). In short, during the denaturing stage of PCR, the hydrogen bonds of double-stranded DNA (ds-DNA) are denatured and separated into single-stranded DNA (ssDNA) by increasing the temperature to 94°C (denaturation temperature). The annealing stage is then carried out at 40-70°C (primer hybridization temperature), where the lower temperature allows for the hydrogen bonds to reform between the ssDNA and primers at the complementary section of the targeted gene. The extension stage is then carried out at a temperature of 72°C (elongation temperature), During this stage, Taq polymerase binds to the primed ssDNA and catalyzes replication by extending the primers from the 3' hydroxyl end and using the dNTPs (dATP, dGTP, dCTP and dTTP) to synthesize new, complementary DNA. Thus, the synthesis of two new dsDNA is completed after one cycle, after which the denaturation, annealing and extension are repeated during the second cycle and so forth. A protocol of between 20 and 40 cycles is needed to synthesize an analysable amount of DNA (~0.1 µg) (Kadri, 2019; van Pelt-Verkuil & te Witt, 2019. Wilson, 2002). The synthesised products (amplicons) can then be characterised by various techniques such as electrophoresis, hybridization, and DNA sequencing (van Pelt-Verkuil & te Witt, 2019).

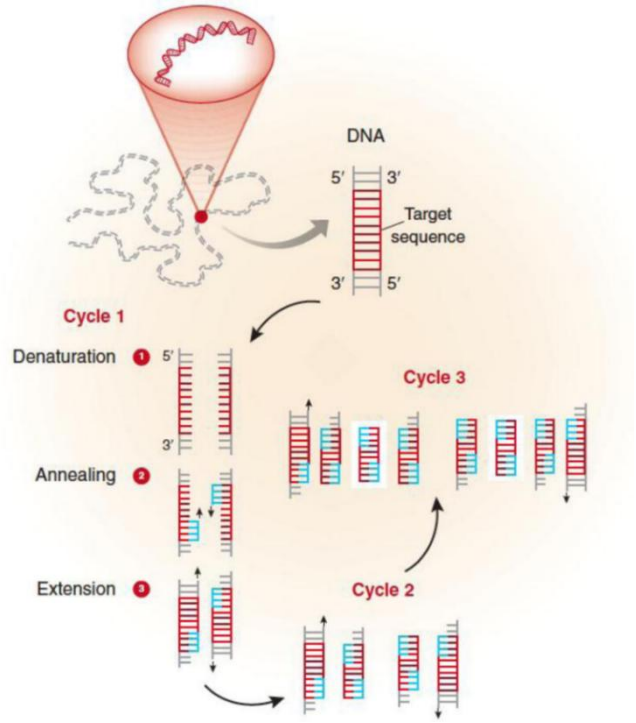


Figure 2.6: Diagram illustrating the principles of polymerase chain reaction (PCR) (Wilson, 2002).

Several molecular techniques are based on PCR technology. Multiplex, nested, and semi-nested PCR approaches allow for the simultaneous and selective detection of different target

genes, species, or toxinotypes. Quantitative PCR (qPCR) uses fluorescently labelled probes (Molecular beacons® and TaqMan®) or intercalating dyes (SYBR® Green) to visualise the fragment amplification during the entire PCR process (Rocha *et al.*, 2015). This method allows for the detection and quantification of target genes in real-time (relative quantification), as well as analysing gene expression levels when combined with reverse transcription (absolute quantification) (Garibyan & Avashia, 2014). Furthermore, many diagnostic methods are based on qPCR techniques. Numerous studies have shown the value and sensitivity of PCR techniques to detect *Clostridium* pathogens in various environmental and food samples (Table 2.2-4). However, there are challenges and limitations when using PCR on samples from agroecosystems (Franklin *et al.*, 2016). One such challenge is the efficacy of DNA extraction from samples. Due to PCR being such a sensitive technique, extraction protocols should be optimized to capture pure DNA from a consortium of bacteria and applied consistently across all samples intended for comparison analysis (Franklin *et al.*, 2016; Garibyan & Avashia, 2014). Any type of sample contamination, even trace quantities of DNA, can lead to inaccurate results (Garibyan & Avashia, 2014). In addition, the design of primers for PCR can only be done from prior DNA sequence data, resulting in the detection of only known pathogens or genes in environmental samples (Franklin *et al.*, 2016; Garibyan & Avashia, 2014). Primers can also anneal non-specifically to sequences that are similar, but not identical to the target DNA. Furthermore, while extremely rare, the DNA polymerase can insert erroneous nucleotides into the PCR sequence (Garibyan & Avashia, 2014). Another drawback with PCR-based diagnostics is the exceptionally large, but poorly understood microbial diversity in agroecosystems, especially soil (Palmer, 2019). In the “black-box” of soil ecology there are many undiscovered species that could have unknown influence on the interpretation of PCR results (Cortois & De Deyn, 2011; Palmer, 2019). Many rare species remain undetectable, and unidentified species with significant genetic homology that might hinder the sensitivity and accuracy of the results (Palmer, 2019). It is, therefore, recommended that PCR products are sequenced to verify the amplification of the targeted gene when working with environmental samples (Franklin *et al.*, 2016).

2.6.4.2. Sequencing

Since Watson and Crick first described the three-dimensional structure of DNA in 1953, the science of sequencing has grown in popularity and importance. To date, sequencing technology has experienced three generations of evolution, especially in DNA and RNA sequencing (Mohammadi & Bavi, 2021). First-generation sequencing generally refers to Sanger sequencing, which uses fluorescently labelled dideoxy-nucleotides and capillary electrophoresis to produce sequencing reads of up to a few hundred nucleotides in length

(Park & Kim, 2016). The term "Next-generation sequencing" (NGS) is used as an umbrella term for second-generation (short-read sequencing) and third-generation (long-read sequencing) technologies (Satam *et al.*, 2023). These advances have increased throughput, sequencing millions of DNA fragments at the same time, while reducing turnaround time and associated costs. NGS has shown to be a powerful discovery tool in genomic research, especially when investigating the whole genome (DNA sequencing) and transcriptomic responses (RNA sequencing) in an organism (Hu *et al.*, 2021; Satam *et al.*, 2023).

Genotyping an organism via sequencing its whole genome can generate a detailed blueprint of its genetic makeup which includes all the genes, regulatory regions and non-coding elements that are present (Kockum *et al.*, 2023). This makes it possible to detect genetic differences, such as single-nucleotide polymorphisms (SNPs) and structural variations like insertions, deletions, and rearrangements, even without a reference sequence (Satam *et al.*, 2023). Additionally, whole-genome sequencing (WGS) of clinically relevant pathogens has transformed the understanding of antibiotic resistance and outbreak investigations (Gajdács *et al.*, 2017). A study done by Wen *et al.* (2022) used WGS to genomically link 43.8% (110/251) of *C. difficile* strains from a clinical setting to prior infection cases over a 4-year period. Furthermore, whole-genome analysis showed the acquisition of fluoroquinolone resistance as a key genetic change linked to the transcontinental dissemination of two lineages of epidemic *C. difficile* (027/BI/NAP1) (He *et al.*, 2013). Additionally, RNA sequencing is a widely used method for studying the behaviour of genes under different biological conditions (Evans *et al.*, 2018). Sequencing the mRNA molecules in an organism can provide a comprehensive snapshot of the expressed genes and various biological processes. The data generated can be used to identify and quantify differential gene expression, discover novel transcripts, and study gene expression dynamics over time (Mohammadi & Bavi, 2021; Satam *et al.*, 2023). This all has made investigating bacterial genome and transcriptome sequencing more practical and convenient.

Short-read sequencing approaches are currently the most frequently used form of NGS and are represented by technology platforms such as Illumina (sequencing by synthesis) and Ion Torrent (semiconductor sequencing) (Hu *et al.*, 2021; Ye *et al.*, 2015). The shared characteristic of these technologies is the extensive sequencing of short DNA molecules (250–800 bp) that are clonally amplified and sequenced in parallel. These approaches generally begin with (i) library preparation, followed by (ii) sequencing, and lastly, (iii) analysis of the data.

i. Good library preparation is a prerequisite for DNA and RNA sequencing when using short-read sequencing platforms. The library preparation for DNA sequencing involves

the fragmentation of DNA, end-repair, ligation of platform-specific adapters (enables downstream processing and sample identification), and size selection. Sample preparation for RNA sequencing generally includes total RNA isolation, target RNA enrichment, chemical fragmentation of remaining RNA, end-repair, adaptor-ligation and random priming for reverse transcription of RNA into complementary DNA (cDNA) (Hu *et al.*, 2021).

ii. Before sequencing can commence, clonal amplification of the DNA fragments is performed to generate strong, detectable signals during sequencing. These fragments then bind to flow cell surfaces (Illumina) or beads (Ion Torrent) and are amplified by "bridging" PCR (Illumina) or emulsion PCR (Ion Torrent) to synthesise millions of spatially separated template fragments simultaneously. The large volumes of sequenced fragments, referred to as reads, can then be reassembled and analysed with various bioinformatic tools (Hu *et al.*, 2021).

iii. Streamlined bioinformatics analysis and management of sequence data is essential when using DNA or RNA sequencing approaches. Therefore, the standard workflow includes primary, secondary, and tertiary analyses.

For DNA sequencing data, the primary analyses involve quality control measures such as filtering and trimming low-quality reads and can be done by the instrumental software after sequencing or by tools such as FastQC and Trimmomatic (Bolger *et al.*, 2014). The short reads (single-end or paired-end) are then stored as FASTQ files and used for secondary analysis, which involves read alignment and variant calling against reference genomes (reference-based alignment) or *de novo* assembly of reads without any reference genomes. Bioinformatic tools such as Bowtie2 and SPAdes are generally used for short read alignments and *de novo* assembly, respectively (Bankevich *et al.*, 2012; Langdon, 2015). These programs use algorithms to align overlapping of reads into contigs (larger contiguous sequences) and then arrange these contigs to form scaffolds (a framework of the sequenced genome), which are then stored as a binary alignment/map (BAM) or sequence alignment/map (SAM) file (El-Metwally *et al.*, 2013; Hu *et al.*, 2021). The quality of the genome assemblies can then be evaluated by a variety of metrics. The N50 metric is commonly used to assess the contiguity of assemblies and represents the length of the smallest contig or scaffold for which longer and equal length contigs cover at least 50 % of the assembly (Alhakami *et al.*, 2017). Tertiary analysis involves the annotation and interpretation of assembled genomes by determining their biological and pathological functions (Ekblom & Wolf, 2014; Hu *et al.*, 2021). These types of analyses consist of two steps, namely (i) structural annotation, where functional elements such protein-coding sequences (CDS) are identified either by *ab initio* algorithms that are trained on gene predicting models, or by curated non-redundant protein and transcript

databases (NCBI, RefSeq, UniProt); and (ii) functional annotation, where biological relevant information (biological function, biochemical function, gene ontology terms, etc.) is assigned to the predicted CDS (Dominguez Del Angel *et al.*, 2018; Ekblom & Wolf, 2014). Although there are several different tools available for structural and functional annotation, most of them run individually and do not combine results. However, there are automatic pipelines, such as NCBI Prokaryotic Genome Annotation Pipeline and Rapid Annotation using Subsystem Technology (RAST) that perform both structural and functional annotation (Aziz *et al.*, 2008; Tatusova *et al.*, 2016).

Similar to the DNA sequencing analysis, RNA sequencing data analysis starts with base calling (primary analysis), reads mapping and transcriptome reconstruction (secondary analysis) and quantification and differential expression analyses (tertiary analysis). However, analysing RNA sequencing data poses greater challenges due to the intricacies of alternative splicing and the dynamic range of gene expression. Read mapping and alignment are crucial for transcriptome profiling and use tools such Bowtie2 or STAR (Bankevich *et al.*, 2012; Dobin & Gingeras, 2015). Transcripts are then generated from mapped reads either through a reference-guided approach, using tools such as Cufflinks, or a reference-independent approach (*de novo* reconstruction) using Trinity (Goff *et al.*, 2012; Grabherr *et al.*, 2011). After a dataset has been mapped or assembled, quality-checking tools such as FASTQC can be utilised to assess the quality of the reads and the analysis. Additionally, normalisation and counting methods can be applied to characterise coverage and gene numbers (Conesa *et al.*, 2016). For the functional annotation of assembled transcripts, which include transcript quantification and differential expression analysis, tools such as edgeR, and DESeq2 are commonly used (Liu *et al.*, 2021). This data is usually visually presented through heatmaps and clustering. However, for a more detailed understanding of differentially expressed genes (DEGs), advanced analyses such as gene ontology (GO), pathway, and network enrichment analysis are necessary (Rosati *et al.*, 2024).

As previously stated in section 2.2 (Taxonomy of *Clostridium* genus), there have been considerable changes in the taxonomy of anaerobic bacteria such as *Clostridium*. The use of NGS has provided new opportunities to revisit the taxonomy of clostridia beyond the standard of 16S rRNA gene sequences. These approaches can enable comprehensive taxonomic and evolutionary analysis and provide much-needed insight into whether the *Clostridium* genus is truly a monophyletic group or needs to be redefined taxonomically (Cruz-Morales *et al.*, 2019). Although NGS approaches have become more affordable over the years, the associated cost

may still hinder such large-scale studies. Another drawback is the requirement for additional bioinformatics skills to analyse the sequences (Baker, 2002).

2.7 Chapter summary

The chapter thoroughly explores the *Clostridium* genus, a diverse and significant group of anaerobic bacteria. It begins by outlining the genus's historical discovery, unique characteristics, and wide distribution across environments, including soil, water, and the gastrointestinal tracts of animals. Key traits like endospore formation, motility mechanisms, and metabolic adaptability enable *Clostridium* species to thrive in diverse and often extreme conditions. Despite their ubiquity, the genus's taxonomy has undergone extensive revisions due to challenges in phenotypic classification, with molecular techniques like 16S rRNA sequencing now playing a pivotal role in defining and reclassifying its phylogeny.

This review delves into the pathogenicity of *Clostridium*, emphasising its production of potent toxins that cause life-threatening diseases such as tetanus, botulism, and gas gangrene. Pathogenic species are categorised into neurotoxic, enterotoxic, and histotoxic groups based on their toxin activity. Beyond its clinical significance, the genus's impact on agroecosystems is examined, highlighting its beneficial roles in nutrient cycling and plant growth promotion and its detrimental effects as a plant pathogen. The document also highlights the widespread prevalence of *Clostridium* in soil, water systems, and fresh produce, illustrating its importance in public health and agriculture.

Antibiotic resistance in *Clostridium* is a central concern, driven by the overuse of antibiotics in agriculture and healthcare. Resistant strains, including multidrug-resistant *C. difficile*, are increasingly challenging to treat, emphasising the urgent need for new antibiotics and resistance monitoring. The chapter also evaluates traditional and advanced methods for cultivating and identifying *Clostridium* species, from culture-based techniques to molecular approaches like PCR and next-generation sequencing. These tools enhance understanding of *Clostridium*'s genomic and ecological diversity, contributing to better management of its risks and benefits across various domains.

CHAPTER 3: INSIDE ENVIRONMENTAL *CLOSTRIDIUM PERFRINGENS* GENOMES: ANTIBIOTIC RESISTANCE GENES, VIRULENCE FACTORS AND GENOMIC FEATURES

3.1 Abstract

Numerous research studies exist on *Clostridium perfringens* in clinical settings; however, its pathogenicity has been greatly neglected in environmental research. Environmental genomes were used to investigate possible antibiotic resistance, and the presence of virulence traits in *C. perfringens* strains from raw surface water. *In-silico* assembly was conducted on the DNA of three *C. perfringens* strains and generated almost complete genomes, setting their length ranging from 3.4 - 3.6 Mbp with GC content of 28.18%. An average of 3,175 open reading frames were identified, with the majority associated with carbohydrate and protein metabolisms. The genomes harboured several antibiotic-resistance genes for glycopeptides, macrolide-lincosamide-streptogramin B, β-lactam, trimethoprim, tetracycline and aminoglycosides. Also, the presence of several genes encoding for polypeptides and multidrug resistance efflux pumps and 35 virulence genes. Some of these genes encode for hemolysins, sialidase, hyaluronidase, collagenase, perfringolysin O and phospholipase C. All three genomes contained sequences indicating phage, antibiotic resistance and pathogenic islands integration sites. Genomic comparison of these three strains confirmed high similarity and shared core genes with clinical *C. perfringens* strains, highlighting their health security risks. This study provides the much-needed genomic insight into the potential pathogenicity of *C. perfringens* present in the environment and emphasises the importance of monitoring this niche in the future.

Keywords: Antibiotic resistance genes, *Clostridium perfringens*, Genetic features, Virulence genes, Water environment, Whole-genome sequencing

3.2 Introduction

Clostridium perfringens (previously known as *Bacillus welchii*) is a strictly anaerobic, Gram-positive, rod-shaped bacterial pathogen (Hassan *et al.*, 2015). This rapid-growing bacterium has the ability to form endospores that are extremely resilient to toxic chemical and environmental stressors, such as heat or radiation (Paredes-Sabja *et al.*, 2008). As a result, *C. perfringens* inhabits diverse environments including soil, water, sewage, food and even the gastrointestinal (GI) tract of mammals (Sathish and Swaminathan, 2009). In the GI tract, *Clostridium perfringens* does not infect healthy cells directly but rather acts by producing toxins and enzymes that cause a number of enteric and systemic diseases in the infected host (Kiu & Hall, 2018; Gross *et al.*, 1989).

Pathogenic *C. perfringens* strains have been found to produce and secrete more than 20 different extracellular toxins and/or hydrolytic enzymes (Revitt-Mills *et al.*, 2015). They are classified into seven groups (toxinotypes A-G) according to the combination toxin types they produce (α-toxin, β-toxin, ε-toxin and ι-toxin, enterotoxin (CPE) and NetB) (Kiu & Hall, 2018). Clinically, the toxinotype A strain is the most common disease-causing agent responsible for gas gangrene (clostridial myonecrosis), necrotic enteritis, as well as mild diarrhoea (Brynestad & Granum, 2002). Although these toxins and enzymes each play a specific role in the disease process, it is the synergistic actions of both on the host that could potentially be the key virulence factors involved in its pathophysiology (Revitt-Mills *et al.*, 2015; Rood, 1998).

Besides virulence factors in *C. perfringens*, another trait, such as antibiotic resistance, poses a danger to clinically treated patients, mainly due to their ability to render antibiotic treatment of severe and life-threatening infections ineffective (Kiu & Hall, 2018). Therefore, extensive research has been conducted on antibiotic resistance profiles in *C. perfringens* using phenotypic methods (e.g. testing of minimal inhibitory concentration) (Akhi *et al.*, 2015; Kock *et al.*, 1998). However, there are only a few studies that have used genomic methods (i.e. WGS- whole-genome sequencing) to investigate antibiotic resistance genes (ARGs) (Kim *et al.*, 2017; Li *et al.*, 2017).

Considering the clinical relevance *C. perfringens* holds, and its wide distribution in nature, the current state of knowledge on the pathogenicity of *C. perfringens* or its non-clinical environment is still poor when compared to other more well-known pathogens (Shimizu *et al.*, 2002). New technologies, such as next-generation sequencing (NGS), have become the new golden standard in *in-silico* analysis. This is because it (NGS) helps to understand bacterial pathogenesis, identify and characterise genes coding for virulence factors, toxins or antibiotic resistance in pathogens, especially *C. perfringens* strains, which are hard to grow under

laboratory conditions (Bakour *et al.*, 2016). Thus, the aim of this study was to expand on existing knowledge of *C. perfringens* through the analysis of assembled genomes from surface water and identifying the presence of virulence and antibiotic resistance genes.

3.3 Material and methods

3.3.1 Bacterial isolation, antibiotic susceptibility testing and genomic DNA extraction

Clostridium perfringens was isolated from water of the Schoonspruit River (26° 40 '46.0"S, 26° 34' 58.7"E) in the North West Province (South Africa) in March 2016. A modified Fung's double tube method, along with tryptose-sulphite-cycloserine agar (Oxoid, UK) was used for the bacterial isolation from collected samples (Fourie *et al.*, 2019). The isolates were purified through streaking and grown under anaerobic conditions at 44 ºC and subjected to antibiotic susceptibility testing as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016). The minimal inhibitory concentrations (MICs) of five clinically relevant antibiotics (ampicillin, tetracycline, clindamycin, chloramphenicol and metronidazole) were determined by the agar dilution method (Table S3.2). Thereafter, multidrug-resistant (MDR) isolates were selected for genomic DNA extraction. gDNA extraction from overnight cultures was done using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany) according to the instructions provided by the manufacturer. Species confirmation was done by amplifying and sequencing the 16S rRNA gene. The 27F (5'- AGA GTT TGA TCM TGG CTC AG- 3') and 1492R (5'- GG TTA CCT TGT TAC GAC TT- 3') (Inqaba Biotec; SA) primers were used (Jiang *et al*., 2006). The reaction started with a denaturing step of 94°C for 2 minutes, where after 35 cycles commenced. These cycles consisted of denaturing at 94°C for 30 seconds, annealing at 54°C for 60 seconds and extension at 72°C for 1 minute. The reaction was concluded by an additional extension at 72°C for 5 minutes. Subsequently, three MDR *C. perfringens* isolates with high DNA purity were selected for whole-genome sequencing.

3.3.2 Genome sequencing, assembly and annotation

DNA libraries were performed using the Nextera XT kit (Illumina Inc., San Diego, Ca) according to the instructions provided by the manufacturer and sequenced using an Illumina's MiSeq300 in paired-end reads. All short (< 50 bp) or low-quality value (QV < 15) fragments were removed. The quality assessment and trimming were done in Trimmomatic (v.0.36) (Bolger *et al.*, 2014), followed by *de novo* assembly in SPAdes (v.3.9.0) to generate scaffolds and contigs (Bankevich *et al.*, 2012). Gene prediction and annotation was conducted using

the NCBI Prokaryotic Genome Annotation Pipeline (v.4.3) along with Rapid Annotation using Subsystem Technology (RAST) server (Tatusova *et al.*, 2016; Overbeek *et al.*, 2014). Subsequently, the obtained draft genome sequences used in this study were then deposited into the NCBI database (Fourie *et al.*, 2019).

3.3.3 In-silico analysis of antibiotic resistance, virulence factors, genomic islands and prophages

Antibiotic resistance genes and virulence factors were identified within the genomes of all three *C. perfringens* strains by using BLASTx comparison to the virulence factors database (VFDB) and ARGs database in deepARG (v.2.0) (Chen *et al.*, 2016; Arango-Argoty *et al.*, 2018). The presence of genomic islands (GIs) within the assembled genomes was predicted using the VRprofile (v.2.0) (Li *et al.*, 2018). Prophage regions were identified, characterised and located using PHASTER (PHAge Search Tool – Enhanced Release) (Arndt *et al.*, 2016). Default parameters were used for all software, unless otherwise specified.

3.3.4 Genomic comparison

Average nucleotide identity (ANI) was determined by using the OrthoANI algorithm (v.1.4) to analyse genomic relatedness between the three analysed and reference *C. perfringens* strains. OrthoANI percentages were calculated and a UPGMA dendrogram was constructed (Lee *et al.*, 2015). Annotated protein sequences were submitted to OrthoVenn2 to identify unique and/or shared orthologous clusters among three *C. perfringens* genomes, as well as three reference genomes (Xu *et al.*, 2019). Reference strains used for comparison were *C. perfringens*: ATCC 13124 (Genbank: NC_008261), Str 13 (Genbank: BA000016), and FORC_025 (Genbank: NZ_CP013101).

3.4 Results and discussion

3.4.1 Genomic annotations

The three assembled *C. perfringens* strains (SC4-C13, SC4-C17 and SC4-C24) that were originally obtained from surface water produced genome sizes of 3.6, 3.5 and 3.4 Mbp, respectively (Table S3.1) and were organised in 205 (both SC4-C13 and SC4-C17) and 110 (SC4-C24) contigs (Fourie *et al.*, 2019). The GC content between the three strains averaged at around 28.1%. Annotation of the genomes indicated the presence of open reading frames

(ORFs) ranging between 3,079 and 3,245 for all three strains (Table S3.1), consistent with published data on this bacterium. The average genome size known for *C. perfringens* varies from 3.0 – 4.1 Mbp, with a GC content ranging between 27 – 28% (Kui & Hall, 2018; Kui *et al.*, 2017). The number of coding sequences present in their genome averaged between 2,500 and 3,600 (Kui & Hall, 2018).

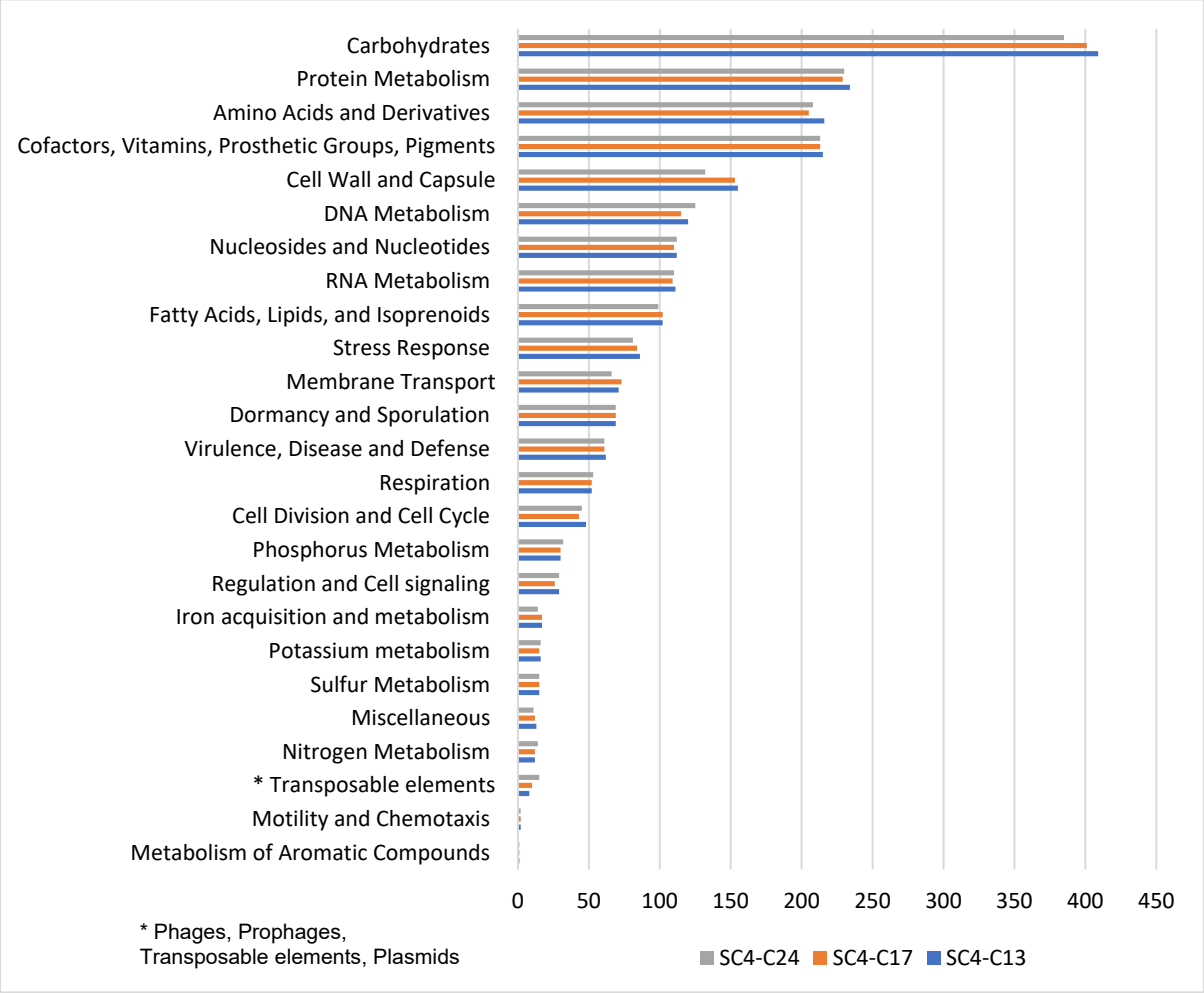


Figure 3.1: ORFs from three analysed *C. perfringens* genomes (blue SC4-C13; orange SC4-C17 and grey SC4-C24) annotated and sorted into subsystems by rapid annotation system technology (RAST) together with counts per each category for each genome.

RAST was used to functionally annotate the open reading frames of each genome into 25 subsystem categories (Figure 3.1). Based on the categories generated, all three stains showed similar amounts of assigned ORFs, for each category. The majority was allocated to "carbohydrates", thus showing the importance of carbohydrates for these pathogens, since they play a vital role in their growth and sporulation (Sacks, 1983). This was then followed by

“protein metabolism”, “amino acids and derivatives” and “cofactors, vitamins, prosthetic groups and pigments”. The less abundant ORFs belonged to the “metabolism of aromatic compounds” category, along with “motility and chemotaxis”, “transposable elements” and “nitrogen metabolism”.

3.4.2 Antibiotic resistance

The deepARG tool applies a deep-learning approach to identify and annotate antibiotic resistance genes from genomic data. It does this by creating a dissimilarity matrix based on ARGs categories and comparing it against the three major datasets currently available, namely Comprehensive Antibiotic Resistance Database (CARD), Antibiotic Resistance Genes Database (ARDB) and Universal Protein Resource (UNIPROT) (Arango-Argoty *et al.*, 2018). This approach identified a total of 11 ARGs in the genomes of the three *Clostridium perfringens* strains (Table 3.1). Strain SC4-C13 and SC4-C17 both possess genes that encode for resistance against seven different classes of antibiotics (glycopeptide, β-lactamase, macrolide-lincosamide-streptogramin B (MLSB), tetracycline, trimethoprim, kasugamycin and bacitracin), whereas strain SC4-C24 only harboured resistance genes against 6 classes, with the absence of the β-lactam resistance gene (*bla*2). Furthermore, all three strains hold two or more genes that encode for multidrug resistance.

Glycopeptides encompass a group of antibiotics that are vital in combating infections caused by antibiotic-resistant bacteria, with vancomycin still the first choice of treatment against Gram-positive bacteria since its introduction 50 years ago (Blaskovich *et al.*, 2018). Vancomycin works by binding to the D-Ala-D-Ala C-terminal of the growing pentapeptide chain during cell wall synthesis and inhibits further elongation and cross-linking of the peptidoglycan chain blocking through transglycosylation and transpeptidation (Sanakal & Kaliwal, 2011). However, resistance to this antibiotic has become a common occurrence, especially in vancomycin-resistant enterococci (VRE) and even in methicillin-resistant *Staphylococcus aureus* (MRSA) (Willems *et al.*, 2005). The resistance in these bacteria has been confirmed by the presence of several vancomycin resistance genes (*van*). Our strains possessed many of these genes (*van*HD, *van*TrL, *van*RB, *van*RG and/or *van*RI), which encode for enzymes that produce synthetic precursors that replace the D-Ala-D-Ala C-terminal, altering the vancomycin binding site (Sanakal & Kaliwal, 2011). Quite a few studies have reported vancomycin resistance genes in *Clostridium* species (Chia *et al.*, 2017; Peltier *et al.*, 2013). However, *Clostridium perfringens* has not been one of the few to show such resistance, since they are normally susceptible to vancomycin (Chia *et al.*, 2017; Camacho *et al.*, 2008; Citron *et al.*, 2005). The

presence of these genes could suggest a new resistance trait in these species if they are expressed.

Table 3.1: Antibiotic resistance genes predicted in three *C. perfringens* strains (SC4-C13; SC4-C17 and SC4-C23) using DeepARG. '+' marks if the gene was present in the genome while '–' marks the absence of the gene in the genome.

Antibiotic			**SC4-C13**	**SC4-C17**	**SC4-C24**
class	*name*	*gene*			
glycopeptide	Vancomycin	*van*HD	–	–	+
		*van*TrL	–	–	+
		*van*RB	–	+	–
		*van*RG	+	+	+
		*van*RI	+	+	+
beta-lactam	Penicillin	*bla2*	+	+	–
MLSB*	Erythromycin	*erm*Q	+	+	+
tetracyclines	Tetracycline	*tet*B(P)	+	+	+
trimethoprim	Trimethoprim	*dfr*K	+	+	+
aminoglycosides	Kasugamycin	*ksg*A	+	+	+
polypeptides	Bacitracin	*bac*A	+	+	+
multidrug	System regulators	*arl*R	+	+	+
	ATP-binding cassette	*vga*B	+	+	–
	transporters efflux pump	*mep*A	+	+	+

* Macrolide-Lincosamide-Streptogramin B

Further study of the results presented in Table 3.1 shows that all three *C. perfringens* genomes each harboured the same gene that encodes resistance for MLSB (*erm*Q), tetracycline (*tet*B(P)), trimethoprim (*dfr*K), kasugamycin (*Ksg*A) and bacitracin (*bac*A). MLSB antibiotics work by inhibiting protein synthesis in both Gram-positive and Gram-negative bacteria. Resistance to this antibiotic due to 23S rRNA methylases (encoded by *erm* genes) that prevent MLSB antibiotics from binding to the ribosome. Several *erm* genes have been detected in

Clostridium species, such as *C. perfringens* and *C. difficile* (Spigaglia & Mastrantonio, 2002). However, the *erm*Q gene was first identified in *C. perfringens* and found to be a distinct class of MLSB resistance determinant (Shoemaker *et al.*, 2001; Berryman *et al.*, 1994).

Tetracyclines are broad-spectrum antibiotics and the second most used antibiotics group after β-lactams. Bacterial resistance to tetracycline is achieved by harbouring one or more of the 36 known *tet* genes (Sheykhsaran *et al.*, 2019). These *tet* genes follow one of three resistance mechanisms: energy-dependent efflux pumps, ribosomal protection proteins (RPPs), or drug target alteration and enzymatic inactivation (van Hoek *et al.*, 2011). The *tet*B(P) gene found in our strains along with *tet*A(P) (not present), are originally two overlapping genes within the Tet P determinant of *C. perfringens* (Sloan *et al.*, 1994). However, because the Tet P determinant has only been found in *Clostridium* species and is often associated with conjugative and non-conjugative plasmids, it has shown the capacity to spread rapidly across the whole genus (Vidor *et al.*, 2019; Roberts, 2011).

Clostridium species are known to show endogenous resistance to trimethoprim (Huovinen *et al.*, 1995). However, the presence of the *drf*K gene in the three *C. perfringens* genomes suggests it could have been acquired from an external source since it has mainly been associated with enterococci and staphylococci species (López *et al.*, 2012). While the *drf*K gene may not be vital for trimethoprim resistance in *Clostridium*, its presence may have implications for further dissemination in the environment, or to other bacteria that are primarily susceptible to trimethoprim.

Antibiotics such as kasugamycin and bacitracin are widely used in agriculture and animal husbandry as growth promoters, with bacitracin also being used as a topical ointment for skin infections (Charlebois *et al.*, 2012; Duffin & Seifert, 2009). Resistance to the aminoglycoside antibiotic kasugamycin, is due to the presence of *Ksg*A gene, which produces a predicted dimethyltransferase. Whereas an ATP-dependent ABC type efflux system consisting of three BcrA, -B and -C proteins is responsible for bacitracin resistance (Duffin & Seifert, 2009; El Ghachi *et al.*, 2004). Studies have shown that the use of antibiotics in agriculture and growth promoters in animal breeding have a direct correlation to the development and dissemination of antibiotic-resistant bacteria and genes into the surrounding water sources. This happens by means of surface runoff, where antibiotic resistance can be further intensified via horizontal gene transfer (HGT) (Kümmerer, 2004).

Genes for efflux pumps (*mep*A), ATP-binding cassette transporters (*vga*B) and system regulators (*arl*R), which are associated with multidrug-resistance, were found in our genomes as well. The *arl*R is part of a two-component regulatory system, a transmembrane sensor and

its associated response regulator (*arl*S-*arl*R). This regulator was found to increase expression of efflux pumps associated with *Staphylococcus aureus* (Fournier *et al.*, 2000). The *vga*B gene is one of the ATP transporter genes and can increase the level of resistance to pristinamycin, a mixture of streptogramin A and streptogramin B compounds via efflux pumps (Chesneau *et al.*, 2005; Roberts, 2002). The *mep*A encodes for a multidrug resistance efflux pump in *S. aureus,* which has reported low-level resistance to quaternary ammonium compounds, chlorhexidine, pentamidine and tigecycline (Costa *et al.*, 2013).

When comparing the genotypic characteristics with the phenotypic profiles (Table S3.2) of these three strains, some conclusions can be drawn. All the strains showed to be susceptible to chloramphenicol, along with the absence of any associated resistance genes to support this. Two of the strains, SC4-C13 and SC4-C17, showed phenotypical resistance to ampicillin (β-lactam) and possessed the *bla*2 gene, whereas SC4-C24 showed intermediate resistance with no genes to confirm resistance in its genome. Mobile genetic elements such as plasmids have been shown to carry β-lactam resistance genes (Partridge *et al.*, 2018). If such plasmid-mediated resistance genes were to be present in *C. perfringens* and expressed, it could translate to phenotypical resistance to β-lactam antibiotics. Alternatively, this low level of resistance might also suggest an increase in the CLSI-defined susceptibility breakpoint for ampicillin in *C. perfringens*. Noticeably, all three strains also showed phenotypic resistance to tetracycline, clindamycin and metronidazole. The presence of genes such as *erm*Q and *tet*B(P) could explain the phenotypical resistance to clindamycin and tetracycline, respectively. However, no genes associated with metronidazole were identified in any of the assembled genomes. Previous studies have reported phenotypic resistance to metronidazole in *C. perfringens* (Akhi *et al.*, 2015; Tansuphasiri *et al.*, 2005). However, the understanding of its resistance mechanism in *Clostridium* species is still unclear (Lynch *et al.*, 2013). It is also important to add that although the assembly delivered almost near genomes, the lack of genetic confirmation for phenotypic resistance may be due to incomplete gene reconstruction or of the region where the gene occurs.

3.4.3 Virulence factors

The genes that are associated with virulence in *C. perfringens* strains were analysed using the VFDB as reference. A total of 35 different virulence genes were successfully annotated with this database, of which 79% are typically associated with *C. perfringens* strains. These include genes that are responsible for adherence, such as fibronectin-binding protein, chaperonin GroEL and type IV pili, as well as the two members of the double component VirR/VirS regulon (sensor histidine kinase and DNA-binding response regulator). There are

also 19 genes present in all three strains that are capable of producing 8 different toxins, namely alpha-clostripain, phospholipase C, collagenase, hyaluronidase, perfringolysin O, sialidase and hemolysin (Table 3.2). Interestingly, the remaining 21% of virulence genes are usually associated with other Gram-positive bacteria, namely *Listeria ivanovii, Streptococcus pyogenes, Mycoplasma penetran*s and *Enterococcus faecium.* This might imply their potential influence or contribution to the expanding pathogenicity in these three strains as bile-resistance and adherence factors (Table 3.2). Among the three *C. perfringens* strains described in this study, SC4-C13 and SC4-C17 strains only possess 94.7% of the above-mentioned virulence factors, while for SC4-C24, it is 100%.

Table 3.2: Virulence factors identified in *C. perfringens* strains based on the virulence factor database (VFDB).

Virulence factor			Reference species	Genbank
Class	**Name**	**Gene**		
Adherence	fibronectin-binding protein	*fbp*	*	WP_011590967
		*fbp*A	*	WP_011010006
	chaperonin GroEL	*groEL*	*	WP_003462314
	elongation Factor Tu	*tuf*	**	WP_011076858
	enterococcal surface protein	*esp*	***	WP_014387145
	type IV pili	*pil*A1	*	WP_011010863
		*pil*B	*	WP_011010636
		*pil*B2	*	WP_011010862
		*pil*C	*	WP_011010635
		*pil*C2	*	WP_01101086
		*pil*D	*	WP_003462279
		*pil*M	*	WP_011010859
		*pil*N	*	WP_011010858
		*pil*T	*	WP_003451114
	streptococcal plasmin receptor/GAPDH	*plr*/*gap*A	+	WP_002986042
Regulation	sensor histidine kinase	*vir*S	*	WP_011590863
	DNA-binding response regulator	*vir*R	*	WP_003449818

Table 3.2: Virulence factors identified in *C. perfringens* strains based on the virulence factor database (VFDB) (*continue*).

Virulence factor			Reference species	Genbank
Class	**Name**	**Gene**		
Toxin	alpha-clostripain	*clo*SI	*	WP_011590397
	phospholipase C (Alpha-toxin)	*plc*	*	WP_011590041
	Kappa-toxin (collagenase)	*col*A	*	YP_697499
	Mu-toxin (hyaluronidase)	*nag*H	*	NP_561107
		*nag*J	*	NP_562150
		*nag*K	*	NP_562195
		*nag*L	*	NP562439
	perfringolysin O (theta-toxin/PFO)	*pfo*A	*	NP_561079
	sialidase	*nan*H	*	YP_695432
		*nan*I	*	WP_011590331
		*nan*J	*	NP_561469
	hemolysin	*hly*B	*	NP_561353
		*hly*C	*	WP_003454634
		*hly*D	*	NP_562734
		*hly*E	*	WP_011010677
Bile resistance	bile-salt hydrolase	*bsh*	++	YP_004855791

* *Clostridium perfringens;* ** *Mycoplasma penetrans*; *** *Enterococcus faecium*; **+** *Streptococcus pyogenes*; **++** *Listeria ivanovii* subsp. *Ivanovii;*

Clostridium perfringens can produce a variety of more than 20 different extracellular toxins and hydrolytic enzymes, giving it the ability to cause various histotoxic infections in humans and animals (Kiu & Hall, 2018). However, not all strains are able to produce all these toxins.

This is mainly due to some toxins being strain-specific (Kiu *et al.*, 2017). The *C. perfringens* strains in this study possess genes able to code a vast array of toxins and enzymes. However, only a combination of several typing toxins is traditionally used to determine the toxinotype of a species. All three strains were identified as toxinotype A, due to the presence of the *plc* gene which encodes for α-toxin and the absence of the other typing toxin genes. Furthermore, *C. perfringens* toxinotype A strains are known to be human pathogens, causing diseases such as gas gangrene (clostridial myonecrosis), necrotic enteritis, as well as mild diarrhoea (Brynestad and Granum, 2002). The presence of perfringolysin O (theta-toxin) has also been shown to have a synergistic effect with α-toxin in the pathology of gas gangrene. Even on its own, this cholesterol-dependent cytolysin can form pores on cell membranes and lyse red blood cells, further highlighting the significant role perfringolysin O has in disease development (Kiu & Hall, 2018; Awad *et al.*, 2001). However, these two toxins, along with the or κ-toxin gene (*colA)*, are tightly regulated by specific regulatory systems in *C. perfringens* (Ohtani & Shimizu, 2016). The VirS/VirR two-component system is one of the most important systems and consists out of two genes: the response regulator (*vir*R*)* and the sensor histidine kinase (*vir*S*)* (Ma *et al.*, 2011). Still, this system assumes responsibility for coordinating the pathogenicity of *C. perfringens* type A strains.

The hydrolytic enzymes produced by *C. perfringens* also play an important role during *C. perfringens*-mediated tissue infection. Sialidase is encoded by several genes that contribute to internal virulence by improving the adherence of *C. perfringens* to host cells, enhancing the production, binding, and activity of certain toxins that are responsible for intestinal infections and contribute to intestinal colonisation by *C. perfringens* (Li *et al.*, 2016). Mu-toxin (hyaluronidase) on the other hand, are enzymes that facilitate the breakdown of hyaluronate substrates, improving contact between *C. perfringens* and the specific cell wall receptors. It has also been found to promote the spread of α-toxin and in doing so, potentiate its cytolytic activity (Hynes & Walton, 2000).

As in most pathogens, adherence plays an essential role in the pathogenesis of *C. perfringens*. Several adherence genes were identified in the genomes of *C. perfringens*, each of which encodes for a different approach to adherence and promoting colonisation. Fibronectin-binding protein (*fbp*A) for instance, makes it possible for *C. perfringens* to bind to fibronectin found in all human tissues and organs, including intestinal epithelial cells and in doing so, invade the host cells (Katayama *et al.*, 2009). Type IV pili are elongated, flexible filaments which extend from the bacterial cell surface and implicated in attaching to and invading of host cells, attachment to abiotic surfaces, biofilm formation, and bacteriophage susceptibility (Rodgers *et al.*, 2011).

3.4.4. Genomic islands

Genomic islands (GIs) are regions of the bacterial genome that are acquired through horizontal gene transfer (HGT) (Klein *et al.*, 2018; Dobrindt *et al.*, 2004). Antibiotic resistance islands (ARIs) and pathogenicity islands (PAIs) are two subgroups of GI that aid and contribute to the pathogenesis of an organism (Yoon *et al.*, 2015). Figure 3.2A shows the distribution of ARIs and PAIs present in the three *C. perfringens* strains. All strains analysed possessed PAIs and ARIs homologous to those found in most enteropathogens. A total of 65 ARIs were identified in all three strains that were related to genes from *Staphylococcus aureus* (24/65), *Acinetobacter baumannii* (19/65), *Pseudomonas aeruginosa* (10/65), *Campylobacter coli* (3/65), *Shigella flexneri* (3/65), *Klebsiella pneumoniae* (2/65), *Proteus mirabilis* (2/65), *Escherichia coli O157H7* (1/65) and *Staphylococcus hominis* (1/65) (Table S3.3). This included genes that encode for putative enzymes, such as acyl-CoA dehydrogenase, DNA topoisomerase III and enoyl-CoA hydratase/isomerase, as well as regulatory proteins such as KDP operon transcriptional regulatory protein (*Kdp*E), propionate catabolism operon regulator (*prp*R), putative transcriptional and response (*Arm*R) regulators, to name a few. Genes encoding for proteins and enzymes contributing to antibiotic resistance (*Tet*A class A, truncated tetracycline resistant protein and streptothricin acetyltransferase) and tolerance to heavy metals (mercuric reductase and cadmium efflux system protein B and C) were also identified. Heavy metals such as mercury and cadmium are commonly used as antimicrobial metal compounds in healthcare (Pal *et al.*, 2017). However, the presence of genes encoding for resistance to antibiotics and heavy metals might suggest possible adverse implications for infectious cases of *C. perfringens*. Iron plays a key role in the growth and survival of many pathogens in any niche. It is utilised for important biological processes such as DNA synthesis, generating energy and protection against reactive oxygen species (Choo *et al.*, 2016). All the *C. perfringens* strains possess ARIs related to *Shigella flexneri* that encode for proteins responsible for the binding and transport of ferric iron (III) (*Fec*C, *Fec*D, and *Fec*E) (Table S3.3). Genes associated with iron acquisition have previously been linked to GIs in *Clostridium perfringens* (Myers *et al.*, 2006)

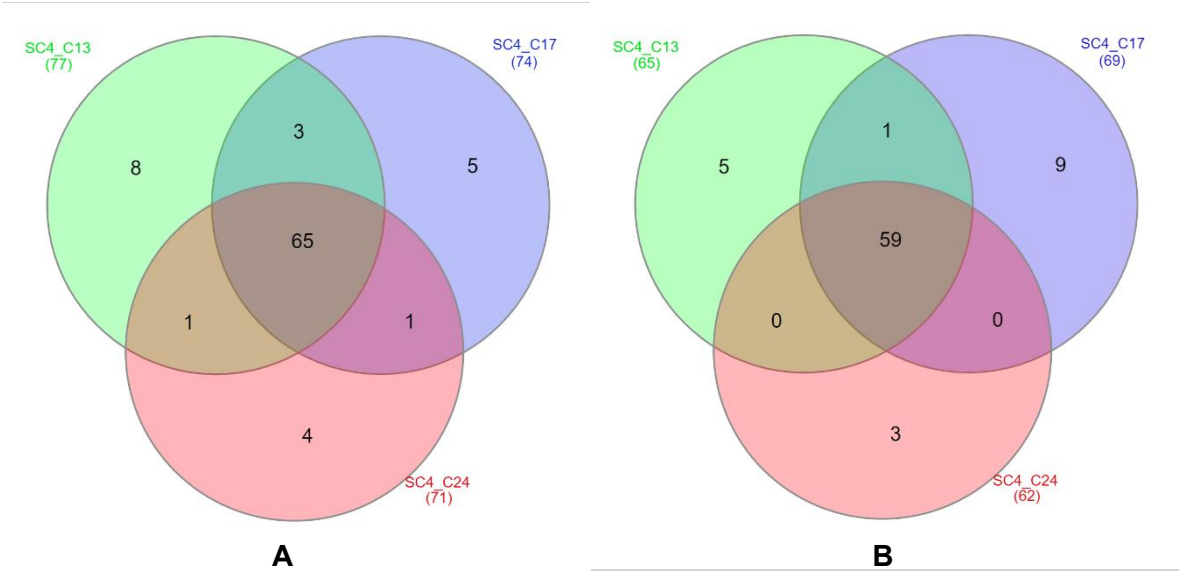


Figure 3.2: Venn diagrams showing the ARIs (A) and PAIs (B) identified in the genomes of the three *C. perfringens* strains.

The distribution of all PAIs identified among the *C. perfringens* strains is visualised in Figure 3.2B. The various PAIs shared among the three strains ranged from hypothetical or unnamed proteins related to *Yersinia pestis*, *Escherichia coli*, *Edwardsiella tarda* and *Streptomyces lividans* (Table S3.4). Other examples include PAIs encoding for *Epd*, integrase, Orf17, Orf29, Orf34, Orf41, Orf59, *Pgk* and *Tkt*A from *Photorhabdus luminescens*; dihydrofolate reductase type Ib (plasmid-encoded), dihydropteroate synthase, *Glc*D protein, *Gsp*E type II secretion protein, hemolysin B, phosphoglycerate transport protein, putative ABC transporter ATP-binding and membrane proteins, putative lysil-tRNA synthetase (*Lys*U) and short-chain dehydrogenase from *Escherichia coli*; ATPases with chaperone activity ATP-binding subunit, serine/threonine protein phosphatase and transcriptional regulator from *Vibrio alginolyticus* and glutamate racemase from *Helicobacter pylori*. Additionally, sequences similar to ones encoding for *Hrc*N and *Hrp*B6 in *Xanthomonas* species were also present. These two genes encode for the type III secretion system (TTSS) proteins, a requirement for the pathogenicity of several plant pathogens (Villela *et al.*, 2019).

Among the three *C. perfringens* strains, SC4-C13 and SC4-17 had the most unique PAIs and ARIs (Table S3.3 and S3.4). Noticeably, both these strains harboured genes encoding for putative transposase. Strain SC4-C13 possessed multiple transposases for IS285 (*Y. pseudotuberculosis*), ISSau4-like, and IS256/Tn4001 (*Staphylococcus aureus*), whereas SC4-C17 harboured a transposase protein for IS30 (*Staphylococcus aureus*). Additionally, all strains possessed two or more GIs containing integrase genes from either *Photorhabdus*

luminescens, *Acinetobacter baumannii* or *Staphylococcus aureus*. The presence of genes associated with transposases and integrases has been shown to infer a high potential for genetic gain or loss in *C. perfringens* genomes (Kiu *et al.*, 2017).

A previous study identified over 300 GIs in the genomes of several clinical *C. perfringens* type A strains, of which almost all were chromosomally encoded (Myers *et al.*, 2006). Less is known about the occurrence of GIs in strains isolated from environmental sources (Klein *et al.*, 2018). Pathogens such as *C. perfringens* are found in various ecological niches, which also include diverse microbial communities that can contribute to HGT events (Kiu *et al.*, 2017).

3.4.5 Prophages

The Phage Search Tool Enhanced Release (PHASTER) program identifies and characterises prophage regions into 3 categories based on the completeness scores, namely intact (>90), questionable (70-90), or incomplete (<70). All three *C. perfringens* strains used in this study contained two or more prophage regions in their genome (Table 3.3). Strain SC4-C24 was shown to harbour three prophage regions, of which one was confirmed to be intact and the other two showed incomplete and questionable phage-related sequences. Another intact prophage was identified in strain SC4-C17, along with an additional incomplete region. Stain SC4-C13 showed putative fragments of two phages, one of which scored relatively high (90). Each prophage region varies in size, number of CDS, and GC content. The two intact prophages in SC4-C17 and SC4-C24 strains have sizes of 49.6 Kbp and 42.2 Kbp, respectively, with both containing the second-highest number of CDS (57). Functional annotation of the genes present in these prophage regions showed protein sequences encoding for phage-related elements such as integrase, terminase, portal, capsid, head, tail, and/or transposase (Table S3.5). Phages play a very important role in the evolution of bacterial genomes (Ramisetty and Sudhakari, 2019). Their interactions with each other have impacted both their survival and persistence. This is due to several advantages prophages have to offer in the way of inversion, deletion and insertion via horizontal gene transfer (HGT) of genetic material (Darmon & Leach, 2014). This is because prophages are known to be ‘hotspots’ for carrying significant genes such as those involved in virulence, antibiotic resistance and metabolic pathways (Ramisetty and Sudhakari, 2019). This led to further investigation into functional protein sequences in the identified prophage regions of the three *C. perfringens* genomes. However, only the presence of hypothetical proteins was discovered in these regions and could suggest that these genes encode for unknown functions from remote sources.

Table 3.3: Prophages regions identified in *C. perfringens* strains using PHASTER.

Strain	size (Kb)	CDS	GC content (%)	Complete-ness	score	Phage	
						name	Genbank
SC4-C13	54.4	53	27.75	**+/–**	90	Clostr_vB_CpeS_CP51	NC 021325
	6.1	14	33.54	**–**	30	Clostr_c_st	NC 007581
SC4-C17	43.6	40	28.10	**–**	50	Clostr_PhiS63	NC 017978
	49.6	57	28.10	+	100	Clostr_vB_CpeS_CP51	NC 021325
SC4-C24	40.6	59	28.41	+/–	90	Clostr_vB_CpeS_CP51	NC 021325
	42.2	57	28.26	+	104	Clostr_phiSM101	NC 008265
	7.6	9	30.11	**–**	40	Sphing_PAU	NC 019521

+ complete; **–** incomplete; **+/–** questionable

3.4.6 Genomic comparisons

The average nucleotide identity for all *C. perfringens* genomes was successfully calculated using OrthoANI (Figure 3.3). ANI analyses of the three strains revealed them to be almost identical to each other as indicated by the ANI of >99% between the strains. Although the differences between the three strains appear negligible, based on the dendrogram, strains SC4-13 and SC4-C17 share relatively more similarities between them, as opposed to strain SC4-24 (Figure 3). When comparing the three *C. perfringens* genomes to that of the reference genomes (ATCC 13124, Str 13, and FORC_025), another high similarity was observed amongst the genomes, with our genomes sharing ANI values of between 98.49% to 98.63% with the reference genomes. The dendrogram also showed the three strains were clustered more closely to FORC_025, followed by the ATCC 13124 strain, which weas derived from clinical environments (Kiu *et al.*, 2017; Myers *et al.*, 2006), whereas only strain 13 was originally isolated from a natural environment (soil) (Shimizu *et al.*, 2002).

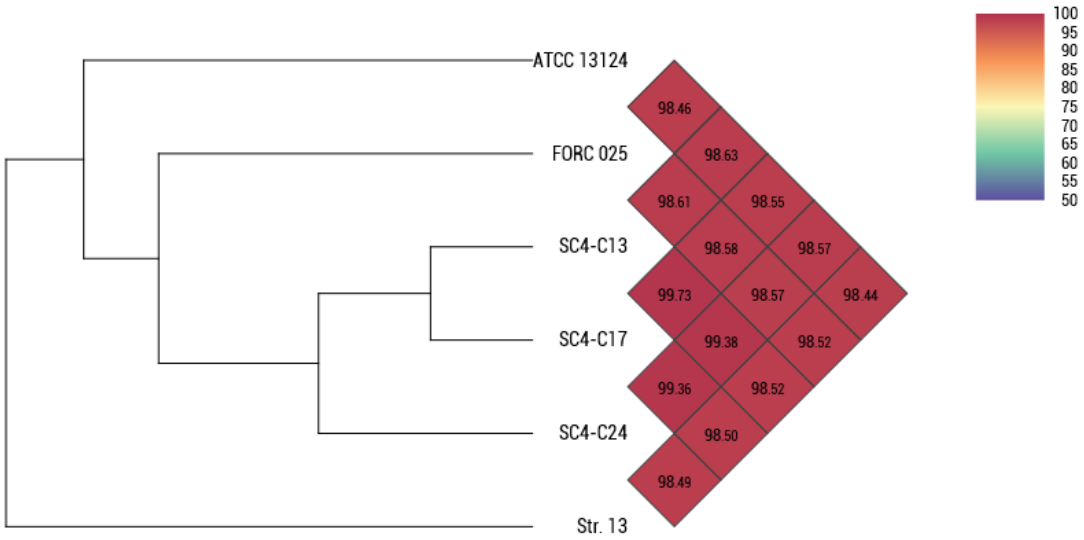


Figure 3.3: Dendrogram of *C. perfringens* strains relatedness calculated with the OrthoANI algorithm, clustered using UPGMA, and shown with the corresponding pairwise identity heat map.

Orthologous genes originate from a single gene present in the last common ancestor through a series of speciation events and usually still have the same biological function in the present-day organism (Xu *et al.*, 2019). Therefore, for further analysis of the three *C. perfringens* strains, a multi-species comparison of the shared core orthologous gene clusters was performed without (Figure 3.4A) and with reference genomes (Figure 3.4B). Results show a large number of (2,779) core orthologous genes shared between the three *C. perfringens* strains (Figure 3.4A). These clusters were all revealed to be associated with biological processes, molecular functions and cellular components within their core genomes (Table S3.6). The orthologous analysis also supported the higher similarity between SC4-C13 and SC4-C17 based on them sharing over 130 orthologous clusters, while SC4-C24 has the highest number of unique orthologous clusters of the three strains. When comparing the core orthologous clusters of the three strains with those of the reference strains, they showed to share 2,300 clusters (Figure 3.4B). Altogether, only 8 orthologous clusters were identified to be unique amongst all the genomes. Interestingly, these clusters were found in strains SC4-C13 and SC4-C24. Strain SC4-C13 had 2 of the 8 unique clusters, one of which was responsible for glycosyltransferase and the other for the biological response (movement, secretion, enzyme production or gene expression) to external cold stimuli of the cell. Strain SC4-C24 possessed the other 6 unique clusters; three clusters were responsible for the initiation of DNA-template transcription, hydrogen peroxide catabolic, and antigen biosynthetic processes, while the other 3 clusters did not have assigned functions. Although only two of the three *C. perfringens* strains showed unique orthologous clusters when compared to the

reference genomes, this may be attributed to the possibility of isolate niche adaptation (Braga *et al.*, 2016).

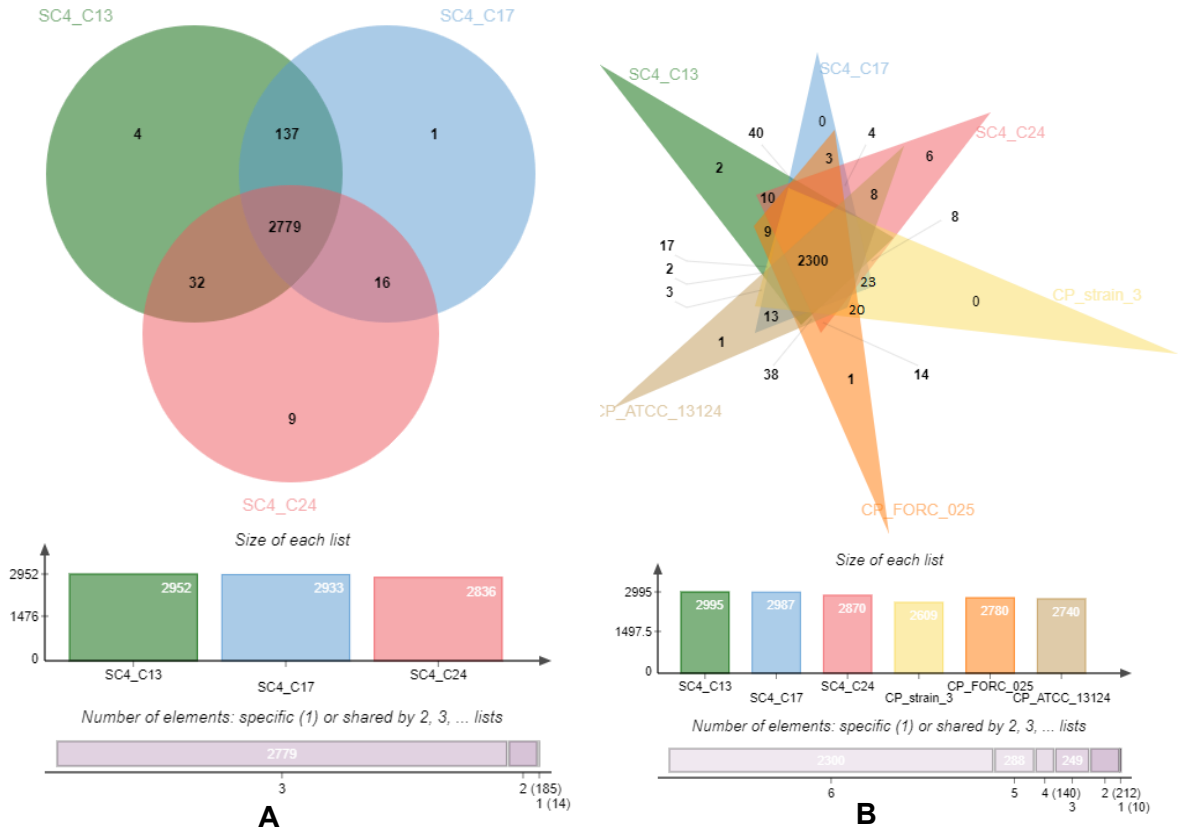


Figure 3.4: Venn diagram showing the core genome of A) the three *C. perfringens* strains (SC4-C13, SC4-C17 and SC4-C24), B) the three *C. perfringens* strains and reference strains (ATCC 13124, FORC_025 and Str.3). The numbers represent the orthologous protein clusters shared by corresponding *genomes.*

3.5 Conclusion

The current study has shown that *C. perfringens* strains from a water environment possess similar genomic features as published clinical *C. perfringens*. The *in-silico* analysis of the three genomes revealed putative genes encoding for virulence factors normally responsible for pathogen adherence to host cells, production of toxins and extracellular enzymes and the presence of key systems regulators managing the pathogenicity of *C. perfringens*. The strains were also classified as toxinotype A, which commonly consists of human pathogens only. Furthermore, the presence of antibiotic resistance genes encoding for several classes of

antibiotics and multidrug resistance efflux-pumps could contribute to the emergence of pathogenic multidrug-resistant *C. perfringens* strains in this environment. Interestingly, the presence of several genes normally associated with other pathogenic genera, as well as a few intact prophages and genomic islands (ARIs and PAIs) might suggest the transfer of genetic material between bacteria through HGT within the water reservoir, thus indirectly implicating the presence of toxic substances in the water (cadmium, copper, mercury etc.) which are associated with HGT in bacteria. Moreover, expression analyses should be the next step of investigation. Furthermore, comparative genomics showed great similarities between the environmental *C. perfringens* strains with those of clinical origin, highlighting the importance of monitoring these bacteria for effective epidemiological surveillance. Genomic studies on *C. perfringens* in natural environments are currently very limited and should be explored further.

CHAPTER 4: EFFECT OF SUB-INHIBITORY CONCENTRATION OF ANTIBIOTICS ON THE TRANSCRIPTOMIC RESPONSE OF ENVIRONMENTALLY OBTAINED *CLOSTRIDIUM PERFRINGENS*

4.1 Abstract

This study investigates the transcriptomic responses of an environmentally derived *Clostridium perfringens* strain to sub-inhibitory concentrations of five antibiotics: clindamycin, sulfamethoxazole, erythromycin, chloramphenicol, and cefoxitin. RNA sequencing was used to investigate gene expression changes. Cefoxitin induced the most extensive transcriptomic alterations, affecting genes associated with antibiotic resistance, stress adaptation, and virulence. Key findings include the up-regulation of resistance genes such as *mprF* and *vmlR* under clindamycin stress, demonstrating adaptive mechanisms like membrane remodelling and ribosomal protection. The *tetW* gene, typically linked to tetracycline resistance, was also up-regulated in response to other antibiotics, suggesting a generalised stress response. Sub-inhibitory antibiotic exposure modulated virulence-related genes, including those involved in toxin production, adherence, and environmental adaptation, potentially enhancing pathogenicity. Metabolic pathway analysis revealed suppression of energy-intensive processes, such as lipid and carbohydrate metabolism, alongside the up-regulation of DNA replication and repair pathways, emphasising a survival-oriented response to stress. These findings highlight the ecological and clinical risks of low-dose antibiotic exposure, which fosters the persistence and adaptability of resistant and virulent bacterial populations. The study highlights the necessity of responsible antibiotic usage to mitigate selective pressures that enable such adaptive bacterial responses.

Keywords: Transcriptomics, *Clostridium perfringens*, Sublethal Antibiotic Concentrations, Antibiotic Resistance, Virulence Modulation

4.2 Introduction

Antibiotic resistance among anaerobic pathogens, such as *C. perfringens*, is increasing worldwide (Khademi & Sahebkar, 2019). This is due to the widespread use of antibiotics as a therapeutic agent in both clinical and agricultural settings. This poses a serious health risk to humans and by extension, the natural environment (Ben *et al.*, 2019). Antibiotics are not fully metabolized and are then introduced into water and land environments through various pathways (Chow *et al.* 2021; Ashbolt *et al.*, 2013). Anthropogenic sources such as the discharge of sewage effluent, agricultural run-off from the use of livestock manure fertilizer, and aquaculture contribute to the presence of antibiotics in the natural environment (*Fouz et al.*, 2020; Andersson & Hughes, 2014; Ashbolt *et al.*, 2013). Even though the half-life of most antibiotics is relatively short, ranging from a couple of hours to hundreds of days, it can still accumulate in the environment and have significant biological effects on native bacteria (Chow *et al.* 2021; Cycoń *et al.*, 2019). This is of concern since research has shown that the presence of antibiotics, even at subinhibitory concentrations (sub-MICs), can still exert their impact on the microbial community in different ways: as selectors of resistance (enrichment for pre-existing and de novo resistance in bacteria), as generators of genetic and phenotypic variability (increasing the rate of mutations, recombination, and horizontal gene transfer) and as signalling molecules (inducing physiological responses that affect the fitness, virulence, and gene expression of bacteria) (Andersson & Hughes, 2014). Recently, several studies have provided insight into the physiological effects of sub-inhibitory concentrations of various antibiotics in major *Clostridium* pathogens such as *C. difficile* and *C. botulinum* (Yutani *et al.*, 2021; Mehr *et al.*, 2019; Zarandi *et al.*, 2017), while only a few have focused on *C. perfringens* (Charlebois *et al.*, 2014; Sakurai & Oda, 2011). The described responses induced by the different antibiotics were highly diverse among the species. However, all the strains were solely from clinical origins, leaving the effects on environmental strains unexplored. It is important to understand how antibiotic residues in the environment affect microbial communities, particularly regarding how they drive resistance and influence bacterial behaviour. This is especially true for environmental strains of anaerobic pathogens such as *C. perfringens*, where studies on their responses to subinhibitory antibiotic concentrations remain limited.

Although sequencing technologies have made significant advancements that have allowed for the thorough analysis of complete genomes, the understanding of complex molecular interactions that drive physiological processes in bacteria under antibiotic stress remains incomplete (Glass *et al.*, 2006). However, the development of high-throughput next-generation sequencing (NGS) technology and methods, such as RNA sequencing (RNA-seq), has made

meticulous transcriptome analysis possible by simultaneously expressing and analysing all genes within bacteria under various conditions (Westermann & Vogel, 2021). The advancements made in this technology have addressed numerous challenges that were previously encountered with hybridization-based microarrays and Sanger sequencing approaches, which were formerly employed to assess gene expression (Kukurba & Montgomery, 2015). Therefore, this study aimed to examine the effects of sub-inhibitory concentrations of various antibiotic treatments on a highly resistant *C. perfringens* strain obtained for the environment.

4.3 Materials and methods

4.3.1 Bacterial strain, growth conditions and minimum inhibitory concentrations (MIC)

Clostridium perfringens used in this study was isolated from a surface water system in the North West province of South Africa by utilising a modified Fung's double tube method (Fourie, 2017). The isolate was then routinely cultured on tryptose sulfite cycloserine agar (Oxoid, UK) and incubated anaerobically at 44 °C. The identity of the isolate was confirmed by Sanger sequencing of the 16S rRNA and *plc* gene following amplification. Antibiotic susceptibility testing was performed on the *C. perfringens* isolate. The minimum inhibitory concentration (MIC) of 9 different antibiotics was determined through agar dilution, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016). The strain showed to be highly resistant to 5 antibiotics and able to grow at sub-MIC (½ x MIC) values of: Clindamycin: 512 µg/ml, Sulfamethoxazole: 256 µg/ml, Erythromycin: 128 µg/ml, Chloramphenicol: 64 µg/ml and Cefoxitin: 64 µg/ml.

4.3.2. Growth curve

Clostridium perfringens cells were initially grown in Robertson's Cooked Meat Medium (Oxoid, UK) for 18 h at 37°C. For each antibiotic exposure assay, a sterile 96-microwell culture plate was used, where 20 µl of culture was inoculated into 180 µl medium (1:9 ratio) supplemented with the appropriate antibiotic to achieve a final concentration of ½ x MIC. A control treatment without any antibiotics was prepared alongside each exposure assay. To achieve anaerobioses, 50 µl sterile mineral oil was added to each well, covering the inoculum mixture. Bacterial growth was then accessed by measuring the optical density (OD_{600}) every 15 min for 12 h at 37°C by using a BioTek PowerWave HT Microplate Spectrophotometer instrument (Agilent Technologies, US). The microwell plate was automatically shaken for 1 min before

each measurement. Each assay was performed in replicates of 5, on separate days. Growth curves were then constructed from the data of each antibiotic exposure assay and fitted to the modified Gompertz equation (Zwietering *et al.*, 1990) to obtain significant biological growth parameters (lag time: λ, maximum growth rate: μ_{max}, and doubling time: t_D), of *C. perfringens* under the different stress conditions.

4.3.3. Treatment of *C. perfringens* with sub-MIC of antibiotics

Fresh overnight culture of *C. perfringens* was added to six McCartney glass bottles containing 25 ml Robertson's Cooked Meat Medium (Oxoid, UK), each supplemented with an antibiotic to reach a final concentration of ½ × MIC and a control without any antibiotic. All treatments were then incubated anaerobically at 37°C with an OD_{600} of 0.3. This process was repeated to achieve three technical replicates for each antibiotic treatment. Based on the data gathered from the different growth curves, each treatment and control were removed from the incubator and immediately placed on ice when it reached the late-exponential phase of growth. Samples were then processed for RNA extraction.

4.3.4. Transcriptome analysis by RNA-seq

Total RNA from treated *C. perfringens* was extracted and purified using Quick-RNA Miniprep Kit (Zymo research, US), according to the manufacturer's instructions. The quality and integrity of the extracted RNA were evaluated by NanoDrop Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, US) and Bioanalyzer 2100 (Agilent Technologies, US) systems. A total of 18 RNA samples were sent to NovoGene Corporation Inc. (China) for mRNA isolation and library preparation using the NEBNext® Ultra Directional RNA Library Prep Kit (New England Biolabs, US). Sequencing was then performed on the Illumina NovaSeq platform with 150 bp paired-end chemistry. Subsequently, the raw sequencing reads used in this study were then deposited into the NCBI database (SRR29819246, SRR29819250, SRR29819247, SRR29819251, SRR29819249, SRR29819248).

In silico analysis of RNA-seq data was performed. The quality of reads obtained from sequencing was evaluated using FastQC (v.0.11.9; http://www.bioinformatics.babraham.ac.uk/projects /fastqc/) and MultiQC (v.1.15; Ewels *et al.*, 2016). This was followed by de novo assembled using Trinity (v.2.15.0; Grabherr *et al.*, 2011) with normalisations (default cut off: 200 transcripts per site). The reads were trimmed using Timmomatic (v.0.36; Bolger *et al.*, 2014). Functional annotation of transcripts was done using Trinotate v4.0.2 and TransDecoder v5.7.1. Each transcript's predominant open reading frame

(ORF) was selected for further analysis. The nucleotide and protein sequences were compared against the NCBI Nr database (a comprehensive collection of protein sequences) and the UniProtKB/SwissProt repository (a curated protein sequence database) using the BLASTx and BLASTp algorithms with an e-value cut-off of 1e-05. Functional domains were detected using HMMER v3.4 in and compared with the Pfam domain database. The transcript abundance was estimated using Salmon v1.10.3 (Patro *et al.* 2017). Differentially expressed genes (DEGs) analysis was performed in R (v3.3.3) using the DESeq2 package. Transcripts with $FDR < 0.05$ and $\log2(FC) \geq \pm 1.5$ were designated as DEGs. Gene Ontology (GO) functions for the differentially expressed transcripts, whether up-regulated or down-regulated, were annotated via WEGO 2.0 (Ye *et al.*, 2018). Analysis of protein Clusters of Orthologous Groups (COGs) was conducted utilising eggNOG, a database dedicated to orthologous gene groups (http://eggnogdb.embl.de/). Each transcript was assigned to various pathways by utilising the KEGG Automatic Annotation Server to search the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. KEGG pathways were reconstructed using KEGG mapper (Kanehisa *et al.*, 2022). DEGs were annotated in the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/) and the Virulence Factor Database (VFDB; https://www.mgc.ac.cn/VFs/) to determine antibiotic resistance and virulence genes, respectively.

4.3.5. Statistical analysis

Biological parameters obtained from modified Gompertz equation were determined via non-linear regression on growth data using QurvE (Wirth *et al.*, 2023). Analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) ($p < 0.05$) test analysis were performed. The STATISTICA 13 (StatSoft, US) software was used.

4.4 Results

4.4.1. Growth curves of *C. perfringens* during antibiotic-induced stress

In this study, *Clostridium perfringens* was subjected to subinhibitory concentrations of 5 antibiotics to ascertain its growth kinetics response. In particular, the antibiotics used included Clindamycin (Lincosamides): 512 µg/ml, Sulfamethoxazole (Sulfonamides): 256 µg/ml, Erythromycin (Macrolides): 128 µg/ml, Chloramphenicol (Phenicols): 64 µg/ml and Cefoxitin (Cephalosporins): 64 µg/ml. The effects of these antibiotics on the growth of *C. perfringens* over 12 h can be seen in Figure 4.1. Based on the growth curves, untreated *C. perfringens*

(control) clearly showed to enter the exponential phase of growth by 0.5 h and OD values stabilising at 1.75 h indicating the start of the stationary phase. This trend was also observed with cells treated with sulfamethoxazole. Additionally, the addition of clindamycin, erythromycin and cefoxitin showed similar initial starting points of the exponential phase, approximately at the 0.75 h mark, then diverged to reach the stationary phase by 2.75 h, 3 h and 3.75 h, respectively. Compared to the other treatments, chloramphenicol resulted in a prolonged exponential phase, with *C. perfringens* only reaching the stationary phase after 6.75 h.

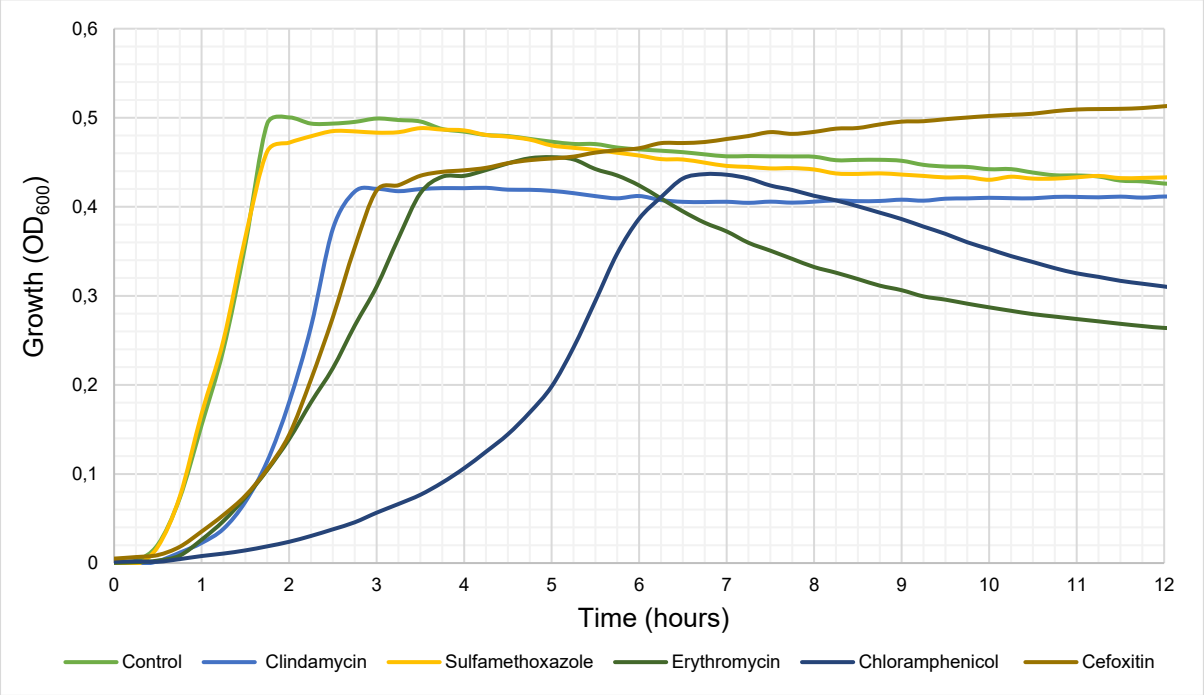


Figure 4.1: Growth curves of *C. perfringens* generated under different antibiotics stress. OD values were measured at 600 nm over 12 h. Values represent means of five replicates grown on different occasions. Sub-MIC of Clindamycin: 512 µg/ml, Sulfamethoxazole: 256 µg/ml, Erythromycin: 128 µg/ml, Chloramphenicol: 64 µg/ml and Cefoxitin: 64 µg/ml. Control contained no antibiotics.

Variable antibiotics exposure also affected the growth kinetics of *C. perfringens* (Table 4.1), which reports the growth parameters obtained from using the Gompertz equation. Chloramphenicol at sublethal concentration had the greatest effect on the growth of *C. perfringens*. When compared to the control, it showed a 3.5-fold reduction in the growth rate, a 4.6-fold increase in lag time and a 3.5-fold increase in doubling time, whereas sulfamethoxazole showed to have no significant effect on its growth. Furthermore, all the

antibiotics, apart from sulfamethoxazole, induced an extension of the lag phase duration (λ), a decrease in the maximum growth rate (μ_{max}), and an increase in doubling time (t_D).

Table 4.1: Mean values and standard deviation of the duration of the maximum specific growth rate (μ_{max}), lag phase (ʎ), and doubling time (t_D) of *C. perfringens* exposed to ½ x MIC of antibiotics; a, b, c, d: homogeneous groups according to statistical analysis.

Antibiotic	μ_{max}			ʎ			t_D		
	Mean	SD		Mean	SD		Mean	SD	
Control	0,63	0,30	c	0,76	0,01	a	1,11	0,03	a
Clindamycin	0,48	0,10	d	1,52	0,01	c	1,46	0,02	a
Erythromycin	0,27	0,10	b	1,35	0,02	b,c	2,57	0,05	b
Chloramphenicol	0,18	0,20	a	3,48	0,10	d	3,88	0,27	c
Sulfamethoxazole	0,57	0,80	c	0,70	0,02	a	1,23	0,08	a
Cefoxitin	0,22	0,20	a,b	1,22	0,04	b,c	3,16	0,13	b

4.4.2. Transcriptome analysis of antibiotics treated *Clostridium perfringens*

Structural annotation (de novo transcriptome assembly)

To obtain a comprehensive analysis of the effects of the 5 antibiotics on *C. perfringens*, 18 cDNA libraries (5 treatments and 1 control, each in triplicate) were constructed and sequenced. All the libraries were sequenced successfully, except for one of the three erythromycin replicates. Table 4.2 shows the summary RNA-Seq data and de novo assemblies of *C. perfringens*. The quality of the raw reads was confirmed using FastQC. RNA-Seq generated on average between 7 770 876 and 11 769 078 raw reads for the different treatments and control (a total of 157 218 170 reads from 17 libraries). Trimmomatic was used to remove adaptors and low-quality sequences and generated on average between 7 677 214 and 11 712 670 clean reads from the five treatments and control. The GC content varied between 31% and 35% among the treatments and control. De novo assembly was performed using Trinity, and a total of 58 430 transcripts were constructed from all the replicates, with the highest average number of transcripts generated from the cefoxitin treatment (5 001) and the lowest from sulfamethoxazole (1 728).

Table 4.2: Summary of RNA-Seq and de novo assembly of *C. perfringens* for each antibiotic treatment results.

Treatment	Replicates	Raw reads[a]	Trimmed reads	N50 (bp)	GC (%)	Total transcripts
Control	K1	8 079 615	7 990 207	2 374	35,0	3 939
	K2	7 895 717	7 812 293	2 701	35,0	3 822
	K3	8 221 150	8 113 524	2 443	35,0	4 205
Average		**8 065 494**	**7 972 008**	**2 506**	**35,0**	**3 989**
Chloramphenicol	CH1	10 090 878	10 070 664	3 430	34,0	2 889
	CH2	8 294 801	8 199 909	2 796	35,0	3 218
	CH3	8 327 736	8 197 083	2 681	35,0	3 271
Average		**8 904 472**	**8 822 552**	**2 969**	**34,7**	**3 126**
Clindamycin	C1	8 912 155	8 817 375	2 250	35,0	3 928
	C2	6 793 000	6 694 989	2 271	35,0	3 649
	C3	7 607 472	7 519 279	2 248	35,0	4 010
Average		**7 770 876**	**7 677 214**	**2 256**	**35,0**	**3 862**
Cefoxitin	OX1	10 890 680	10 859 271	2 067	35,0	5 163
	OX2	10 185 834	10 114 607	2 265	35,0	4 792
	OX3	9 308 466	9 281 312	2 013	35,0	5 048
Average		**10 128 327**	**10 085 063**	**2 115**	**35,0**	**5 001**
Sulfamethoxazole	S1	10 654 026	10 569 209	16 648	31,0	1 741
	S2	8 879 444	8 834 382	16 242	31,0	1 633
	S3	9 539 041	9 479 108	15 610	31,0	1 811
Average		**9 690 837**	**9 627 566**	**16 167**	**31,0**	**1 728**
Erythromycin	E1	14 839 778	14 817 055	15 052	30,0	2 916
	E2	8 698 377	8 608 285	13 340	30,0	2 395
Average		**11 769 078**	**11 712 670**	**14 196**	**30,0**	**2 656**

[a]Reads = read 1 + read 2

PCA analysis was performed with the normalised expression counts (based on the Trinity output) to investigate if the replicates from each treatment cluster together (Figure 4.2). As a result, the first two principal components (PCs) explained more than 88% of the variability among the different treatments. Four antibiotic treatments (chloramphenicol, cefoxitin, erythromycin and sulfamethoxazole) were grouped in distinct clusters, indicating clear differences among their expression levels. However, the three control and clindamycin treatment replicates showed similar transcriptome profiles by clustering close together.

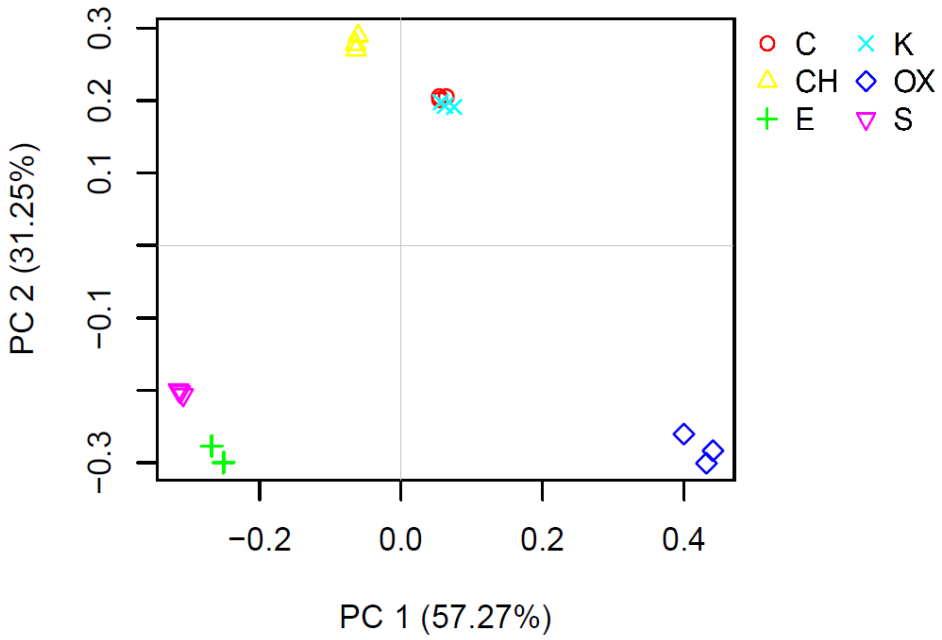


Figure 4.2: PCA scatter plot of normalised expression counts for *C. perfringens* during exposure to sublethal concentrations of five antibiotics. K: Control, C: Clindamycin, CH: Chloramphenicol, OX: Cefoxitin, E: Erythromycin and S: Sulfamethoxazole. The PCA plot shows the variance of the three replicates of each antibiotic treatment, except for erythromycin (two replicates). The percentages on each axis represent the percentages of variation explained by the principal components and determine 88.52 % of the total variance.

4.4.3. Functional annotations of transcripts

Since most of the antibiotics used in this study had an effect on the growth of *C. perfringens* at sublethal concentrations (Figure 4.1), the gene expression profiles of *C. perfringens* grown in media supplemented with ½ × MIC antibiotics were compared with a control without any antibiotic by RNA-seq. As shown in Figure 4.3, a total of 2 002 transcripts showed significantly different expression when subjected to five antibiotic treatments (FC of log2 > 1.5, FDR-corrected p-value < 0.05, Figure 4.3: A-E). Differentially expressed transcripts showed distinct response patterns in response to the five different antibiotics (Figure S4.1). Variance analysis revealed distinct differential expression patterns for cefoxitin, sulfamethoxazole, and erythromycin. In contrast, in the presence of clindamycin and chloramphenicol, the transcriptional patterns of *C. perfringens* displayed profiles with the lowest variance compared to those of the control sample (without antibiotic). Exposure to sublethal concentration of cefoxitin had the greatest effect on the expression profile of *C. perfringens*, with 343 transcripts shown to be up-regulated and 383 transcripts down-regulated (Figure 4.3A). Sulfamethoxazole had the second-highest expression profile with 265 and 189 transcripts being up- and down-regulated, respectively (Figure 4.3B). This was followed by erythromycin with 222 up-regulated and 175 down-regulated transcripts (Figure 4.3C) and then chloramphenicol with 36 and 190 transcripts up- and down-regulated, respectively (Figure 4.3D). Although clindamycin had the highest sublethal concentration of all the antibiotic treatments (512 µg/ml), the stress response it induced in *C. perfringens* showed the lowest effect, with only 38 transcripts shown to be up-regulated and 60 transcripts down-regulated (Figure 4.3E).

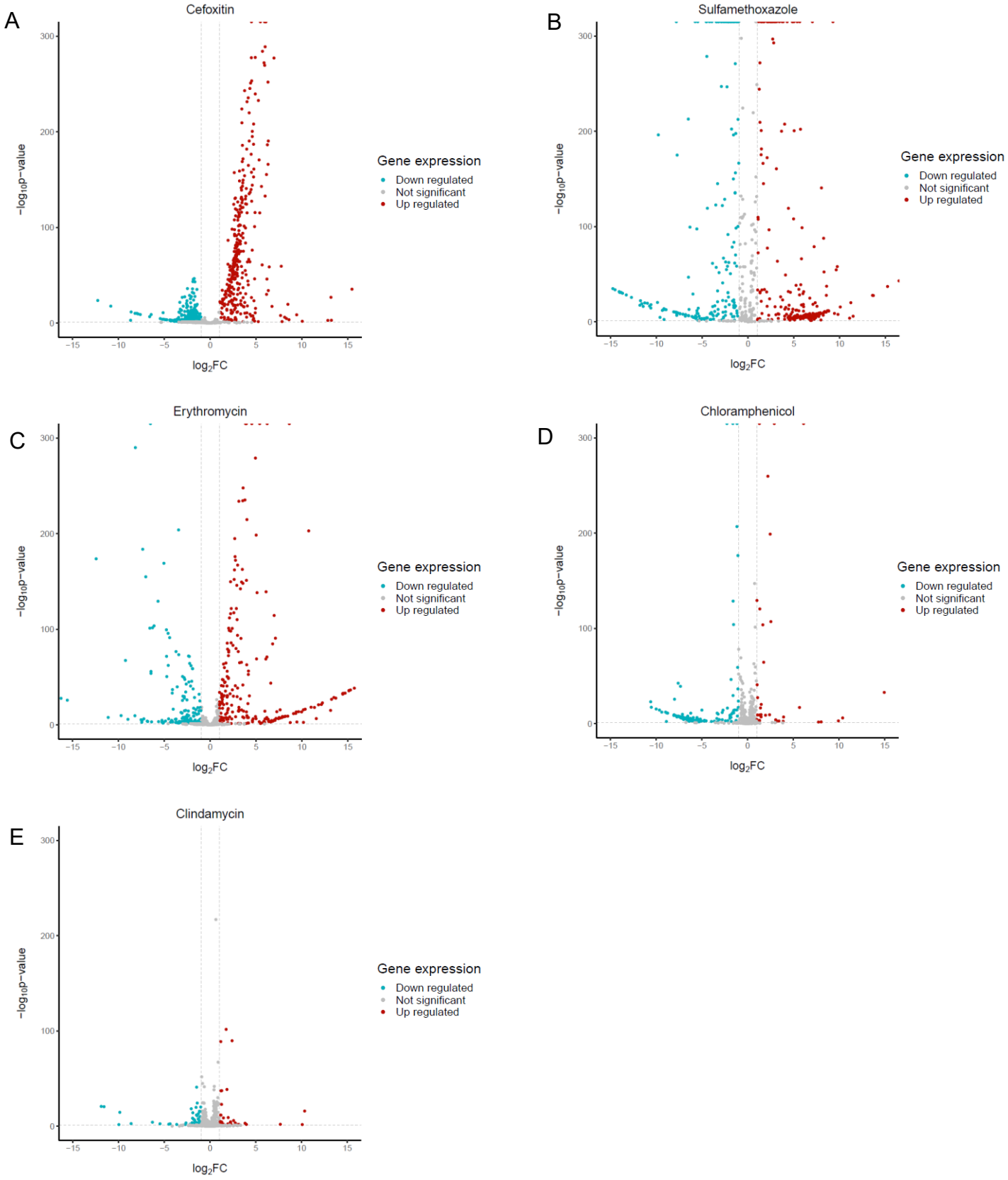


Figure 4.3: Volcano plots of the differentially expressed transcripts in the *C. perfringens* under A: cefoxitin (64 µg/ml), B: sulfamethoxazole (256 µg/ml), C: erythromycin (128 µg/ml), D: chloramphenicol (64 µg/ml) and E: clindamycin (512 µg/ml) stresses. The x-axis represents the fold change in gene expression between two samples, calculated as the ratio of the expression level in the treated sample to that in the control sample. The y-axis represents the *P* value, which measures the statistical significance of the gene expression difference. A higher *P* value indicates a more significant difference in expression. Each point on the graph corresponds to a specific transcript. Red points indicate significantly up-regulated transcripts, blue points represent significantly down-regulated transcripts, and grey points signify transcripts with no significant differences.

4.4.3.1. Cluster of orthologous groups (COG)

A cluster of orthologous groups of proteins analysis was done to evaluate the functional categorisation of *C. perfringens* transcripts. The results in Figure 4.4 show the number of differently expressed transcripts from the five antibiotic treatments that were grouped into the different COG categories. Transcripts from chloramphenicol, clindamycin and sulfamethoxazole treatments were classified into 17 COG categories, whereas the transcripts for cefoxitin and erythromycin were grouped into 18 COG categories (Figure S4.1). The relative abundance of transcripts assigned to the "Function unknown" group was observed across all five antibiotic treatments. Clindamycin and erythromycin treatments shared similar annotations, as did sulfamethoxazole and chloramphenicol (Figure 4.4). Most of the transcripts from these four antibiotic treatments were assigned to three functional categories, namely "Cell wall/membrane/envelope biogenesis", "Carbohydrate transport and metabolism" and "Replication, recombination and repair". Additionally, these treatments also showed the lowest counts of differentially expressed transcripts involved in "Coenzyme transport and metabolism", "Cell motility" and "Intracellular trafficking, secretion, and vesicular transport". Exposure to cefoxitin showed the most deviation in COGs associated with biological processes, with an increased number of transcripts assigned to most of the COG categories, specifically to "Amino acid transport and metabolism", "Coenzyme transport and metabolism", "Energy production and conversion", "Translation, ribosomal structure and biogenesis", "Replication, recombination and repair", "Cell wall/membrane/envelope biogenesis" and "Inorganic ion transport and metabolism". Additionally, cefoxitin and erythromycin were the only two treatments that grouped transcripts to the "Post-translational modification, protein turnover, chaperones" category. Based on the COG analysis of the differently expressed transcripts, "Signal transduction mechanisms", "Cell wall/membrane/envelope biogenesis", "Transcription" and "Defence mechanisms" might play key roles in antibiotic-resistance mechanisms in *C. perfringens*.

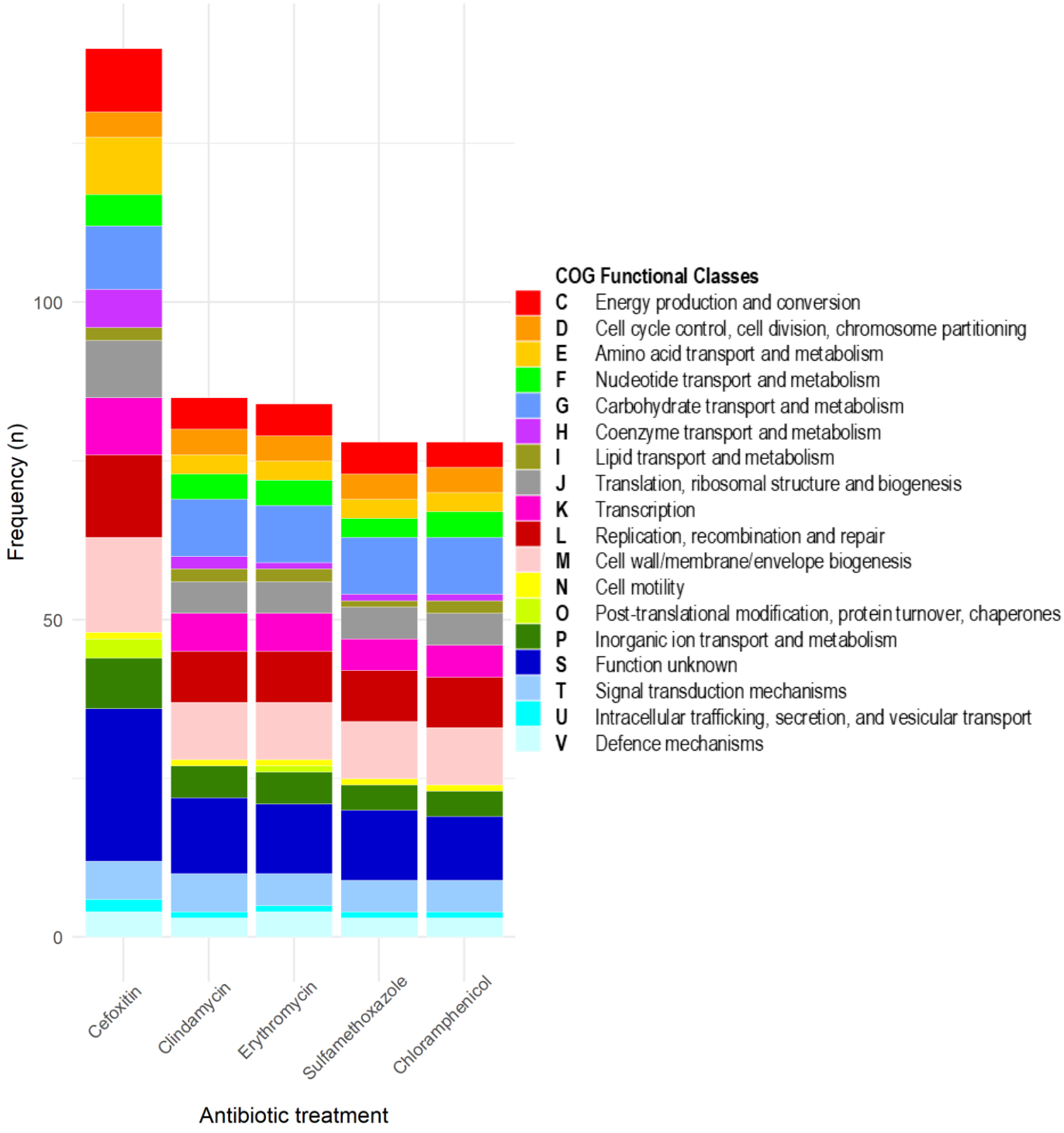


Figure 4.4: Clusters of Orthologous Groups (COG) classification of differentially expressed transcripts from *C. perfringens* exposed to five antibiotics. The vertical axis represents the number of transcripts assigned to the different COG categories, whereas the horizontal axis represents the different antibiotic treatments.

4.4.3.2. Gene Ontology (GO) Functional Enrichment Analysis

GO enrichment analysis was performed (Figure S4.2-6) to further understand the function of the differentially expressed transcripts underlying the effect of sublethal concentrations of 5 antibiotics on *C. perfringens*. Based on sequence homology, transcripts were assigned to one or more GO terms and categorised into the three main function categories (biological process, molecular function, and cellular component). Upon GO functional enrichment of all up-regulated transcripts, a total of 519, 432, 643 specific GO terms in biological process, cellular component and molecular function were identified, respectively (Table S4.2). For all down-regulated transcripts, 613, 526 and 737 GO terms were assigned to biological process, cellular component and molecular function, respectively. Cefoxitin had the greatest number of assigned GO terms (615 up-regulated and 796 down-regulated), and clindamycin had the lowest (73 up-regulated and 108 down-regulated).

In the detailed functional analysis of up-regulated GO terms (Figure 4.5A), the most represented functional categories at the transcriptional level in all antibiotic treatments were related to binding [referring to the binding of heterocyclic compounds (GO:1901363), organic cyclic compounds (GO:0097159) and carbohydrate derivatives (GO:0097367)], catalytic activities [involving activities such as hydrolase (GO:0016787), transferase (GO:0016740) and catalase on DNA (GO:0140097), RNA (GO:0140098) and proteins (GO:0140096)], and metabolic processes [involving functions related to cellular processes (GO:0044237), biosynthesis of cellular components (GO:0009058), nitrogen metabolism (GO:0006807), organic substance metabolic processes (GO:0071704) and small molecule metabolic processes (GO:0044281)]. Although the GO categories of down-regulated transcripts (Figure 4.5B) were similar to those which were up-regulated, cellular components of cell parts, including cell periphery (GO:0071944) and plasma membrane (GO:0005886), were shown to be significant.

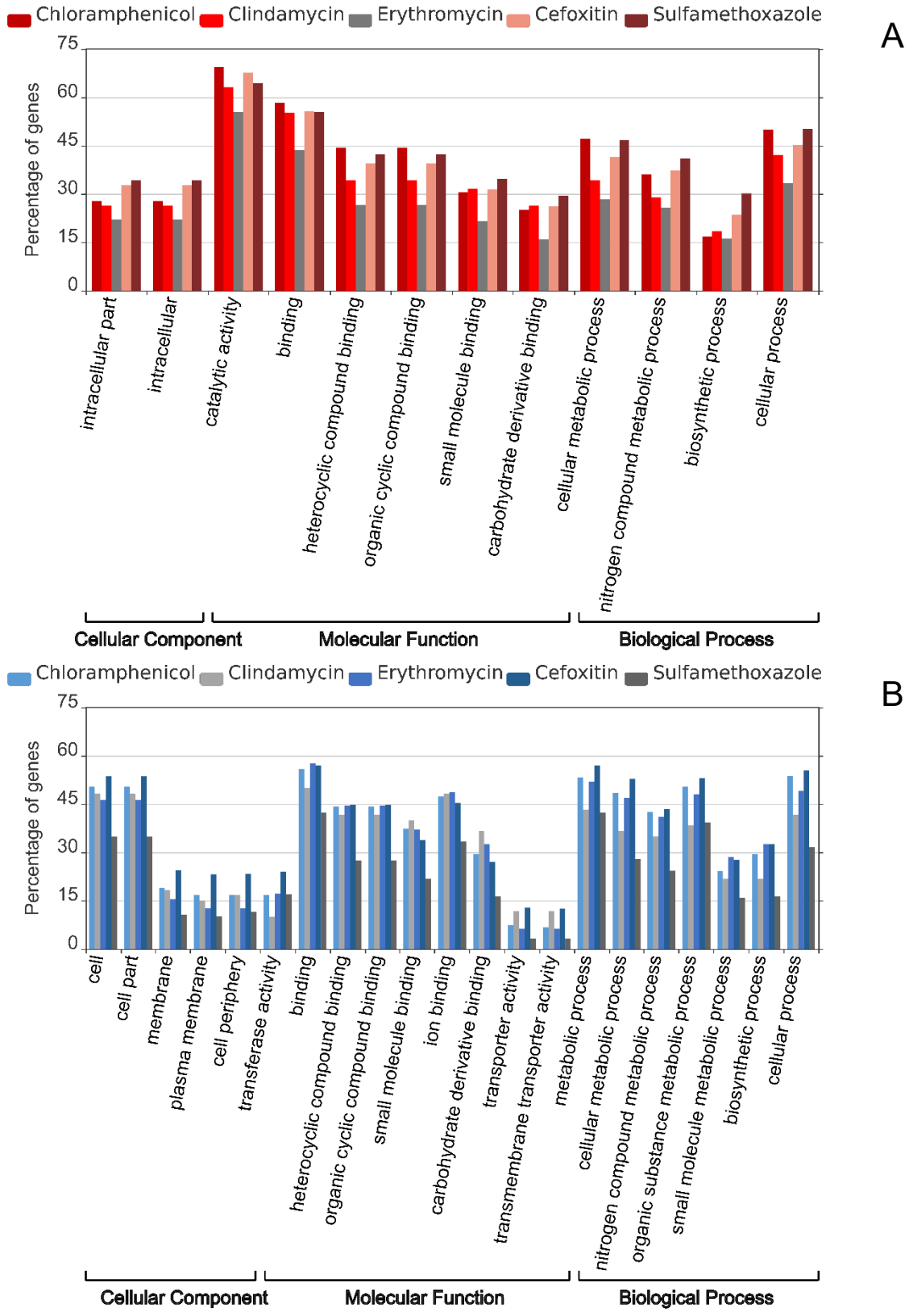


Figure 4.5: Gene Ontology (GO) analysis of differentially expressed transcripts identified in *Clostridium perfringens* when exposed to sublethal concentrations of five antibiotics. The graphs show the statistically significant (P < 0.5) GO terms in the three main categories: cellular component, molecular function and biological process. A. up-regulated transcripts B. down-regulated transcripts.

4.4.3.3. KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathways

The analysis of differently expressed transcripts in the KEGG database allowed for the identification of active biological pathways in *C. perfringens*. KEGG analysis classified 166 transcripts (73 up-regulated and 93 down-regulated) to 26 different pathways (Figure 4.6, Table S4.3-6). Both up and down-regulated pathways predominantly involve broad metabolic functions, such as “Metabolic pathways”, “Carbohydrate metabolism”, “Biosynthesis of secondary metabolites”, “Energy metabolism”, “Nucleotide metabolism” and “Glycan biosynthesis and metabolism”. Interestingly, a high number of down-regulated transcripts were assigned to the “Lipid metabolism” pathway. This pathway, along with other down-regulated metabolic pathways, such as “Carbohydrate metabolism”, could indicate a suppression of energy-intensive processes in *C. perfringens* when exposed to antibiotics. The results also showed three metabolic pathways which were only associated with down-regulated transcripts, namely “Fatty acid metabolism”, “Biosynthesis of nucleotide sugars” and “Biosynthesis of other secondary metabolites”.

Focusing on the environmental information processing category, the same number of up-and down-regulated transcripts were mapped to the “Membrane transport” pathway. This could possibly indicate a dual role in nutrient uptake and resistance mechanisms in *C. perfringens*. However, the “Signal transduction” pathway was shown to be more associated with down-regulated transcripts, suggesting a decrease in cellular communication in favour of immediate survival strategies. The results also showed both up-and down-regulated transcripts mapped to pathways affect genetic information processing, however, their functional focus differs**.** Down-regulated pathways exhibit reductions in transcription, translation, and protein folding processes, signifying a general decrease in cellular biosynthetic systems. However, pathways such as “Replication and repair” which were more up-regulated could highlight a prioritised response to mitigate potential antibiotic-induced genetic damage. Pathways linked to bacterial infectious diseases and antimicrobial resistance are modulated in both up- and down-transcripts. While these pathways are moderately down-regulated in some respects, their up-regulation suggests an adaptive response to antibiotic stress, thereby allowing bacteria to survive better under these stress conditions.

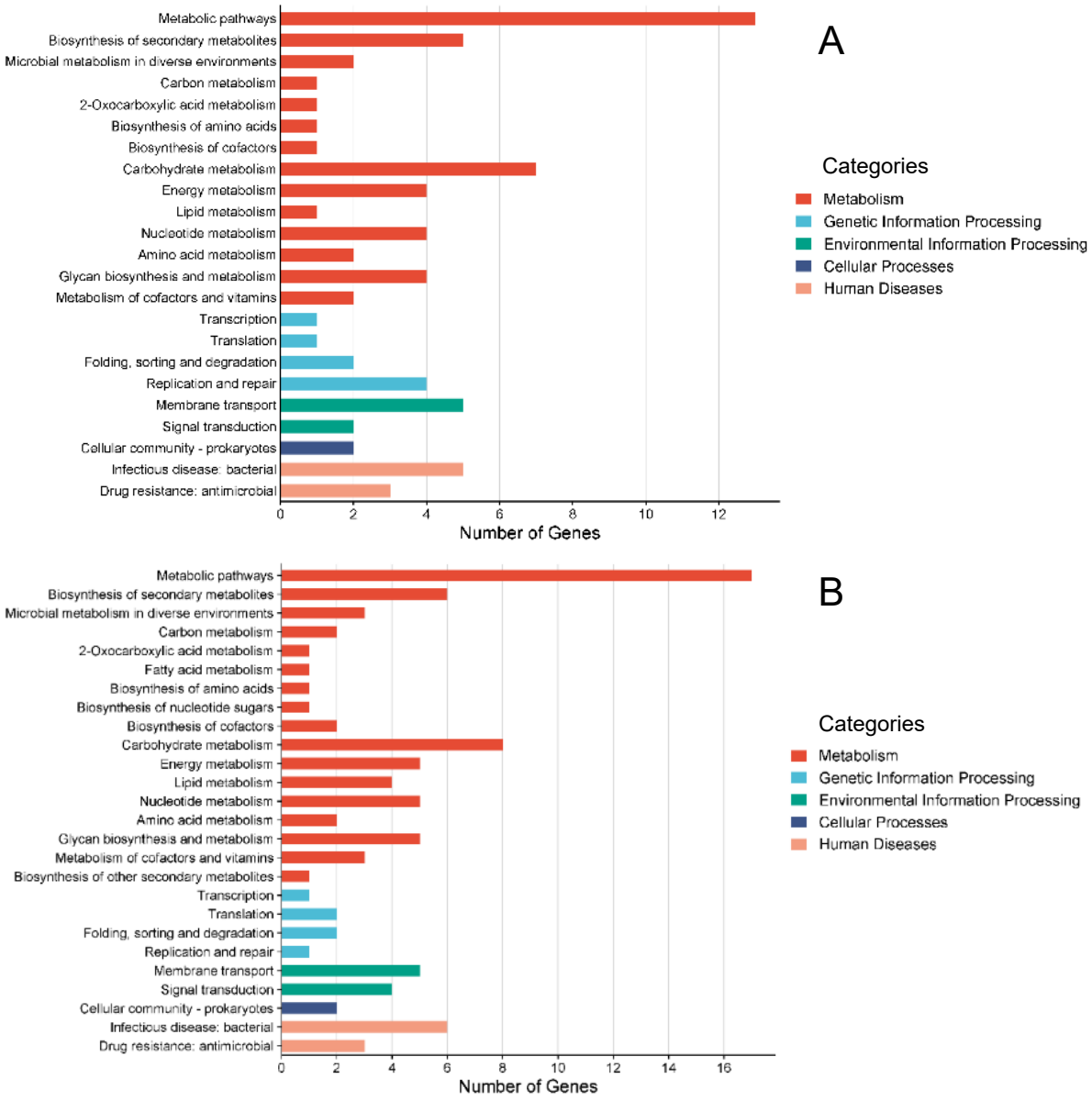


Figure 4.6: KEGG pathways associated with transcripts that were significantly differentially expressed (P value of 0.05) in *Clostridium perfringens* exposed to sublethal concentrations of five antibiotics. Bar graph (A) depicts the up-regulated pathways (73 genes) and (B) the down-regulated pathways (93 genes) assigned to the five main pathways categories: metabolism, genetic information processes, environment information processes, cellular processes and human diseases, are summarized. The x-axis presents the number of genes in the categories pathway, and the y-axis presents the categories pathways. Weakly modulated components (not significant) within the pathways are not displayed.

4.4.3.4. Effects of antibiotics on antibiotic resistance genes and virulence factors

Of the 25 antibiotic-resistance genes identified in *C. perfringens*, only 11 genes were expressed differently during the five antibiotic treatments (Table 4.3). The cefoxitin treatment showed the highest number of DE transcripts related to antibiotic resistance, with nine resistance genes being down-regulated and two up-regulated. Antibiotic treatments such as sulfamethoxazole and clindamycin only showed three DE transcripts. Chloramphenicol and erythromycin treatments showed the lowest number of DE transcripts (only two), all of which were down-regulated. The genes encoding for phosphatidylglycerol lysyltransferase (*mpr*F) and ABC-F subfamily ATP-binding cassette ribosomal protection proteins (*Vml*R) were both up-regulated during exposure to clindamycin, whereas both genes were down-regulated during cefoxitin exposure. The penicillin-binding protein 1A (PBP1) showed to be up-regulated during cefoxitin and sulfamethoxazole treatments and down-regulation during chloramphenicol and erythromycin treatments. However, the gene encoding for the tetracycline resistance protein (*tet*W) was up-regulated during cefoxitin and sulfamethoxazole treatments, but down-regulation during exposure to erythromycin.

Table 4.6: Effects of sub-MIC of antibiotics on antibiotic resistance genes identified in *C. perfringens.*

Class and Gene	Gene annotation	Antibiotic treatment				
		OX	S	CH	E	C
	Aminoglycoside					
aph(3')-Ia	Aminoglycoside 3'-phosphotransferase	↓ 6.218	↓ 10.615	ns	ns	ns
gyrB	Aminocoumarin resistant	ns	ns	ns	ns	ns
ant(6)	Aminoglycoside nucleotidyltransferase	ns	ns	ns	ns	ns
	Amphenicol					
catA1	Chloramphenicol acetyltransferase (CAT	ns	ns	ns	ns	ns
cfr(C)	23S rRNA methylase	ns	ns	ns	ns	ns
	Beta-lactam					
blaC	Beta-lactamase	↓ 5.1367	ns	ns	ns	ns
blaZ	BlaZ beta-lactamase	ns	ns	ns	ns	ns
PBP1	Penicillin-binding protein 1A	↑ 4.405	↑ 55.3072	↓ 5.6779	↓ 8.6952	ns
PBP2	Penicillin-binding protein 2A	↓ 1.2322	ns	ns	ns	ns
*bla*TEM-181	TEM beta-lactamase	ns	ns	ns	ns	ns
	Diaminopyrimidine					
dfrC	Trimethoprim resistant dihydrofolate reductase	ns	ns	ns	ns	ns
	Fluoroquinolone					
norA	Major facilitator superfamily (MFS) antibiotic efflux pump	↓ 3.0256	ns	ns	ns	ns
norC	Major facilitator superfamily (MFS) antibiotic efflux pump	ns	ns	ns	ns	ns

Table 4.6: Effects of sub-MIC of antibiotics on antibiotic resistance genes identified in *C. perfringens* (*continue*).

Class and Gene	Gene annotation	Antibiotic treatment				
		OX	S	CH	E	C
	MLSB					
cplR	ABC-F subfamily ATP-binding cassette ribosomal protection proteins	ns	ns	ns	ns	ns
ermQ	Erm 23S ribosomal RNA methyltransferase	ns	ns	ns	ns	ns
lnuC	Lincosamide nucleotidyltransferase (LNU)	ns	ns	ns	ns	ns
vmlR	ABC-F subfamily ATP-binding cassette ribosomal protection proteins	↓ 1.4031	ns	ns	ns	↑ 1.2718
	Multidrug					
bmrA	Multidrug resistance ABC transporter ATP-binding/permease protein	↓ 3.1129	ns	ns	ns	ns
	Penicillin					
femA	Aminoacyltransferase	↓ 2.9068	ns	ns	ns	ns
femB	Aminoacyltransferase	↓ 1.607	ns	↓ 5.2704	ns	↓ 1.1385
	Peptide					
mprF	Phosphatidylglycerol lysyltransferase	↓ 1.7807	ns	ns	ns	↑ 3.1385
	Pseudomonic acid					
mupA	Antibiotic-resistant isoleucyl-tRNA synthetase (ileS)	ns	ns	ns	ns	ns
	Tetracycline					
tet(44)	Tetracycline-resistant ribosomal protection protein	ns	ns	ns	ns	ns
tetA(P)	Tetracycline efflux protein	ns	ns	ns	ns	ns
*tet*W	Tetracycline resistance protein	↑ 2.1801	↑ 36.9195	ns	↓ 15.193	ns

↑: Significantly up-regulated, ↓: Significaly down-regulated, ns: No significant change

OX: Cefoxetin, S: Sulfamethoxazol, CH: Chloramphenicol, E: Erythromycin, C: Clindamycin

Using the Virulence Factor Database (VFDB), a total of 37 genes related to virulence in *Clostridium perfringens* were identified. Of the 37 identified genes, 15 genes showed to be differentially expressed during the five antibiotic treatments (Table 4.4). These included genes associated with virulence classification such as adherence, toxin production, acid resistance, intracellular survival, antiphagocytosis, immune evasion, iron uptake, secretion system, and stress adaptation. Sulfamethoxazole showed 10 virulence genes that were differentially expressed, of which eight were up-regulated and two were down-regulated. Cefoxitin and erythromycin showed ten genes (5 down-regulated, 5 up-regulated) and 9 genes (7 down-regulated, 2 up-regulated) that were differentially expressed, respectively. All 6 virulence genes that were differentially expressed during chloramphenicol exposure were down-regulated. Clindamycin showed the lowest number of differentially expressed virulence genes, with only 3 down-regulated and 2 up-regulated.

Table 4.4: Effects of sub-MIC of antibiotics on virulence factors identified in *C. perfringens.*

Virulence class and factors	Gene	Antibiotic treatment				
		OX	S	CH	E	C
Adherence						
Fibronectin-binding protein	*fbpA*	ns	ns	ns	ns	ns
Chaperonin GroEL	*groEL*	↓ 1.8917	↑ 6.0661	↓ 6.722	↓ 2.6102	↓ 1.53
Bifunctional autolysin	*altE*	ns	↑ 5.2238	↓ 5.382	ns	ns
Elastin binding protein (*Staphylococcus*)	*ebp*	ns	ns	ns	ns	ns
Elongation Factor Tu	*tuf*	↑ 3.453	ns	ns	↓ 16.8545	ns
Regulation						
Sensor histidine kinase	*virS*	ns	ns	ns	ns	ns
DNA-binding response regulator	*virR*	ns	ns	ns	ns	ns
Toxin						
Alpha-clostripain	*cloSI*	ns	↓ 1.0176	na	↑ 1.5828	↑ 2.4037
Phospholipase C (Alpha-toxin)	*plc*	ns	ns	ns	ns	ns
Hemolysin		ns	ns	ns	ns	ns
Kappa-toxin (collagenase)	*colA*	ns	↑ 6.7345	ns	↓ 7.4625	↑ 1.7574
Mu-toxin (hyaluronidase)	*nagH*	↑ 1.3913	ns	ns	ns	ns
	nagJ	↑ 1.9328	↑ 1.5641	ns	↓ 1.4551	ns
Perfringolysin O (theta-toxin/PFO)	*pfoA*	ns	ns	ns	ns	ns
Sialidase	*nanH*	↑ 3.3465	↓ 14.5242	ns	↑ 15.2912	ns
Acid resistance						
Urease subunit alpha	*ureA*	↓ 3.0487	↑ 6.5974	↓ 5.3819	↓ 2.5709	↓ 1.9368
Intracellular survival						
Lipoate protein ligase A1(Listeria)	*lplA1*	ns	↑ 7.1911	↓ 5.9765	ns	ns
Anaerobic respiration						
Nitrate reductase (*Mycobacterium*)	*narH*	ns	ns	ns	ns	ns
Antiphagocytosis						
Capsule (*Klebsiella*)	*gnd*	↓ 2.5857	ns	ns	ns	ns
Enzyme						
Lipase (*Staphylococcus*)	*geh*	ns	ns	ns	ns	ns
	lip	ns	ns	ns	ns	ns
Serine V8 protease (*Staphylococcus*)	*sspA*	ns	ns	ns	ns	ns
Streptococcal enolase (*Streptococcus*)	*eno*	ns	ns	ns	ns	ns
Thermonuclease (*Staphylococcus*)	*nuc*	ns	ns	ns	ns	ns
Fimbrial adherence determinants						
Fim (*Salmonella*)	*fimZ*	ns	ns	ns	ns	ns

Table 4.4: Effects of sub-MIC of antibiotics on virulence factors identified in *C. perfringens* (*continue*).

Virulence class and factors	Gene	Antibiotic treatment				
		OX	S	CH	E	C
Immune evasion						
Capsule (*Staphylococcus*)	*capM*	ns	ns	ns	ns	ns
	capP	ns	ns	ns	ns	ns
Polysaccharide capsule (*Bacillus*)	*galU*	ns	ns	ns	ns	ns
		↑ 3.6977	ns	ns	ns	ns
Invasion						
Lipoprotein promoting entry protein (*Listeria*)	*lpeA*	ns	ns	ns	ns	ns
Iron uptake						
ABC transporter (*Corynebacterium*)	*fagC*	ns	ns	ns	ns	ns
Heme biosynthesis (*Haemophilus*)	*hemL*	↓ 1.2664	↑ 5.1754	↓ 6.4137	↓ 1.7457	ns
Phagosome arresting						
Nucleoside diphosphate kinase (*Mycobacterium*)	*ndk*	ns	ns	ns	ns	ns
Secretion system						
T6SS-II(*Klebsiella*)	*clpB*	ns	↑ 11.223	↓ 10.0077	↓ 2.5064	↓ 2. 0597
Serum resistance and immune evastion						
LPS(*Francisella*)	*wbtE*	ns	ns	ns	ns	ns
Stress adaptation						
Catalase (*Neisseria*)	*katA*	↓ 2.6622	ns	ns	ns	ns
Surface protein anchoring						
Lipoprotein diacylglyceryl transferase (*Listeria*)	*lgt*	ns	ns	ns	ns	ns

↑: Significantly up-regulated, ↓: Significaly down-regulated, ns: No significant change

OX: Cefoxetin, S: Sulfamethoxazol, CH: Chloramphenicol, E: Erythromycin, C: Clindamycin

4.5. Discussion

In this study, the transcriptomic response of a water-borne *Clostridium perfringens* strain exposed to 5 different antibiotics was investigated. Although antibiotic resistance in *C. perfringens* has been the focus of several studies (Charlebois *et al.*, 2014; Khademi &

Sahebkar, 2019; Sakurai & Oda, 2011), the effects elicited by various antibiotics at sublethal concentrations remain poorly reported on. The results of the RNA-seq data showcased the up- and down-regulated genes involved in antibiotic resistance, virulence factors, as well as alterations in metabolic and stress-related pathways. These responses shed light on how *C. perfringens* is able to survive under antibiotic stress.

The results showed that at a sublethal concentration, chloramphenicol had the greatest effect on the growth kinetics of *C. perfringens*. Chloramphenicol is a broad-spectrum antibiotic that inhibits bacterial growth by targeting the 50S ribosomal subunit and preventing peptide bond formation during protein synthesis (Brook, 2016). This bacteriostatic mode of action disrupts essential protein production, resulting in a slower growth rate and replication of *C. perfringens*. Although antibiotics such as clindamycin and erythromycin also target the 50S ribosomal subunit (Wilson & Cockerill, 1987), they showed a lesser effect on the growth kinetics of *C. perfringens* than chloramphenicol. The bacterial growth was also affected by cefoxitin, a second-generation cephalosporin (β-lactam antibiotic). This effect is due to its ability to inhibit cell wall synthesis by binding to penicillin-binding proteins (PBPs). Of all the antibiotic treatments used in this study, sulfamethoxazole affected the growth of *C. perfringens* the least. This class of antibiotic (sulfonamide) limits bacterial growth by preventing the synthesis of dihydrofolic acid, which is essential for the production of DNA and proteins (Tačić *et al.*, 2017).

Several resistance genes were identified in *C. perfringens* that encode for beta-lactamase resistance (*blaC, blaZ, TEM-181, mrcA* and *mecA*). Among these genes, the *mrcA* gene encoding for penicillin-binding protein 1A (PBP1) exhibited significant up-regulation in cells treated with cefoxitin and sulfamethoxazole. PBP1 plays an important role in *C. perfringens* resistance to cefoxitin and sulfamethoxazole, highlighting its significance in maintaining cell wall integrity under antibiotic stress. Cefoxitin, a β-lactam cephalosporin, targets PBPs by inhibiting their transpeptidation and transglycosylation activities, both of which are essential for the biosynthesis of peptidoglycan (Sauvage & Terrak, 2016). This inhibition compromises the bacterial cell wall, leading to lysis of the cell (Livermore, 1995). In response, *C. perfringens* up-regulates *mrcA*, increasing PBP1 expression to mitigate the effects of cefoxitin. Similarly, sulfamethoxazole, a sulfonamide that targets the folate biosynthesis pathway, indirectly impacts the precursors of peptidoglycan synthesis by inhibiting folate metabolism, which is critical for nucleotide and cell wall component biosynthesis (Yaeger *et al.*, 2023). Thus, the up-regulation of *mrcA* under sulfamethoxazole treatment suggests a compensatory mechanism to sustain cell wall synthesis.

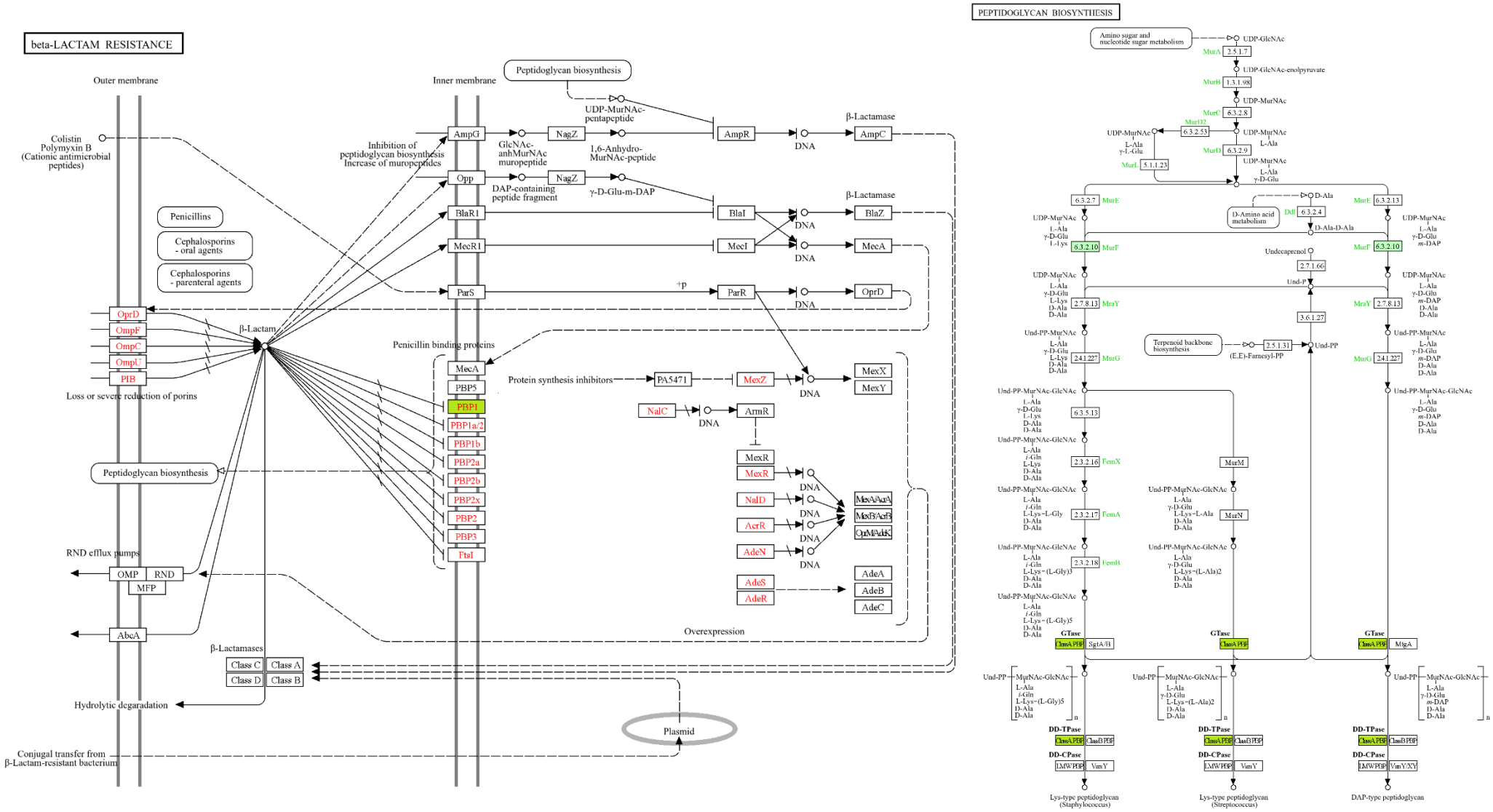


Figure 4.7: KEGG pathways associated with penicillin-binding protein 1A (PBP1) in *Clostridium perfringens* exposed to cefoxitin and sulfamethoxazole. Up-regulated genes are highlighted in green and represent pathway-related precursor proteins in *C. perfringens*. Pathways were reconstructed using KEGG mapper (Kanehisa *et al.*, 2022).

The overexpression of PBP1 acts as a primary resistance mechanism by allowing sufficient enzymatic activity for peptidoglycan biosynthesis even in the presence of antibiotics (Figure 4.7). In cefoxitin-treated cells, this target overproduction reduces the antibiotic's inhibitory efficiency by increasing the availability of PBPs that are not bound to the drug. This adaptation aligns with mechanisms previously observed in other bacterial species, such as *Escherichia coli* and *Staphylococcus aureus*, which utilise increased PBP expression to resist β-lactam antibiotics (Gardete *et al.*, 2004; Hugonnet *et al.*, 2016). Additionally, PBP1 may also contribute to the resistance by altering peptidoglycan structure or composition, thereby reducing antibiotic-binding affinity. Although structural changes were not examined in this study, such adaptations are well-documented in resistant strains (Sethuvel *et al.*, 2023; Van Heijenoort, 2001). The *mrcA* up-regulation may also reflect the activation of cell envelope stress responses, such as two-component regulatory systems, which coordinate the expression of cell wall synthesis and repair genes in response to antibiotic-induced damage (Wilson, 2014). This interconnected regulatory network highlights the role of PBP1 as a central mediator of cell wall adaptation under antibiotic stress.

During the annotation process, multiple genes encoding for resistance against tetracycline were identified (*tet*(44), *tetA*(P) and *tetW*). Although tetracycline was not one of the antibiotic treatments used in this study, the *tetW* resistance gene was expressed differentially in *C. perfringens* when exposed to cefoxitin, sulfamethoxazole and clindamycin. This could indicate a complex adaptive response that extends beyond its traditional association with tetracycline resistance. The *tetW* gene encodes for a ribosomal protection protein that mitigates the effects of tetracyclines by dislodging them from the ribosome (Sionov & Steinberg, 2022). However, its increased expression in response to cefoxitin, a β-lactam antibiotic, and sulfamethoxazole, a sulfonamide, suggests a broader role in bacterial stress responses. The activation of *tetW* under these conditions likely reflects a generalised transcriptional response to antibiotic-induced stress, mediated by global regulatory networks such as cell envelope and metabolic stress responses (Piddock, 2006). The up-regulation of *tetW* may also contribute to cross-resistance, wherein exposure to one antibiotic induces pathways that enhance survival against others. Its expression under cefoxitin and sulfamethoxazole exposure suggests a potential role in stabilising ribosomal activity or supporting translation under stress, resulting in enhanced bacterial resilience (Baym *et al.*, 2016). This phenomenon emphasises the risks of sublethal antibiotic exposure, which can select for the expression of resistance genes even in the absence of their corresponding antibiotic target.

Of all the antibiotic treatments, clindamycin was shown to affect gene expression the least. Although several resistance genes were identified in *C. perfringens*, the up-regulation of the *mprF* and *vmlR* genes shows the adaptive mechanisms that enhance the survival of *C.*

perfringens under this antibiotic stress. Clindamycin is a lincosamide antibiotic that inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, disrupting peptide elongation and leading to bacteriostatic effects (Wilson, 2014). However, the up-regulation of these genes suggests that *C. perfringens* employs both membrane remodelling and ribosomal protection strategies to counteract clindamycin-induced stress.

The *mprF* gene encodes for phosphatidylglycerol lysyltransferase, an enzyme that modifies membrane phospholipids by adding lysine to phosphatidylglycerol, resulting in lysyl-phosphatidylglycerol (L-PG) (Figure 4.8). This modification reduces the negative charge of the bacterial membrane, decreasing the binding affinity of cationic antimicrobial peptides and other positively charged molecules such as antibiotics (Ernst & Peschel, 2011). The up-regulation of *mprF* in response to clindamycin suggests that membrane remodelling is a key defence mechanism in *C. perfringens*. By altering the membrane charge, the bacteria may reduce the interaction of clindamycin with the cell membrane, indirectly limiting its access to intracellular targets. Furthermore, membrane modifications can enhance resistance to immune defences, contributing to bacterial survival during host-pathogen interactions (Hurdle *et al.*, 2011).

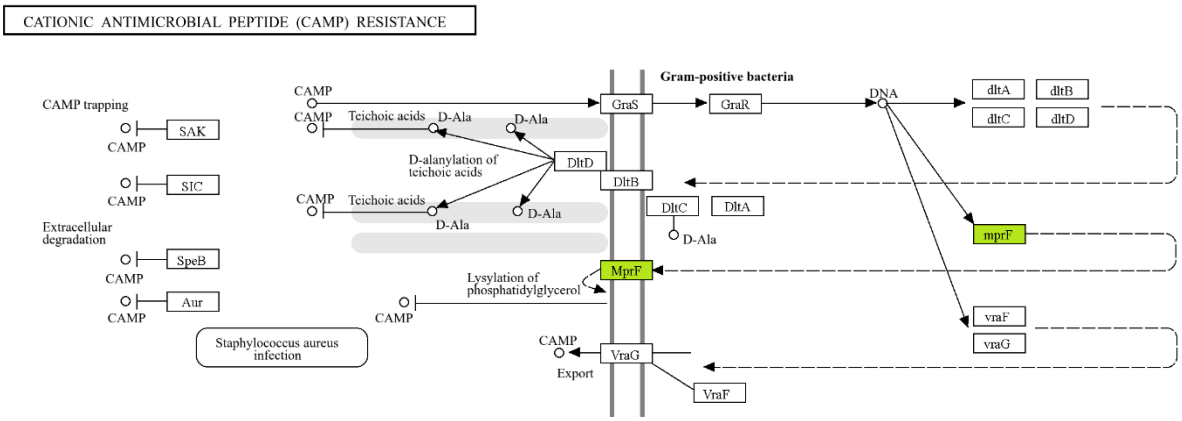


Figure 4.8: KEGG pathway associated with cationic antimicrobial peptides (CAMP) resistance in *Clostridium perfringens* exposed to clindamycin. Up-regulated genes are highlighted in green and represent pathway-related precursor proteins in *C. perfringens*. Pathways were reconstructed using KEGG mapper (Kanehisa *et al.*, 2022).

The *vmlR* gene encodes for a ribosomal protection protein that belongs to the ABC-F subfamily of ATP-binding cassette transporters. These proteins protect the ribosome by dislodging antibiotics from their binding sites or altering ribosomal conformation to prevent antibiotic binding, thus preserving translational activity (Sharkey *et al.*, 2016). The up-regulation of *vmlR* in clindamycin-treated cells directly counteracts the antibiotic's primary mode of action. By maintaining protein synthesis, *C. perfringens* can sustain vital cellular

functions and repair damage caused by antibiotic stress. Similar ribosomal protection mechanisms have been documented in other pathogens, including *Staphylococcus aureus*, highlighting the widespread importance of the ABC-F subfamily in antibiotic resistance (Brdová *et al.*, 2024). The simultaneous up-regulation of *mprF* and *vmlR* could also suggest a coordinated adaptive response that combines membrane remodelling with ribosomal protection, resulting in a dual defence strategy. This synergistic response likely provides a significant survival advantage to *C. perfringens* under clindamycin pressure.

Sublethal concentrations of antibiotics significantly influence the expression of virulence genes in *C. perfringens*, showcasing its ability to adapt to environmental stressors while potentially altering its pathogenic profile. The antibiotics tested elicited distinct transcriptional responses among virulence-related genes. For adherence-related genes, fibronectin-binding protein (*fbpA*) showed no significant changes across treatments, suggesting that adherence mechanisms crucial for host colonisation are maintained under antibiotic stress. This stability aligns with previous studies indicating that *C. perfringens* preserves its adhesion capacity even under adverse conditions (Llanco *et al.*, 2017). Stress-response genes essential for protein folding, such as *groEL*, displayed variable expression depending on the antibiotic used. Cefoxitin and chloramphenicol significantly down-regulated *groEL*, indicating suppressed stress response activation. Conversely, sulfamethoxazole induced up-regulation, likely reflecting increased protein-folding demands caused by metabolic disruptions from inhibited folate biosynthesis (Lin *et al.*, 2008). This observation aligns with studies on *C. perfringens* demonstrating that metabolic inhibitors often up-regulate stress response pathways to counteract intracellular damage (Abo-Remela & Shimizu, 2012; Camargo *et al.*, 2024). Cell wall-related genes, including *altE* (encoding bifunctional autolysin), showed contrasting responses. Sublethal cefoxitin and chloramphenicol exposure significantly down-regulated *altE*, suggesting a reduced need for autolysin activity when peptidoglycan synthesis is inhibited. Conversely, sulfamethoxazole up-regulated *altE*, possibly due to metabolic stress driving cell wall remodelling to adapt to environmental challenges. Previous research on *Staphylococcus aureus* has shown that autolysin activity is tightly regulated and influenced by environmental stresses, including antibiotic exposure (Memmi *et al.*, 2012). Toxin-related genes exhibited notable responses under ribosome-targeting antibiotics. Clindamycin exposure up-regulated toxin-associated genes, potentially through stress-induced pathways that enhance toxin production, a phenomenon similarly reported in studies of *C. perfringens* where stress conditions increased toxin gene expression (Gohari *et al.*, 2021; Stevens *et al.*, 1987). Erythromycin elicited a similar but less pronounced effect, suggesting a conserved response mechanism to ribosomal inhibition.

These findings are consistent with broader studies on *C. perfringens* that illustrate how environmental stressors, including antibiotics, influence its transcriptional landscape. For example, Shojadoost *et al.* (2012) demonstrated that antibiotic-induced stress alters virulence factor regulation, enabling *C. perfringens* to maintain its pathogenicity. The differential modulation of virulence genes observed in this study highlights the dual role of antibiotics in selecting for resistance and reshaping bacteria's pathogenic potential. Sublethal antibiotic concentrations, frequently present in agricultural or environmental settings, can promote survival and potentially enhance virulence through up-regulation of genes such as *altE* and *groEL* (Andersson & Hughes, 2014; Cycoń *et al.*, 2019). These conditions may also facilitate horizontal gene transfer, further disseminating resistance and virulence determinants (Blázquez *et al.*, 2012). Understanding these transcriptional adaptations is crucial for assessing the risks posed by low-dose antibiotic exposure in both clinical and environmental contexts.

4.6. Conclusion

This study investigated the transcriptomic response of an environmentally obtained *Clostridium perfringens* strain to sub-inhibitory concentrations of antibiotics. The findings revealed that antibiotics at these concentrations induce significant physiological and molecular changes in *C. perfringens*, including alterations in growth kinetics, resistance mechanisms, and virulence factor expression. Chloramphenicol was found to have the greatest impact on growth, extending the lag phase and reducing growth rates, while sulfamethoxazole showed minimal effects. Cefoxitin elicited the most pronounced transcriptomic changes, including up-regulation of stress and resistance-associated pathways. The study also identified key resistance mechanisms such as the up-regulation of *mprF* and *vmlR* under clindamycin stress, indicating membrane remodelling and ribosomal protection strategies. Interestingly, the *tetW* gene, associated with tetracycline resistance, was up-regulated in response to other antibiotics, highlighting a generalized adaptive response. Sub-lethal antibiotic exposure also modulated virulence-related genes, including those involved in toxin production and stress adaptation, potentially enhancing bacterial pathogenicity. Metabolic pathway analysis indicated that energy-intensive processes were suppressed, while DNA replication and repair pathways were up-regulated, suggesting a survival-focused adaptive strategy. These findings highlight the risks associated with sub-inhibitory antibiotic concentrations in environmental settings, which not only promote resistance but may also enhance bacterial virulence. This

study highlights the need for careful management of antibiotic use to prevent the evolution and spread of resistant, highly adaptable bacterial strains.

CHAPTER 5: THE EFFECTS OF IRRIGATION ON THE SURVIVAL OF *CLOSTRIDIUM SPOROGENES* IN THE PHYLLOSPHERE AND SOIL ENVIRONMENTS OF LETTUCE

5.1 Abstract

This study aimed to address the gap in knowledge regarding the fate of foodborne pathogens within agroecosystems. It specifically focused on the surrogate microorganism *Clostridium sporogenes*, which was introduced into lettuce-producing environments via surface and spray irrigation methods, respectively. The concentration of *C. sporogenes* in the rhizosphere, phyllosphere, and non-rhizosphere soil was quantified by quantitative polymerase chain reaction (qPCR) over a 42-day trial. The surface irrigation method exhibited a more noticeable contamination effect on the soil environments, compared to the phyllosphere. The results indicated a noticeable increase in *C. sporogenes* concentrations during the initial 22 days, with a 10.4-fold rise (0.39 to 4.05 log copy numbers/g soil) in the rhizosphere and 1.9-fold increase (2.97 to 5.59 log copy numbers/g soil) in the non-rhizosphere. However, concentrations in both soil environments subsequently decreased, falling below the initial inoculum concentration by the end of the trial. In contrast, the spray irrigation method resulted in most of the contamination being localised on the lettuce phyllosphere, with a high *C. sporogenes* concentration of 9.09 log copy numbers/g leaves on day zero. This concentration exponentially decreased to a minimal 0.019 log copy numbers/g leaves by day 32. Although concentrations in both soil environments decreased over time, trace concentrations of *C. sporogenes* were detectable at the end of the trial, posing a potential hazard to the microbiological safety of post-harvest produce. These findings shed light on the dynamics of *C. sporogenes* in agroecosystems and highlights the importance of irrigation practices that ensure the safety of those who consume fresh produce.

Keywords**:** *Clostridium sporogenes***,** irrigation**,** phyllosphere, soil environments, qPCR

5.2 Introduction

The consumption of fresh produce has increased over the past few decades due to its accompanying health and nutritional benefits (Alam *et al.*, 2014; Machado-Moreira *et al.*, 2019). This has led to the need for mass distribution within the shortest amount of time to meet this growing demand and, as a result, consumers are increasingly exposed to foodborne diseases (Balali *et al.*, 2020). Fresh, ready-to-eat (RTE) produce is likely to be contaminated with disease-causing bacteria through direct contact with soil or animal manure, or irrigation with contaminated water (Gurtler & Gibson, 2022). Wastewater or sewage-polluted surface water is used to irrigate about 10% of the world's crops (Manshadi *et al*., 2013). This is of concern, since most foodborne pathogens originate from the intestinal tract and faecal material of mammals (Hamza *et al.*, 2018). The anaerobic pathogens *Clostridium botulinum* and *Clostridium perfringens* are examples of bacterial hazards; they have environmental and intestinal origins (Rai & Tripathi, 2007). These pathogens impact public health and the economy through their ability to produce several deadly toxins and spores that are highly resistant to environmental stressors, which contributes to their survival, pathogenicity, and transmission (Alegbeleye *et al.*, 2018; Hoffmann *et al.*, 2015; Li *et al.*, 2016; Palmer *et al.*, 2019).

Since approximately 70% of the world's freshwater is used for irrigation, the quality of these water sources is of great importance (Scanlon *et al.*, 2007). In South Africa, routine testing for *Clostridium* pathogens in water sources used for irrigation is not an established practice. A previous study conducted by the authors reported high levels of *Clostridium* pathogens in several surface water systems used for irrigation in the North West Province (Fourie, 2017). Investigation of the genomic features of the pathogenic *C. perfringens* found in these systems showed multidrug resistance, enhanced virulence factors, and environmental adaptation through mobile genetic elements (Fourie *et al.*, 2020). Based on these hazards, the present study focuses on the potential spread of *Clostridium* in the food chain. It is hypothesised that when plants are irrigated with contaminated water, *Clostridium* species can establish and survive in RTE vegetables, such as lettuce, and are able to become endophytic. However, field studies that involve pathogenic *Clostridium* are very restricted due to environmental health and safety concerns (Park *et al.*, 2018). Therefore, to assess how irrigation water transmits *Clostridium* in the preharvest environment of lettuce, a surrogate species, namely *Clostridium sporogenes*, was used in a greenhouse setting. To investigate its survival in the rhizosphere, phyllosphere, and non-rhizosphere soil of lettuce, the concentrations of *C. sporogenes* were determined by using a qPCR assay.

5.3 Materials and methods

5.3.1 Experimental layout

A design plan (Figure S5.1) for the greenhouse trial was followed, which comprised three different treatments. Lettuce seedlings were transplanted into pots containing sterilised soil. Treatment 1 and 2 inoculated *C. sporogenes* into the lettuce-producing environment through simulating surface and spray irrigation, respectively. Treatment 3 served as a control treatment, with no *C. sporogenes* present in irrigation water. Twenty-five replicate pots were used for each treatment. Pots were grouped by treatments to avoid cross-contamination. The trial was conducted at Eco-Rehab in Potchefstroom, South Africa. The biosafety level 1 (BSL-1) greenhouse is equipped with temperature control, LED grow lights, and HEPA filters (Figure S5.2). The greenhouse maintained an average temperature of 26 °C, with a 16:8 h light–dark cycle for the duration of the experiment.

5.3.2 Bacterial strain, growth conditions, and inoculum preparation

For this study, the *C. sporogenes* ATCC 3584 strain was purchased from Microbiologics (Minnesota, USA). The inoculum was firstly grown anaerobically on cooked meat medium (Oxoid, UK) at 37 °C for 24 h to confirm culture purity. Spores of *C. sporogenes* were used as inoculum and were harvested through a modified method described by Rabi *et al* (2017). In short, a single colony was used to inoculate brain heart infusion (BHI) broth (Oxoid, UK) and incubated using a rotary shaker at 100 rpm at 35 °C under anaerobic atmosphere conditions for 48 h. Endospore staining was then performed to determine spore maturation. The spores that were released from the mother cells were collected by centrifugation (4 000 rpm, 15 min); after that, they were washed three times with 50 mM phosphate-buffered saline (PBS, pH 7.3). After each wash, vegetative cells and other cellular debris were removed from the top of the spore pellet to obtain a pure spore suspension. The concentration of spores was estimated by spore staining, followed by direct microscopic count (DMC) (~ 3×10^{11} CFU/ml) and then stored at 4 °C until they were used for inoculation.

5.3.3 Soil collection, lettuce preparation, and sterilisation

Agricultural soil was collected from the Agricultural Research Council: Grain and Crop Institute in Potchefstroom, South Africa. The soil composition was determined by the particle size distribution of the soil, as described by Gerber *et al.* (2015); the results showed that the soil consisted of 39% sand, 30.4% silt, and 29% clay. Based on its composition, the soil was

classified as loamy clay soil (Figure S5.2). The soil was then sterilised through autoclaving it twice (121 °C, 30 min). Seedlings of 'Great Lakes' lettuce (*Lactuca sativa* 'Great Lakes') were purchased from a local nursery in Potchefstroom, South Africa. The soil surrounding the roots of the seedlings was removed, washed with sodium hypochlorite (NaOCl 10%; v/v), and rinsed twice with distilled H_2O. The sterilised soil, as well as the leaves and roots of the lettuce, were then assessed by conventional PCR methods to ensure the absence of *C. sporogenes.*

5.3.4 Irrigation of soil and lettuce with *C. sporogenes*

Each lettuce seedling was transplanted into a separate pot (23 cm diameter) that contained 2 kg of sterilised soil. Three different treatments followed, each in separate areas to prevent any cross-contamination. Treatment 1 simulated surface irrigation: the soil was carefully covered with 100 ml of distilled water containing *C. sporogenes*, limiting any contact between the water and the leaves of the lettuce seedling. Treatment 2 simulated spray irrigation: the leaves were sprayed with a hand-held spray bottle containing 100 ml of distilled water and *C. sporogenes.* Treatment 3 served as the negative control group: 100 ml of distilled water was used to irrigate the soil containing the seedling. The bacterial suspension in irrigation water used for treatments 1 and 2 was measured by using spore staining, followed by DMC and was approximately 9×10^9 CFU/ml for both treatments (Girardin *et al.*, 2005). Each pot used throughout the trail was placed onto a plastic tray and was given bottom watering with distilled water to ensure the soil and lettuce plants were not disturbed during the trial. Distilled water was collected from the laboratory; the pots were watered daily for the first 7 days; thereafter, they were watered every 3 days or when the soil surfaces began to dry. A half-strength Hoagland's solution was also given weekly via bottom watering (Hoagland & Arnon, 1950).

5.3.5 Sample collection

Rhizosphere, non-rhizosphere soil, and phyllosphere samples were aseptically collected from each treatment on days 0, 9, 22, 31, and 42. In order to collect phyllosphere samples, the edible leaves of lettuce were removed by cutting off the whole head of the lettuce with a sterile blade and placing them in sterile plastic bags. Rhizosphere soil was collected by removing the lettuce plant from the soil, gently shaking it, and collecting the soil from the roots. Non-rhizosphere soil was obtained with a sterile spoon. The samples were collected at 7 cm away from the lettuce and 5 cm below the soil surface. All soil samples were collected in separate 50 ml sterile falcon tubes. All samples were immediately transported to the laboratory for further processing and analysis.

5.3.6 Sample preparation, DNA extraction, and qPCR

For the phyllosphere samples, the outermost leaves of each lettuce head were removed and discarded. Five leaves were then chosen at random from the remaining lettuce head layers. These were aseptically ground with a pestle and mortar to ensure more uniform leaf samples across all treatments. Total DNA was then extracted from 1 g of the leaf homogenate by using the DNeasy Plant Pro Kit (Qiagen, DE) and following the manufacturer's instructions. For the soil samples, each collected sample was aseptically sieved (3 mm) to ensure thorough homogenisation before the DNA extraction. The total DNA was then extracted from 1 g of soil using the NucleoSpin® Soil kit (Macherey-Nagel, GE), also following the manufacturer's instructions. A NanoDrop spectrophotometer and a 1% agarose gel electrophoresis were used to evaluate the concentrations and integrity of the DNA extracted from both leaf and soil samples (Thermo Scientific, USA). All DNA samples were stored at -20 °C for downstream analysis.

Absolute quantifications were done by qPCR assay, using the QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) with SYBR® Green fluorescence reagent. Previously described *C. sporogenes* primers were chosen for the detection and quantification of a 96 bp fragment of the *ger*AA gene (Morandi *et al.*, 2015). The primer sequences are as follows: *ger*AA-F CCG CAG GAA TAA ACA ATG TTC TAA and *ger*AA-R CAG CAT AAG CAG CCC CTA AAA. All reactions were performed in a final volume of 25 µl and consisted of 10 µl of 2 × PowerUp™ SYBR Green Master Mix (Applied Biosystems, USA), 1 µl of each primer (0.4 µM each), 11 ul of Nuclease-Free Water and 2 µl of gDNA. The Green Master Mix comprised SYBR Green I dye Dual-Lock Taq DNA Polymerase, dNTPs mix with dUTP/dTTP, heat-labile UDG, passive reference dye ROX, and buffer components. The reactions were carried out in 96-well plates sealed with film. The thermal cycling conditions that were used are as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. All runs included a non-template negative control and *C. sporogenes* ATCC 3584 as the positive control.

5.3.7 Standard curve construction

To construct the standard curve, genomic DNA from a pure culture of *C. sporogenes* (37 °C, 24 h) was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA). This was followed by qPCR amplification of 10-fold serial dilutions of the pure gDNA, with concentrations ranging from 10 ng to 1×10^{-5} ng of gDNA. The data was initially analysed with QuantStudio™ Design and Analysis Software (Version 1.3.1) at a threshold determination of 0.03. Threshold cycle (Ct) values were plotted against the corresponding concentration of

each DNA dilution in order to assess the linear range of detection and reliability of the qPCR assay. The standard curve was calculated as y = −ax + b (a refers to the standard curve slope and b refers to the y-intercept). The amplification efficiency of the reaction (E) was calculated as $E = (10^{-1/a})$, and the percent efficiency was evaluated as (E − 1) × 100%. According to Pfaffl and Bustin (2004) and Rutledge and Côté (2003), amplification efficiencies of standard curves between 90% and 110%, which correspond to a slope between −3.1 and −3.6, and coefficient of determination (R^2) > 0.98 are considered to be reliable comparison trend lines for the quantification of unknown samples. For all the analyses, three technical replicates of each sample were performed.

5.3.8 Data analysis

All qPCR data was initially analysed with QuantStudio™ Design and Analysis Software (Version 1.3.1), where the Ct values were exported into Microsoft Excel Worksheet for further statistical analysis. An Analysis of variance (ANOVA) was conducted to determine statistically significant differences within and between the two irrigation methods (surface and spray irrigation) on *C. sporogenes* copy numbers/g of soil or leaves. A p-value of 0.05 for the ANOVA analysis was considered as significant.

5.4 Results

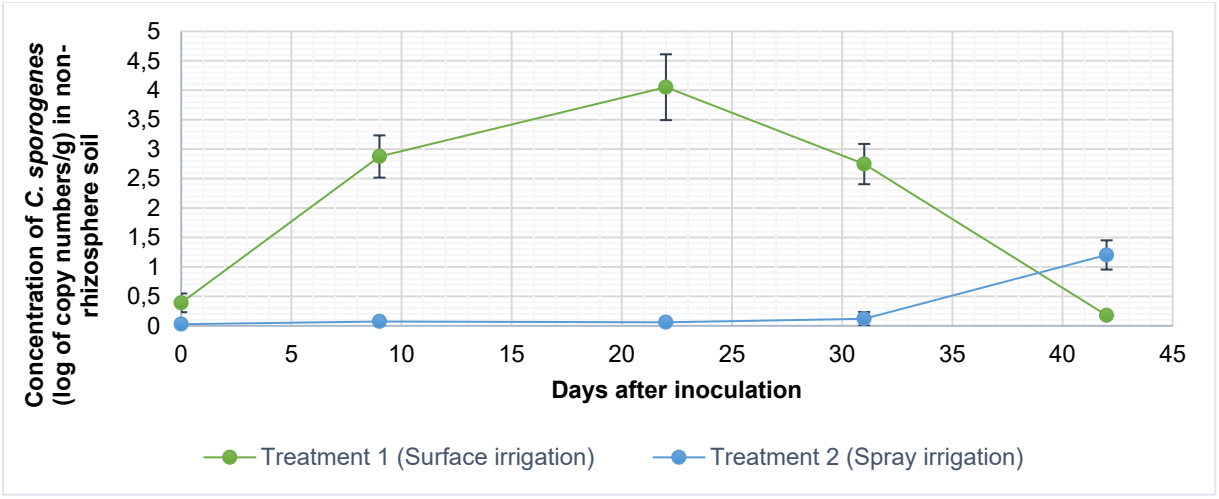
5.4.1 Sensitivity, standard curve, and amplification efficiency of qPCR assay

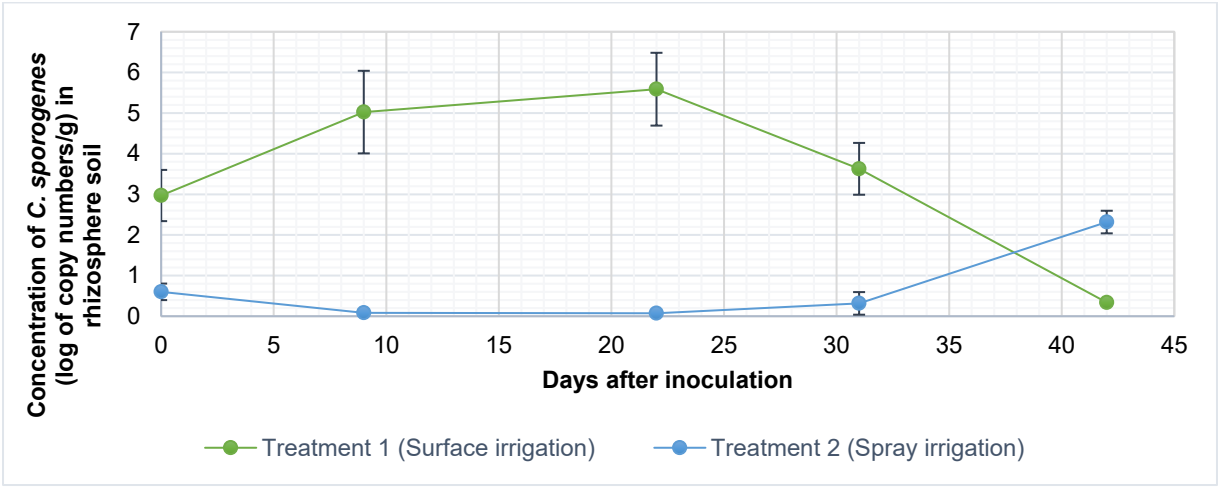
To detect and quantify *C. sporogenes* in environmental samples, a qPCR assay was utilised. The performance and sensitivity of the assay (Figure S5.4) were evaluated by amplifying the *ger*AA gene in a 10-fold serial dilution of pure gDNA at known concentrations (10 ng – 1×10^{-5} ng). The amplification curve showed good reproducibility, and the fluorescence intensity changed consistently with the serial dilution of the gDNA concentration. The results of the constructed standard curve showed a strong linear relationship between the Ct values and the known DNA concentrations over 7 orders of magnitude. The coefficient of determination (R^2) was high at 0.996, indicating a good fit of the data points on the standard curve. The slope of the log-linear phase was −3.51. The Y-intercept was 25.706, and the amplification efficiency (E) was estimated to be 93.1%. According to the melting curve, the 96 bp amplicons that were generated were unimodal and showed a single dissociation peak at 72.1 ± 0.09 °C, indicating

that the primers used in this study exhibited adequate specificity. Additionally, no melting curve was detected in the negative controls.

5.4.2 Survival of *C. sporogenes* in the soil, rhizosphere, and phyllosphere of lettuce

Based on the constructed standard curve (Figure S5.4), the qPCR assay was able to detect and quantify *C. sporogenes* in the non-rhizosphere soil, rhizosphere, and phyllosphere of lettuce after being contaminated with surface (T1) and spray (T2) irrigation over the 42-day duration of this study. *Clostridium sporogenes* was immediately detectable in leaves and soil samples at various concentrations after the inoculated water was applied. The initial concentrations of 2.97 and 0.6 log copy numbers/g soil were reported in non-rhizosphere soil (Figure 5.1A), 0.39 and 0.03 log copy numbers/g soil in rhizosphere soil (Figure 5.1B), and 0.02 and 9.09 log copy numbers/g leaves (Figure 5.1C) after surface and spray irrigation, respectively. However, the DNA extracted from the soil and leaf samples from the control treatment (T3) showed no amplification of the *ger*AA gene at any of the sampling days, which was expected.





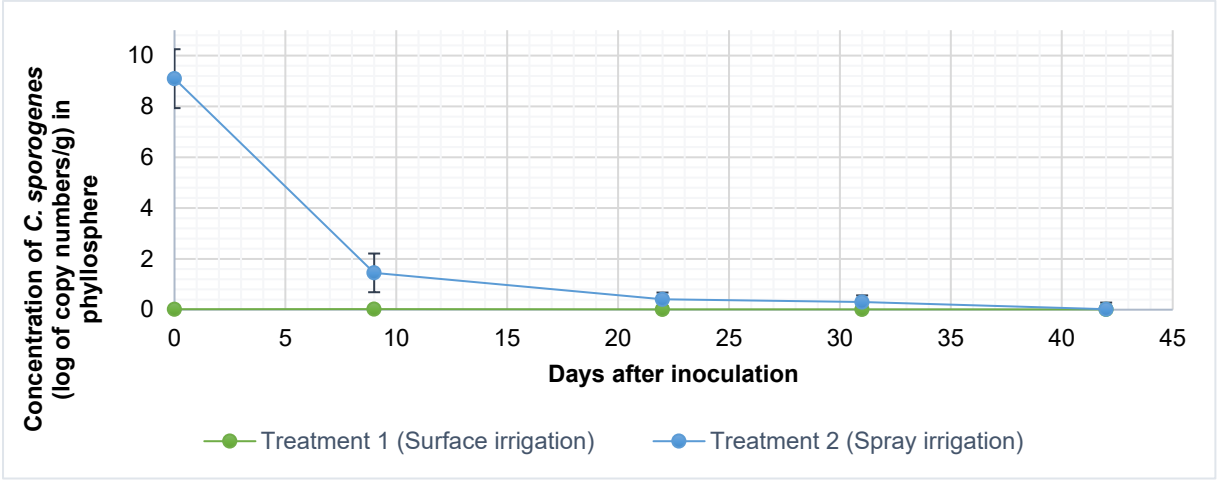


Figure 5.1: Survival of *C. sporogenes* ATCC 3584 in non-rhizosphere soil **(A)**, rhizosphere **(B),** and phyllosphere **(C)** of lettuce over 42 days following two different irrigation treatments. Treatment 1 introduced *C. sporogenes* into the lettuce-producing environments via surface irrigation (Green); whereas Treatment 2 did so by spray irrigation (Blue). Both treatment methods administered a single 100 ml dose of *C. sporogenes* (9×10^9 CFU/ml) on day 0. Afterwards, *Clostridium sporogenes* concentrations were determined in each of the three lettuce environments on different days (0, 9, 22, 31, and 42 days) by means of qPCR; these are reported as log copy numbers/g sample. The results are the means and standard deviation of five replicates.

The detectable amount of *C. sporogenes* in the non-rhizosphere over the 42 days using qPCR (Figure 5.1A) was significantly different between the two irrigation treatments (Table 5.1). For treatment 1, the concentration of *C. sporogenes* showed an increase, reaching a peak of 5.59 log copy numbers/g soil on day 22. However, the concentration gradually decreased to below the initial concentration (2,98 log copy numbers/g soil) at day 42 (0.34 log copy numbers/g soil). The opposite trend was observed for treatment 2, where, over the first 9 days, the concentration decreased from 0.6 to 0.08 log copy numbers/g soil. Following this, the *C. sporogenes* concentrations remained relatively consistent, only showing a 7.25-fold increase from day 31 (0.32 log copy numbers/g soil) to day 42 (2.32 log copy numbers/g soil).

When compared, the survival of *C. sporogenes* in the rhizosphere soil (Figure 5.1B) followed a similar pattern to that of the above-mentioned non-rhizosphere soil (Figure 5.1A). Different trends were observed in the rhizosphere soil of the two irrigation methods. The concentration of *C. sporogenes* in the rhizosphere soil of lettuce following treatment 1 showed an initial 10.4-fold increase from day 0 to 22 (0.39 to 4.05 log copy numbers/g soil) and then a decrease in concentration at day 31 and 42 (2.75 and 0.18 log copy numbers/g soil, respectively). The prevalence of *C. sporogenes* following treatment 2 remained consistently low in the rhizosphere soil for the first 3 weeks of the study, with concentrations of 0.03 to 0.06 log copy numbers/g soil for days 0 to 22, respectively. However, the concentration increased 10-fold during the last 2 weeks of the study, reaching a high of 1.2 log copy numbers/g soil at day 42.

The initial concentration of *C. sporogenes* in the phyllosphere samples (Figure 5.3C) varied greatly between the two irrigation methods: the spray irrigation method used in treatment 2 resulted in a much higher detectable amount on the leaves (9,09 log copy numbers/g leaves) than that of the surface irrigation used in treatment 1 (0,018 log copy numbers/g leaves). However, in treatment 2, the concentrations drastically changed from 9.09 to 1.45 log copy numbers/g leaves during the first 9 days, resulting in a 6.3-fold decrease. The concentration of *C. sporogenes* then continued to gradually decrease until day 42, where only 0.019 log copy numbers/g leaves remained. Additionally, low concentrations of *C. sporogenes* were also detected on the phyllosphere of lettuce following surface irrigation, ranging between 0.012 and 0.02 log copy numbers/g leaves throughout the 42-day trial.

Table 5.1 shows the statistically significant differences between the treatments and lettuce environments. Treatment 1 was significantly different from treatment 2 in both soil environments over the 42-day trial. However, when comparing treatments 1 and 2, the phyllosphere showed initial significant differences up to day 31; whereas there was no significant difference between the two treatments, as both concentrations reduced to 0,01 log copy numbers/g leaves at day 42. Furthermore, statistical differences were observed over the entire duration of the trial among all three lettuce environments from treatment 1. Similar observations were made within treatment 2, except for day 31, with no statistical differences among the concentrations in the non-rhizosphere soil, rhizosphere, and phyllosphere. A two-way ANOVA found that the concentrations in all three environments for both treatments were statistically significant throughout the trial. This means that the method of irrigation (surface or spray irrigation) influenced the transport and survival of *C. sporogenes* in the non-rhizosphere soil, rhizosphere soil, and phyllosphere of lettuce.

Table 5.1: Significant differences between and within treatments 1 and 2 for *Clostridium sporogenes* concentration (log copy numbers /g) in the soil environments and phyllosphere of lettuce over a 42-day trial period.

One-way ANOVA			**Day**		
Between treatments	**0**	**9**	**22**	**31**	**42**
T1-P vs T2-P	<0.001*	0.005*	0.019*	0.009*	0.976
T1-R vs T2-R	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
T1-S vs T2-S	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Within treatment 1					
T1-P vs T1-S	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
T1-P vs T1-R	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
T1-R vs T1-S	<0.001*	<0.001*	0.025*	0.021*	<0.001*
Within treatment 2					
T2-P vs T2-S	<0.001*	0.006*	0.041*	0.970	<0.001*
T2-P vs T2-R	<0.001*	0.005*	0.035*	0.109	<0.001*
T2-R vs T2-S	<0.001*	0.066	0.009*	0.184	<0.001*
Two-way ANOVA					
T1-T2-P vs T1-T2-S	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
T1-T2-P vs T1-T2-R	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
T1-T2-R vs T1-T2-S	<0.001*	<0.001*	0.015*	0.074	<0.001*
T1-P-S-R vs T2-P-S-R	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

* Means they are significantly different from each other ($p < 0.05$).

T1: Treatment 1 (Surface irrigation), **T2**: Treatment 2 (Spray irrigation). **P**: Phyllosphere of lettuce. **S**: Non-rhizosphere soil, **R**: Rhizosphere soil.

5.5 Discussion

Many of the studies that investigated the microbial contamination of vegetable crops adopted a spiking approach to illustrate the potential hazard associated with the consumption of raw vegetables. In such instance, harvested vegetables are spiked with a known concentration of microbial contaminants. This approach does not yield evidence of the potential endophytic attributes of such contaminants, which may confirm their abilities to colonise and form symbiotic partnerships with the host plant. Therefore, for this study, the researchers irrigated the lettuce plants with contaminated water; this method allowed the researchers to monitor the development of the plant–microbe interaction over a period of time and establish the colonisation of the lettuce by the surrogate indicator organism *C. sporogenes*. Furthermore, the researchers were able to investigate the fate of the *Clostridium* in the rhizosphere, phyllosphere, and non-rhizosphere soil, following surface and spray irrigation in a greenhouse setting. A culture-independent approach, using qPCR, was used to determine the survival of *C. sporogenes* in these three environments. Furthermore, the qPCR technique enabled the detection and quantification of the *ger*AA gene present in *C. sporogenes* in all three of the investigation areas. The use of qPCR has been shown to successfully quantify *Clostridium* species in processed vegetables, milk, and dairy products (Chon *et al.*, 2012; Morandi *et al.*, 2015; Sahiner *et al.*, 2022), with great sensitivity and accuracy (MacDougall *et al.*, 2018). The current study successfully achieved a comprehensive quantification of *C. sporogenes* by exclusively using DNA extracted from agriculturally intricate niches, which are recognised for their complexity and susceptibility to diverse biotic and abiotic influences (Wydro, 2022). This methodology facilitated an in-depth exploration into the fate of *Clostridium* within these three environments subsequent to a simulated contamination event induced by irrigation.

Different irrigation methods can greatly affect the degree of microbial contamination on receiving produce. Several studies report that the probability of contaminating the foliage of produce is increased by using overhead sprinkler irrigation (Ganeshan, 2015; Iwu & Okoh, 2019; Kisluk & Yaron, 2012). These studies also suggest that the use of irrigation methods that limit the contact between the water and the produce phyllosphere may decrease the risk of contamination, examples include surface irrigation. A possible explanation for the *C. sporogenes* on the phyllosphere samples that received surface irrigation in this study could be the backsplash of contaminated water or contact with soil during the initial irrigation treatment (Ibekwe *et al.*, 2009). Alternatively, endophytic colonisation of fodder plants with other *Clostridium* species, such as *C. botulinum,* has been reported. However, further research is needed regarding this occurrence. *Clostridium* proliferates in environments such as soil, but little is known about its role as endophytic or plant-associated bacteria (Zeiller *et*

al., 2015). The results of the current study also show an initial high level of *C. sporogenes* detected on the phyllosphere of lettuce after spray irrigation. However, these levels show a drastic decrease over time. A similar study by Ercolani (1997) also report a decline in levels of *C. pasteurianum*, *C. perfringens,* and *C. sporogenes* in the leaves of tomato and basil plants after spray inoculation. This is because spray irrigation covers the crop and the soil with water. The majority of the water that contained *C. sporogenes* was retained in the phyllosphere, resulting in a lesser count of bacteria being introduced directly into the soil through droplets.

It is important to state that the aerial portion of a plant is a harsh and unstable environment for most microorganisms (Chaudhry *et al.*, 2021), especially for obligate anaerobes, such as *Clostridium* species. It is, therefore, expected that *C. sporogenes* on leaves would have a higher death than growth rate. The survival and, subsequently, the colonisation of microorganisms on produce are influenced by direct exposure to multiple environmental factors, such as oxygen, temperature, water availability, and UV radiation, as is the case with endophytes (Alegbeleye *et al.*, 2022; Chaudhry *et al.*, 2021). The nutrient sources on leaves are also sparse in comparison to other environments, such as the rhizosphere soil or the enteric environment of mammals (Chaudhry *et al.*, 2021; Leveau & Lindow, 2001). Although the spores produced by *C. sporogenes* are tolerant to many of these environmental stressors, studies have shown that the presence of oxygen and the lack of essential nutrients, such as L-alanine, greatly affect the germination success of *Clostridium* spores (Fujioka & Frank, 1966; Wang *et al.*, 2017). Although *C. sporogenes* was unable to colonise the phyllosphere of lettuce successfully through the two irrigation methods in this study, it was still detected in low levels after 42 days. If *Clostridium* species and their spores are present on produce during preharvest, it could jeopardise the post-harvest quality and pose a safety hazard for consumers. This is evident in studies that evaluated the microbiological safety of minimally processed RTE vegetables and salads, including spinach, mixed leaf salad, and lettuce that have shown the presence of pathogenic *Clostridium* (Bakri *et al.*, 2009; Eckert *et al.*, 2013).

Regarding the subterranean survival of *C. sporogenes*, it is important to note that even though *Clostridium* species are ubiquitously distributed in soil, it does not imply that they can survive in any soil. Soil structures and soil types greatly affect the prevalence, survival, and movement of bacteria in soil (Palmer *et al.*, 2019). Fine-grained soil, such as the clay loam soil used in this study, has high nutrient values and water-retaining properties that could aid the survival of some enteric pathogens (Jamieson *et al.*, 2002). This is likely the case with *Clostridium* species as well. When pathogens are introduced via water to the topsoil horizon, the water translocates downward through the pore structures of the soil; this disperses bacteria to lower soil horizons, such as the rhizosphere and non-rhizosphere soil (Gessler & Bohnel, 2006). In

the current study, after the water was translocated, the concentrations of *C. sporogenes* in rhizosphere and non-rhizosphere soil showed similar trends in both irrigation treatments. This indicates that the rhizosphere soil environment does not provide better or worse conditions for the proliferation of *C. sporogenes* in comparison with non-rhizosphere soil. Based on the physiology of *Clostridium* species, particularly their nutrient and anaerobic requirements, the soil environments are comparatively less hostile than that of the phyllosphere (Palmer *et al.*, 2019). There is greater availability of organic substrates and lower oxygen levels in the rhizosphere soil than in the phyllosphere, which could account for the survival of *C. sporogenes* in the rhizosphere and surrounding soil. Plant roots modulate anaerobic respiration in soil by consuming the oxygen that is present, thereby, increasing the anaerobic volume in soil and establishing oxygen-free zones (Lecomte *et al.*, 2018). Roots also release more than 25% of their organic matter and exude nutrients, such as carbon sources, at the tips and junction of lateral roots, creating energy-rich micro-pockets/environments where anaerobic bacteria can grow and multiply (Brandl *et al.*, 2004; Jaeger *et al.*, 1999). The aforementioned may be a result of the delayed germination of *C. sporogenes* spores during treatment 2 of this study, which resulted in an increase in the concentration after day 31 of the trial. The *Clostridium* spores initiate germination when they sense the presence of germinants. These germinants are small molecules and may be nutrient signals from the plant roots or the surrounding soil. This enables a signalling cascade that causes the spore membrane and cortex to become degraded, which results in germination (Shen *et al.*, 2019).

When *C. sporogenes* was introduced into the soil through surface irrigation, it resulted in higher concentrations than that of spray irrigation. Surface irrigation causes the water to cover the soil surface completely; the water then siphons through the soil matrix to reach the roots of the plant (Pachepsky *et al.*, 2011). In this study, *C. sporogenes* was detectable in both rhizosphere and non-rhizosphere soil after surface irrigation with contaminated water. Although the concentration of *C. sporogenes* showed an initial increase over the first 22 days in both soil environments, the concentration decreased to below the original inoculation concentration at day 42. The observed trend may be attributed to the use of sterilised agricultural soil. A study done by Garcia and McKay (1969) found a similar growth and survival pattern of *C. septicum* in sterilised soil over 32 days. According to Li *et al.* (2019), the use of sterilised soil results in the recolonisation of a healthier soil microbiome after 6 weeks. Their results suggest that the destruction of the native microbial population brought about by soil sterilisation can rapidly recover due to the microbial activities associated with a plant. In this study, the lack of microbial competition could explain the initial increase of *C. sporogenes* in the soil. However, *C. sporogenes* could not compete with the development of a new, healthier

microbiome seeded by the lettuce endophytes over the weeks and started to decrease in concentration.

5.6 Conclusion

The use of a surrogate microorganism, namely *C. sporogenes*, showed that irrigation water is an important transmission route for *Clostridium,* which enables pathogens to proliferate in neighbouring soil environments. *Clostridium sporogenes* was detectable in the rhizosphere, phyllosphere, and non-rhizosphere soil at every sampling interval over the 42 days, demonstrating the likelihood and persistence of bacterial contamination from irrigation water in these three environments over time. However, the introduction of *C. sporogenes* into the agricultural environment through surface and spray irrigation yielded significantly different results. The application of surface irrigation resulted in much higher *C. sporogenes* contamination in soil environments, whereas contamination from spray irrigation was predominately on the phyllosphere of lettuce. Although *C. sporogenes* was not able to successfully colonise the various soil and plant environments, rhizosphere and non-rhizosphere soil did provide a more favourable environment for it to survive and proliferate. Because this study was conducted in a controlled greenhouse setting, the simulated conditions are not a complete representation of the complex agroecosystem found in open fields. The fate of *Clostridium* can be influenced by external factors, such as weather, UV exposure, and temperature, as well as competition from other microorganisms that are present in these environments. However, the fact that *C. sporogenes* persisted in these three environments highlights the impact that agricultural practices, such as irrigation methods, can have on the spread of foodborne pathogens during preharvest cultivation. Additionally, qPCR has proven to be a useful method for monitoring *C. sporogenes* in various agricultural sources and should be considered for similar future studies. More attention should be given to pathogenic spore-forming bacteria, such as *Clostridium* in agroecosystems.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study comprehensively explores *Clostridium* species, with particular emphasis on *C. perfringens*, within agroecosystems. It investigated their genetic attributes, adaptive responses to environmental pressures, and potential dissemination through agricultural practices. The findings highlight the critical role of water systems as reservoirs and conduits for these pathogens, posing significant environmental and public health threats. The aim of the study was to investigate the genetic characteristics of environmentally obtained *Clostridium* pathogens, their survival in agricultural environments, and the potential associated health risks to humans and animals. These goals were achieved through specific objectives, each addressed by a study chapter.

Objective 1: To investigate antibiotic resistance, virulence factors, and other genomic features in environmentally isolated *C. perfringens* using whole-genome sequencing.

This objective was achieved in Chapter 3, which explores the genomic characteristics of *C. perfringens* isolates from surface water systems. Whole-genome sequencing revealed a rich genetic repertoire, including multiple antibiotic resistance genes (ARGs), virulence factors, and mobile genetic elements. Identified ARGs spanned key antibiotic classes, such as β-lactams, macrolides, and glycopeptides, highlighting the adaptability of these environmental strains and the impact of anthropogenic antibiotic use. Virulence genes encoding toxins like perfringolysin O and phospholipase C emphasised the pathogenic potential of these isolates. The discovery of genomic islands and prophages further suggests active horizontal gene transfer mechanisms that facilitate the dissemination of resistance and virulence traits. These findings demonstrate the dual role of agroecosystems as reservoirs and amplifiers of pathogenic traits and highlight the urgent need for enhanced monitoring and mitigation strategies to curb the spread of these potentially dangerous strains.

Objective 2: To determine the transcriptomic response of multidrug-resistant *C. perfringens* to sublethal concentrations of various antibiotics using RNA-Seq.

This objective was addressed in Chapter 4, which investigated the transcriptomic responses of *C. perfringens* to sublethal antibiotic concentrations, simulating environmental exposure scenarios. The study reveals that even low concentrations of antibiotics, often found in contaminated water systems, can profoundly influence bacterial physiology. Resistance-associated genes were up-regulated, enabling the bacterium to adapt and maintain resilience under stress. Additionally, virulence-related genes involved in toxin production, adhesion, and stress adaptation were modulated, potentially enhancing pathogenicity. The study also observed the down-regulation of energy-intensive metabolic pathways, reflecting a shift toward a survival-oriented physiological state. These findings highlight the ecological risks posed by residual antibiotics in agricultural environments, where sub-MIC exposure fosters resistance, virulence, and adaptability in bacterial populations.

Objective 3: To investigate the survival of *C. sporogenes* in ready-to-eat produce using an experimental greenhouse pot study.

This objective was explored in Chapter 5, which examined the survival and transfer dynamics of *C. sporogenes*, a surrogate for pathogenic *Clostridium* species, in irrigation practices. Contaminated irrigation water was shown to facilitate bacterial persistence in both soil and plant environments. Surface irrigation resulted in significant soil contamination, while spray irrigation primarily impacted the lettuce phyllosphere. Although bacterial concentrations in both environments declined over time, trace amounts persisted, particularly in soil samples, posing potential risks to food safety. These findings highlight the importance of effective irrigation practices and rigorous water quality monitoring to minimise contamination risks and safeguard public health.

Collectively, these findings illustrate the intricate interaction between environmental reservoirs, microbial adaptability, and public health risks. Surface water systems, often contaminated by agricultural runoff and anthropogenic waste, act as reservoirs for antibiotic-resistant and virulent *C. perfringens* strains. Residual antibiotics exacerbate these challenges by promoting bacterial resilience and enhancing pathogenic traits. The use of contaminated water for irrigation facilitates the transfer of these pathogens to crops and soil, creating a direct pathway for human exposure through the food chain. The implications of this research extend beyond agricultural practices, addressing broader ecological and public health concerns.

Strategies such as improved wastewater treatment, stricter regulations on agricultural antibiotic use, and enhanced surveillance systems are essential to mitigate these risks. Furthermore, understanding the long-term ecological impacts of these interactions is critical for developing sustainable agricultural practices that ensure food safety while preserving environmental health.

By combining genomic, transcriptomic, and ecological perspectives, this study provides foundational insights into the behaviour of *Clostridium* species in agroecosystems. It highlights the urgent need for interdisciplinary approaches to address antibiotic resistance and foodborne pathogens. Future research should aim to bridge gaps in knowledge regarding persistence, adaptability, and mitigation of *Clostridium* species in diverse agricultural contexts. These efforts are vital for safeguarding public health, enhancing food security, and promoting sustainable environmental stewardship.

6.2 Recommendations for future studies

Based on the findings of this study, the following recommendations are proposed for advancing research in this field:

- The ecological and physiological effects of sublethal antibiotic residues in agricultural settings require further exploration. Research should focus on how these residues influence bacterial fitness, pathogenicity, and resistance development. Quantifying antibiotic levels in agroecosystems and correlating them with microbial community dynamics will help identify key drivers of resistance.
- Future studies should investigate how *Clostridium* species enter the food chain, focusing on pre- and post-harvest contamination routes. Predictive models should be developed to assess risks associated with contaminated irrigation systems and to design evidence-based mitigation strategies that enhance the safety of fresh produce.
- Research is needed to investigate the effects of climate change, such as increased rainfall variability and temperature shifts, on the prevalence, survival, and behaviour of *Clostridium* species in agroecosystems. Understanding these dynamics will help predict future risks and inform adaptive agricultural practices.
- Collaborative efforts with public health researchers should focus on evaluating the health impacts of *Clostridium* species in agricultural settings, particularly antibiotic-resistant infections linked to foodborne exposure. Multidisciplinary studies can further

elucidate the societal and economic consequences of foodborne illnesses and contribute to comprehensive risk management strategies.

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Appendix I

CHAPTER 3: INSIDE ENVIRONMENTAL *CLOSTRIDIUM PERFRINGENS* GENOMES: ANTIBIOTIC RESISTANCE GENES, VIRULENCE FACTORS AND GENOMIC FEATURES

Table S3.1: General genome features count for three isolated *C. perfringens* strains

Strain	SC4-C13	SC4-C17	SC4-C24
Genome size (bp)	3,604,770	3,514,948	3,437,837
GC content (%)	28.19	28.14	28,21
N_{50} length (bp)	454,71	461,812	356,343
No. of contigs	205	205	110
No. of ORFs	3,245	3,201	3,079
No. of total RNAs	125	124	130
No. of total rRNAs	29	29	34
No. of total tRNAs	92	91	92
No. of pseudogenes	55	66	55
GenBank accession no.	RQNP00000000	RQNQ00000000	RQNR00000000

Table S3.2: MIC ranges and SIR interpretation of 5 clinically relevant antibiotics for three *C. perfringens* strains (SC4-C13, SC4-C17 and SC4-C24)

Class	Antibiotic	CLSI range (µg/ml)			SC4-C13		SC4-C17		SC4-C24	
		S	I	R	MIC (µg/ml)	SIR	MIC (µg/ml)	SIR	MIC (µg/ml)	SIR
Beta-lactam	Ampicillin	≤ 0.5	1	≥ 2	4	R	2	R	1	I
Tetracyclines	Tetracycline	≤ 4	8	≥ 16	256	R	256	R	256	R
MLSB	Clindamycin	≤ 2	4	≥ 8	64	R	64	R	16	R
Phenicols	Chloramphenicol	≤ 8	16	≥ 32	8	S	4	S	4	S
Nitroimidazoles	Metronidazole	≤ 8	16	≥ 32	32	R	32	R	32	R

*** S= Susceptible; I= Intermediate resistance; R= Resistant**

Table S3.3: Summary of antibiotic resistance islands (ARIs) in *C.perfringens* strains as predicted by VRprofile.

Strains	Length (aa)	ARI	Origin	Ha-value	Accesion no.
SC4_C13, SC4_C17, SC_C24	455	propionate catabolism operon regulator prpR	*Pseudomonas aeruginosa*	0.354	12698381
	378	putative acyl-CoA dehydrogenase	*Pseudomonas aeruginosa*	0.254	12698384
	283	2-deoxy-D-gluconate 3-dehydrogenase	*Pseudomonas aeruginosa*	0.208	12698385
	489	putative amino acid/amine transport protein	*Pseudomonas aeruginosa*	0.194	12698393
	384	1,3-propanediol dehydrogenase	*Pseudomonas aeruginosa*	0.367	12698394
	578	potassium-transporting ATPase (A chain)	*Staphylococcus aureus*	0.427	13785455
	889	cadmium resistance protein B	*Staphylococcus aureus*	0.314	14020985
	89	hypothetical protein	*Staphylococcus aureus*	0.326	14020990
	109	hypothetical protein	*Staphylococcus aureus*	0.284	14020994
	413	mercuric reductase	*Staphylococcus aureus*	0.140	14021018
	230	orf4	*Staphylococcus aureus*	0.252	14021020
	259	ATP-binding protein FecE	*Shigella flexneri 2a*	0.375	15808722
	328	FecD	*Shigella flexneri 2a*	0.299	15808723
	337	FecC	*Shigella flexneri 2a*	0.285	15808724
	408	unknown	*Staphylococcus aureus*	0.304	16579875
	206	unnamed protein product	*Staphylococcus hominis*	0.254	18148888
	161	dihydrofolate reductase (plasmid)	*Klebsiella pneumoniae*	0.267	192822647
	244	resolvase (plasmid)	*Klebsiella pneumoniae*	0.262	192822658
	515	type I restriction-modification system DNA methylase	*Staphylococcus aureus PM1*	0.618	229002235
	160	hypothetical protein	*Staphylococcus aureus PM1*	0.287	229002245
	326	conserved hypothetical protein orfYpartial	*Staphylococcus aureus PM1*	0.258	229002247
	232	putative response-regulator ArmR	*Pseudomonas aeruginosa*	0.289	24461541
	258	putative enoyl-CoA hydratase/isomerase	*Pseudomonas aeruginosa*	0.264	24461556
	294	putative transcriptional regulator	*Pseudomonas aeruginosa*	0.246	24461567
	728	putative DNA topoisomerase III	*Pseudomonas aeruginosa*	0.298	24461623
	204	TnpR	*Pseudomonas aeruginosa*	0.328	265509450

	257	rRNA methylase	*Staphylococcus aureus*	0.342	28465856
	245	glycerophosphoryl diester phosphodiesterase homolog	*Staphylococcus aureus*	0.257	28465859
	295	xylose repressor	*Staphylococcus aureus*	0.247	28465863
	119	Cadmium efflux system accessory protein homologcadmium resistance protein C	*Staphylococcus aureus*	0.420	28465871
	159	hypothetical protein	*Staphylococcus aureus*	0.560	28465880
	98	hypothetical protein	*Staphylococcus aureus*	0.450	29539367
	269	dihydropteroate synthase	*Escherichia coli O157H7*	0.279	327185038
	140	streptothricin acetyltransferase	*Campylobacter coli*	0.257	387778851
	652	truncated tetracycline resistant protein	*Campylobacter coli*	0.279	387778859
	1169	pyruvate ferredoxin	*Campylobacter coli*	0.587	387778860
	551	sulphate premease Sup	*Acinetobacter baumannii*	0.247	409973527
	290	integrase	*Acinetobacter baumannii*	0.241	409973533
	408	TetA(A)	*Proteus mirabilis*	0.311	481190476
	150	membrane proteinpartial	*Proteus mirabilis*	0.333	481190508
	380	PsaB	*Acinetobacter baumannii*	0.326	484356514
	220	ItrA2	*Acinetobacter baumannii*	0.395	484356525
	448	Pgm	*Acinetobacter baumannii*	0.289	484356530
	507	LldP	*Acinetobacter baumannii*	0.319	484356531
	501	AspS	*Acinetobacter baumannii*	0.152	484356532
	205	ItrA3	*Acinetobacter baumannii*	0.556	484359534
	232	type 1 capsule synthesis gene	*Staphylococcus aureus*	0.272	506697
	217	type 1 capsule synthesis gene	*Staphylococcus aureus*	0.362	506698
	257	type 1 capsule synthesis gene	*Staphylococcus aureus*	0.311	506699
	637	type 1 capsule synthesis gene	*Staphylococcus aureus*	0.372	506700
	190	type 1 capsule synthesis gene	*Staphylococcus aureus*	0.284	506703
	688	potassium-transporting ATPase (B chain)	*Staphylococcus aureus*	0.548	5360811
	231	KDP operon transcriptional regulatory protein KdpE	*Staphylococcus aureus*	0.355	5360818
	275	Gne2	*Acinetobacter baumannii*	0.262	587656393
	212	QhbA	*Acinetobacter baumannii*	0.317	587656400

	312	TrxB	*Acinetobacter baumannii*	0.394	672940407
	132	ArsC	*Acinetobacter baumannii*	0.263	672940410
	700	ArsC	*Acinetobacter baumannii*	0.343	672940418
	150	orf	*Acinetobacter baumannii*	0.247	672940419
	319	ItrB3	*Acinetobacter baumannii*	0.263	697403905
	306	GalU	*Acinetobacter baumannii*	0.418	697403908
	338	Gne1	*Acinetobacter baumannii*	0.509	697403911
	384	WecB	*Acinetobacter baumannii*	0.461	697403916
	90	hypothetical protein	*Staphylococcus aureus*	0.289	7592617
	690	ATP-dependent DNA helicase	*Acinetobacter baumannii*	0.322	90265357
SC4_C13, SC4_C17	889	Probable cadmium-transporting ATPase (Cadmium efflux ATPase)	*Staphylococcus aureus*	0.313	28465870
	448	Ugd	*Acinetobacter baumannii*	0.263	484359536
	397	site-specific recombinase	*Staphylococcus aureus*	0.446	5360826
SC4_C17, SC4_C24	1087	type I restriction-modification system endonuclease homologue	*Staphylococcus aureus PM1*	0.503	229002233
SC4_C24, SC4_C13	412	hypothetical protein	*Staphylococcus aureus PM1*	0.343	229002234
SC4_C13	150	polypeptide B	*Staphylococcus aureus*	0.200	14021010
	196	resolvase (plasmid)	*Klebsiella pneumoniae*	0.321	192822645
	45	hypothetical protein	*Staphylococcus pseudintermedius*	0.667	221148490
	136	transposase for ISSau4-like	*Staphylococcus aureus PM1*	0.375	229002231
	406	transposase for insertion sequence element IS256 in transposon Tn4001	*Staphylococcus aureus*	0.249	29539378
	56	hypothetical protein	*Pseudomonas aeruginosa*	0.500	46092537
	237	type 1 capsule synthesis gene	*Staphylococcus aureus*	0.266	506709
	251	GtrOC9	*Acinetobacter baumannii*	0.323	697403915
SC4_C17	533	cassette chromosome recombinase B3	*Staphylococcus aureus*	0.285	14020973
	149	putative transposase for IS30	*Staphylococcus aureus*	0.411	29539354
	133	Wzc	*Acinetobacter baumannii*	0.248	484359519
	232	LgaB	*Acinetobacter baumannii*	0.369	697403887
	66	transcription regulator	*Acinetobacter baumannii*	0.258	90265339
SC4_24	189	resolvase	*Proteus mirabilis*	0.333	481190461

	105	Gne2	*Acinetobacter baumannii*	0.367	484359523
	132	GalU	*Acinetobacter baumannii*	0.264	484359535
	500	HsdM protein	*Staphylococcus aureus*	0.313	49257053

Table S3.4: Summary of pathogenicity islands (PAIs) in *C.perfringens* strains as predicted by VRprofile.

Strains	Length (aa)	PAI	Origin	Ha-value	Accesion no.
SC4_C13, SC4_C17, SC_C24	839	DNA gyrase A subunit	*Delftia tsuruhatensis*	0.509	149774749
	214	phosphoglycolate phosphatase	*Delftia acidovorans*	0.300	151500321
	979	DNA gyrase A subunit	*Delftia acidovorans*	0.298	151500324
	269	dihydropteroate synthase	*Escherichia coli*	0.349	331029100
	275	short chain dehydrogenase	*Escherichia coli*	0.255	331029102
	161	dihydrofolate reductase	*Proteus mirabilis*	0.261	429138628
	90	dihydrofolate reductase type Ib (plasmid)	*Escherichia coli*	0.300	950041
	150	single-strand binding protein	*Salmonella enterica*	0.193	AAC26638
	341	QueA	*Pseudomonas syringae pv. tomato*	0.470	AAF71483
	172	unknown	*Staphylococcus aureus subsp. aureus COL*	0.244	AAL04144
	259	ATP-binding protein FecE	*Shigella flexneri 2a*	0.375	AAL08451
	328	FecD	*Shigella flexneri 2a*	0.299	AAL08452
	337	FecC	*Shigella flexneri 2a*	0.285	AAL08453
	290	Int	*Photorhabdus luminescens*	0.250	AAN64203
	482	Orf17	*Photorhabdus luminescens*	0.320	AAO17183
	283	Orf29	*Photorhabdus luminescens*	0.269	AAO17195
	285	Orf59	*Photorhabdus luminescens*	0.270	AAO17213
	397	Pgk	*Photorhabdus luminescens*	0.438	AAO17214
	332	Epd	*Photorhabdus luminescens*	0.422	AAO17215
	314	TktA	*Photorhabdus luminescens*	0.245	AAO17218
	334	Orf34	*Photorhabdus luminescens*	0.246	AAO18059
	217	Orf41	*Photorhabdus luminescens*	0.332	AAO18066
	69	unknown	*Photorhabdus luminescens*	0.522	AAO18076
	465	HrcN	*Xanthomonas citri pv. glycines*	0.262	AAP34339
	260	glutamate racemase	*Helicobacter pylori*	0.265	AAR03910
	62	putative ferredoxin transketolase	*Streptomyces turgidiscabies Car8*	0.419	AAW49307

	445	phosphoglycerate transport protein	*Escherichia coli*	0.366	AAZ04434
	455	VasH	*Aeromonas hydrophila*	0.244	ABG57145
	381	conserved hypothetical protein	*Streptomyces lividans*	0.333	ABP49156
	357	hypothetical protein	*Edwardsiella tarda*	0.378	ABW69089
	160	transcriptional regulator	*Vibrio alginolyticus*	0.194	ACN89292
	814	ATPases with chaperone activity ATP-binding subunit	*Vibrio alginolyticus*	0.342	ACN89306
	239	serine/threonine protein phosphatase	*Vibrio alginolyticus*	0.314	ACN89319
	866	ClpB	*Acinetobacter baumannii*	0.373	AGM48629
	460	HrpB6	*Xanthomonas oryzae pv. oryzae MAFF 311018*	0.265	BAB07852
	256	unnamed protein product	*Yersinia pestis*	0.336	CAA21355
	307	unnamed protein product	*Yersinia pestis*	0.352	CAA21362
	248	unnamed protein product	*Yersinia pestis*	0.306	CAA21363
	489	ansP	*Yersinia pestis*	0.280	CAA21364
	191	unnamed protein product	*Yersinia pestis*	0.403	CAA21366
	294	unnamed protein product	*Yersinia pestis*	0.204	CAA21367
	296	unnamed protein product	*Yersinia pestis*	0.321	CAA21373
	182	unnamed protein product	*Yersinia pestis*	0.275	CAA21405
	293	unnamed protein product	*Yersinia pestis*	0.253	CAA21406
	301	unnamed protein product	*Yersinia pestis*	0.252	CAA21407
	577	ABC transport protein	*Escherichia coli*	0.248	CAC43427
	222	putative ABC transporter ATP-binding protein	*Escherichia coli*	0.333	CAD33755
	269	putative ABC transporter membrane protein	*Escherichia coli*	0.253	CAD33756
	575	hemolysin B	*Escherichia coli*	0.259	CAD33760
	501	putative lysil-tRNA synthetase LysU	*Escherichia coli*	0.443	CAD66193
	444	hypothetical protein	*Escherichia coli*	0.279	CAE85172
	310	hypothetical protein	*Escherichia coli*	0.323	CAE85173
	401	GspF hypothetical type II secretion protein	*Escherichia coli*	0.300	CAE85232
	563	GspE hypothetical type II secretion protein	*Escherichia coli*	0.361	CAE85233
	466	GlcD protein	*Escherichia coli*	0.315	CAE85245

	444	putative DNA binding protein with DNA-dependent ATPase activity	*Yersinia pseudotuberculosis*	0.392	CAF28476
	413	putative oxidoreductase	*Yersinia pseudotuberculosis*	0.306	CAF28571
	413	3-oxoacyl-acyl carrier protein synthase II	*Pseudomonas savastanoi pv. phaseolicola*	0.430	CAI36078
	728	Topoisomerase IA	*Pseudomonas savastanoi pv. phaseolicola*	0.319	CAI36097
SC4_C13, SC4_C17	303	conserved hypothetical protein	*Delftia tsuruhatensis*	0.267	149774732
SC_C13	136	hypothetical protein	*Providencia stuartii*	0.279	108861485
	150	putative transmembrane transport protein	*Streptomyces turgidiscabies Car8*	0.273	AAW49311
	245	putative ATPase	*Yersinia pseudotuberculosis*	0.257	CAF28475
	406	IS285 transposase	*Yersinia pseudotuberculosis*	0.480	CAF28515
	198	superfamily II DNA/RNA helicase	*Pseudomonas savastanoi pv. Phaseolicola*	0.263	CAI36056
SC4_C17	158	chloramphenicol acetyltransferase	*Proteus vulgaris*	0.177	331029084
	286	DNA topoisomerase III (plasmid)	*Klebsiella pneumoniae subsp. pneumoniae Kp13*	0.245	569550113
	53	Integrase	*Staphylococcus aureus*	0.358	AAC28969
	155	terminase	*Staphylococcus aureus*	0.355	AAL67611
	119	unnamed protein product	*Yersinia pestis*	0.244	CAA21342
	143	unnamed protein product	*Yersinia pestis*	0.250	CAA21343
	147	ybtQ	*Yersinia pestis*	0.240	CAA21387
	373	hsdr-like Type I restriction enzyme	*Yersinia pseudotuberculosis*	0.254	CAF28526
	133	ParA-like chromosome partitioning protein	*Pseudomonas savastanoi pv. phaseolicola*	0.309	CAI36042
SC4_C24	177	HrcN	*Pseudomonas syringae pv. tomato*	0.260	AAG33879
	175	ClpB protein	*Aeromonas hydrophila*	0.400	ABG57144
	147	putative ClpA/B-type chaperone	*Vibrio alginolyticus*	0.245	ACN89328

Table S3.5: Phages present in the genomes of *C. perfringens* strains

Strain	Region	Completeness (score)	Specific keyword	Total protein	Phage hit protein	Hypothetical protein	Att site
SC4-C13	1	questionable(90)	integrase,terminase,portal,capsid,head	53	43	10	Yes
	2	incomplete(30)	transposase,capsid	14	6	8	No
SC4-C17	1	incomplete(50)	head,integrase	40	22	16	Yes
	2	intact(100)	head,capsid,portal,terminase,integrase	57	46	11	Yes
SC4-C24	1	questionable(90)	head,capsid,portal,terminase	59	43	16	No
	2	intact(104)	tail,head,capsid,portal,terminase	57	47	10	No
	3	incomplete(40)	transposase,tail	9	7	2	No

Table S3.6: Orthologous cluster associated with biological processes, molecular functions and cellular components within the core genomes of *C. perfringens* strains (SC4-C13, SC4-C17, SC4-C24).

Biological processes		
GO annotation	**Function name**	**Protein count**
GO:0008150	biological_process	635
GO:0008152	metabolic process	604
GO:0044237	cellular metabolic process	471
GO:0009987	cellular process	372
GO:0006807	nitrogen compound metabolic process	370
GO:0044238	primary metabolic process	281
GO:0046483	heterocycle metabolic process	256
GO:0043170	macromolecule metabolic process	245
GO:0006725	cellular aromatic compound metabolic process	237
GO:0006139	nucleobase-containing compound metabolic process	187
GO:0016070	RNA metabolic process	146
GO:0006082	organic acid metabolic process	144
GO:0006793	phosphorus metabolic process	125
GO:0065007	biological regulation	116
GO:0006810	transport	104
GO:0051234	establishment of localization	104
GO:0005975	carbohydrate metabolic process	94
GO:0006412	translation	93
GO:0050896	response to stimulus	89
GO:0032502	developmental process	79
GO:0051186	cofactor metabolic process	77
GO:0009117	nucleotide metabolic process	76
GO:0006396	RNA processing	57
GO:0009116	nucleoside metabolic process	54
GO:0006811	ion transport	50
GO:0051179	localization	47
GO:0019538	protein metabolic process	45
GO:0016043	cellular component organization	44
GO:0006629	lipid metabolic process	42
GO:0044255	cellular lipid metabolic process	42
GO:0006518	peptide metabolic process	36
GO:0043412	macromolecule modification	36
GO:0006259	DNA metabolic process	35
GO:0043603	cellular amide metabolic process	33
GO:0042254	ribosome biogenesis	31
GO:0005976	polysaccharide metabolic process	30

GO:0007154	cell communication	28
GO:0008643	carbohydrate transport	28
GO:0006260	DNA replication	26
GO:0006766	vitamin metabolic process	24
GO:0006066	alcohol metabolic process	23
GO:0006281	DNA repair	22
GO:0032989	cellular component morphogenesis	20
GO:0006464	cellular protein modification process	19
GO:0015031	protein transport	19
GO:0006818	hydrogen transport	14
GO:0009308	amine metabolic process	14
GO:0006091	generation of precursor metabolites and energy	12
GO:0006457	protein folding	11
GO:0006865	amino acid transport	11
GO:0051704	multi-organism process	11
GO:0042440	pigment metabolic process	10
GO:0051301	cell division	9
GO:0006508	proteolysis	8
GO:0007049	cell cycle	8
GO:0022607	cellular component assembly	8
GO:0065003	macromolecular complex assembly	8
GO:0071555	cell wall organization	8
GO:0006112	energy reserve metabolic process	7
GO:0006996	organelle organization	7
GO:0043094	cellular metabolic compound salvage	7
GO:0051276	chromosome organization	7
GO:0051641	cellular localization	7
GO:0006081	cellular aldehyde metabolic process	6
GO:0022411	cellular component disassembly	6
GO:0043101	purine-containing compound salvage	6
GO:0000003	reproduction	5
GO:0006304	DNA modification	5
GO:0009225	nucleotide-sugar metabolic process	5
GO:0009292	genetic transfer	5
GO:0051189	prosthetic group metabolic process	5
GO:0006730	one-carbon metabolic process	4
GO:0007059	chromosome segregation	4
GO:0008655	pyrimidine-containing compound salvage	4
GO:0046903	secretion	4
GO:0051604	protein maturation	4
GO:0009372	quorum sensing	3
GO:0015931	nucleobase-containing compound transport	3
GO:0032196	transposition	3

GO:0032392	DNA geometric change	3
GO:0032501	multicellular organismal process	3
GO:0045229	external encapsulating structure organization	3
GO:0001906	cell killing	2
GO:0006354	DNA-templated transcription, elongation	2
GO:0015074	DNA integration	2
GO:0015849	organic acid transport	2
GO:0015976	carbon utilization	2
GO:0016032	viral process	2
GO:0031640	killing of cells of other organism	2
GO:0040011	locomotion	2
GO:0042710	biofilm formation	2
GO:0045230	capsule organization	2
GO:0045333	cellular respiration	2
GO:0006113	fermentation	1
GO:0006276	plasmid maintenance	1
GO:0006323	DNA packaging	1
GO:0006662	glycerol ether metabolic process	1
GO:0006805	xenobiotic metabolic process	1
GO:0006928	movement of cell or subcellular component	1
GO:0007005	mitochondrion organization	1
GO:0008283	cell proliferation	1
GO:0015833	peptide transport	1
GO:0015949	nucleobase-containing small molecule interconversion	1
GO:0015979	photosynthesis	1
GO:0019748	secondary metabolic process	1
GO:0034622	cellular macromolecular complex assembly	1
GO:0042180	cellular ketone metabolic process	1
GO:0043446	cellular alkane metabolic process	1
GO:0044419	interspecies interaction between organisms	1
GO:0050877	neurological system process	1
GO:0051181	cofactor transport	1
GO:0051258	protein polymerization	1
Molecular functions		
GO annotation	**Function name**	**Protein count**
GO:0003674	molecular function	72
GO:0016787	hydrolase activity	69
GO:0005215	transporter activity	61
GO:0043167	ion binding	55
GO:0016740	transferase activity	50
GO:0003676	nucleic acid binding	47
GO:0008233	peptidase activity	36
GO:0016491	oxidoreductase activity	28

GO:0000166	nucleotide binding	18
GO:0005488	binding	14
GO:0004871	signal transducer activity	12
GO:0048037	cofactor binding	11
GO:0001882	nucleoside binding	8
GO:0008289	lipid binding	4
GO:0003824	catalytic activity	2
GO:0004497	monooxygenase activity	2
GO:0009055	electron carrier activity	2
GO:0016829	lyase activity	2
GO:0016874	ligase activity	2
GO:0001871	pattern binding	1
GO:0004386	helicase activity	1
GO:0005198	structural molecule activity	1
GO:0005515	protein binding	1
GO:0016209	antioxidant activity	1
GO:0019239	deaminase activity	1
GO:0030246	carbohydrate binding	1
GO:0043021	ribonucleoprotein complex binding	1
GO:0051213	dioxygenase activity	1
Cellular components		
GO annotation	**Function name**	**Protein count**
GO:0016020	membrane	57
GO:0044464	cell part	56
GO:0005575	cellular component	4
GO:0005622	intracellular	2
GO:0005739	mitochondrion	1
GO:0030312	external encapsulating structure	1
GO:0030313	cell envelope	1
GO:0031469	polyhedral organelle	1

Appendix II

CHAPTER 4: EFFECT OF SUB-INHIBATORY CONCENTRATION OF ANTIBIOTICS ON THE TRANSCRIPTOMIC RESPONSE OF ENVIRONMENTALLY OBTAINED CLOSTRIDIUM PERFRINGENS

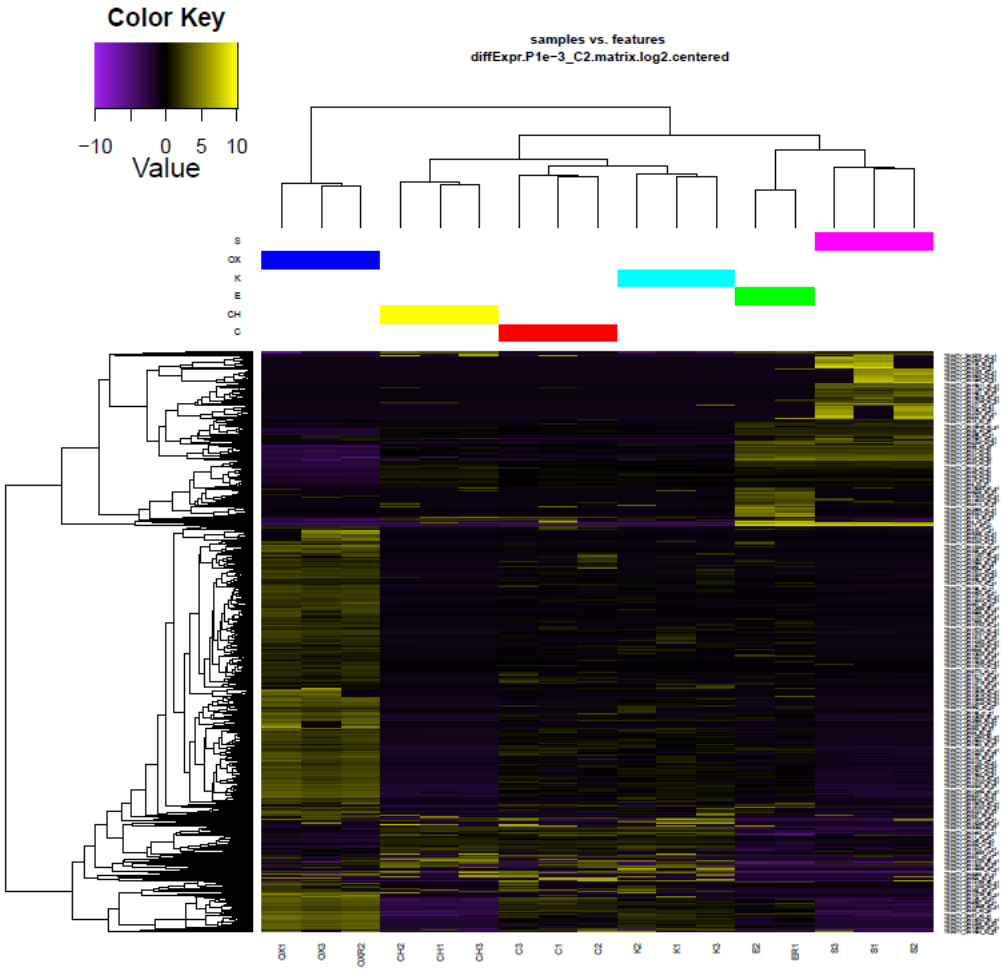


Figure S4.1: Hierarchical clustering of differentially expressed transcripts of *Clostridium perfringens* treated with sublethal concentration of 5 different antibiotics. Heatmap showing the relative expression levels of each transcript (rows) in each sample (columns). Rows and columns are hierarchically clustered. Expression values (FPKM) are log2 –transformed and then median-cantered by transcript. These ate based on the three replicates from each antibiotic treatment. Antibiotics used: cefoxitin (64 µg/ml, replicates: OX1, OXR2, OX3): sulfamethoxazole (256 µg/ml, replicates: S1, S2, S3), erythromycin (128 µg/ml, replicates: ER1, E2), chloramphenicol (64 µg/ml, replicates: CH1, CH2, CH3) and clindamycin (512 µg/ml, replicates: C1, C2, C3).

Table S4.1: Clusters of Orthologous Groups (COG) classification of differentially expressed transcripts from *C. perfringens* exposed to 5 antibiotics.

COGs	Antibiotic treatment									
	Erythromycin		Cefoxitin		Chloramphenicol		Clindamycin		Sulfamethoxazole	
	n	%	n	%	n	%	n	%	n	%
C	5	5.952381	10	7.1428571	4	5.128205	5	5.882353	5	6.410256
D	4	4.761905	4	2.8571429	4	5.128205	4	4.705882	4	5.128205
E	3	3.571429	9	6.4285714	3	3.846154	3	3.529412	3	3.846154
F	4	4.761905	5	3.5714286	4	5.128205	4	4.705882	3	3.846154
G	9	10.714286	10	7.1428571	9	11.538462	9	10.588235	9	11.538462
H	1	1.190476	6	4.2857143	1	1.282051	2	2.352941	1	1.282051
I	2	2.380952	2	1.4285714	2	2.564103	2	2.352941	1	1.282051
J	5	5.952381	9	6.4285714	5	6.410256	5	5.882353	5	6.410256
K	6	7.142857	9	6.4285714	5	6.410256	6	7.058824	5	6.410256
L	8	9.523810	13	9.2857143	8	10.256410	8	9.411765	8	10.256410
M	9	10.714286	15	10.7142857	9	11.538462	9	10.588235	9	11.538462
N	1	1.190476	1	0.7142857	1	1.282051	1	1.176471	1	1.282051
O	1	1.190476	3	2.1428571	0	0	0	0	0	0
P	5	5.952381	8	5.7142857	4	5.128205	5	5.882353	4	5.128205
S	11	13.095238	24	17.1428571	10	12.820513	12	14.117647	11	14.102564
T	5	5.952381	6	4.2857143	5	6.410256	6	7.058824	5	6.410256
U	1	1.190476	2	1.4285714	1	1.282051	1	1.176471	1	1.282051
V	4	4.761905	4	2.8571429	3	3.846154	3	3.529412	3	3.846154

Table S4.2: Summary of Gene ontology (GO) analysis of differentially expressed transcripts from *C. perfringens* exposed to 5 antibiotics.

GO Analysis		Chloramphenicol		Clindamycin		Erythromycin		Cefoxitin		Sulfamethoxazole		Total	
		UP-REG	DOWN-REG	UP-REG	DOWN-REG	UP-REG	DOWN-REG	UP-REG	DOWN-REG	UP-REG	DOWN-REG	UP-REG	DOWN-REG
Gene		36	190	38	60	222	175	343	384	265	189	904	998
Annotated Genes		28	158	32	47	144	136	263	338	211	119	678	798
GO Terms	Biological	21	123	24	31	108	104	201	264	165	91	519	613
	Cellular	17	109	22	33	91	89	157	222	145	73	432	526
	Function	27	142	27	44	138	127	257	310	194	114	643	737
Total		65	374	73	108	337	320	615	796	504	278	1,594	1,876

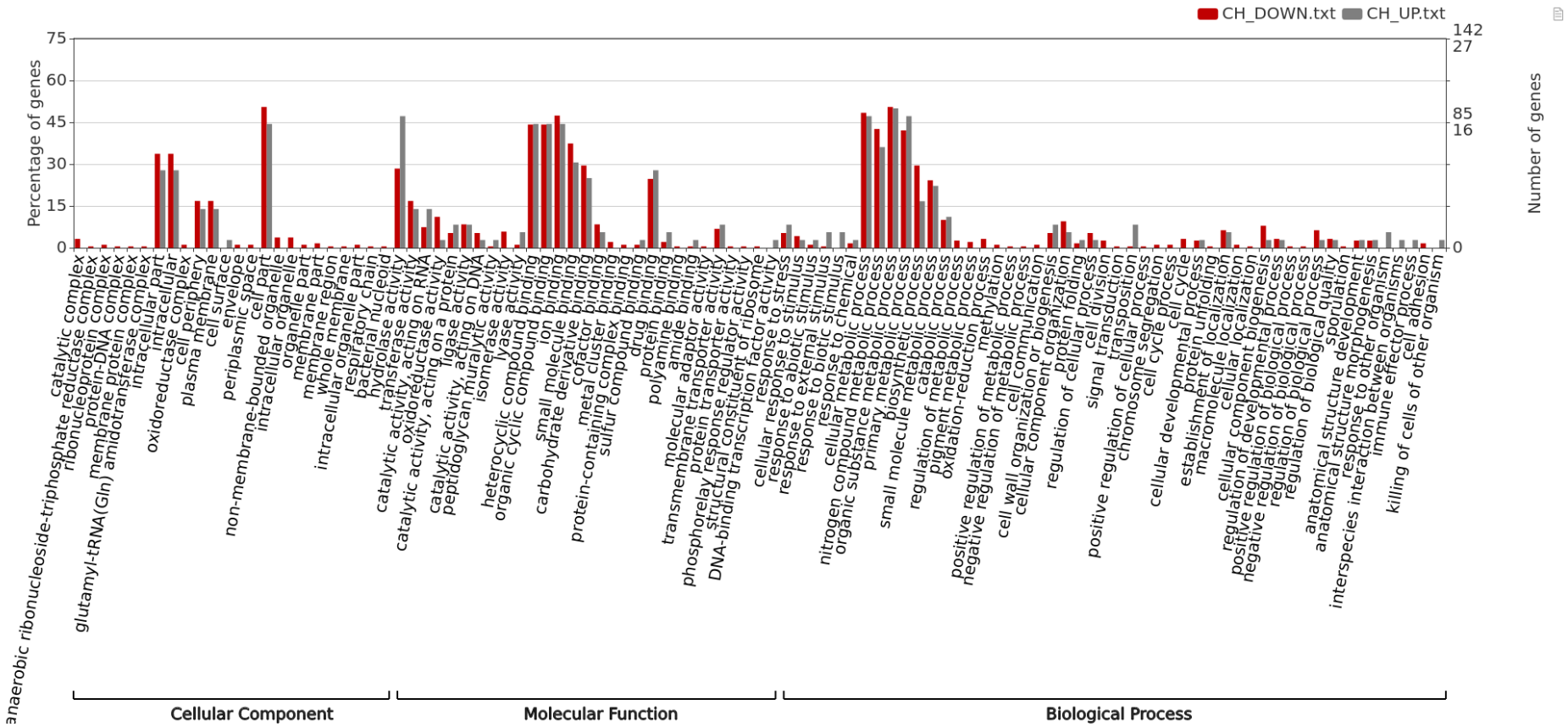


Figure S4.2: Gene Ontology (GO) analysis of differentially expressed transcripts identified in *Clostridium perfringens* when exposed to sublethal concentrations of Chloramphenicol. The graphs show the GO terms in the three main categories: cellular component, molecular function and biological process. RED: up-regulated transcripts GREY: down-regulated transcripts.

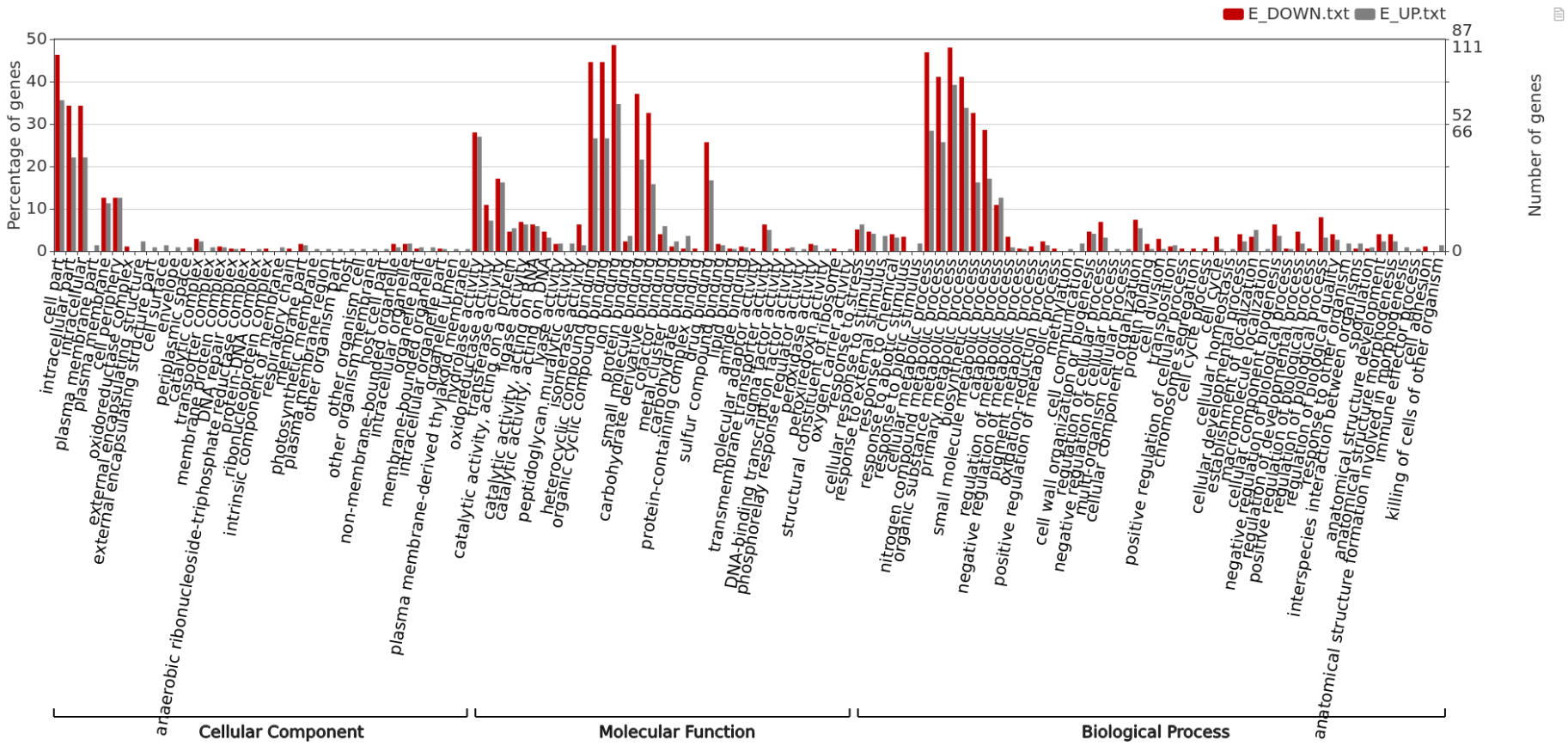


Figure S4.3: Gene Ontology (GO) analysis of differentially expressed transcripts identified in *Clostridium perfringens* when exposed to sublethal concentrations of Erythromycin. The graphs show the GO terms in the three main categories: cellular component, molecular function and biological process. RED: up-regulated transcripts GREY: down-regulated transcripts.

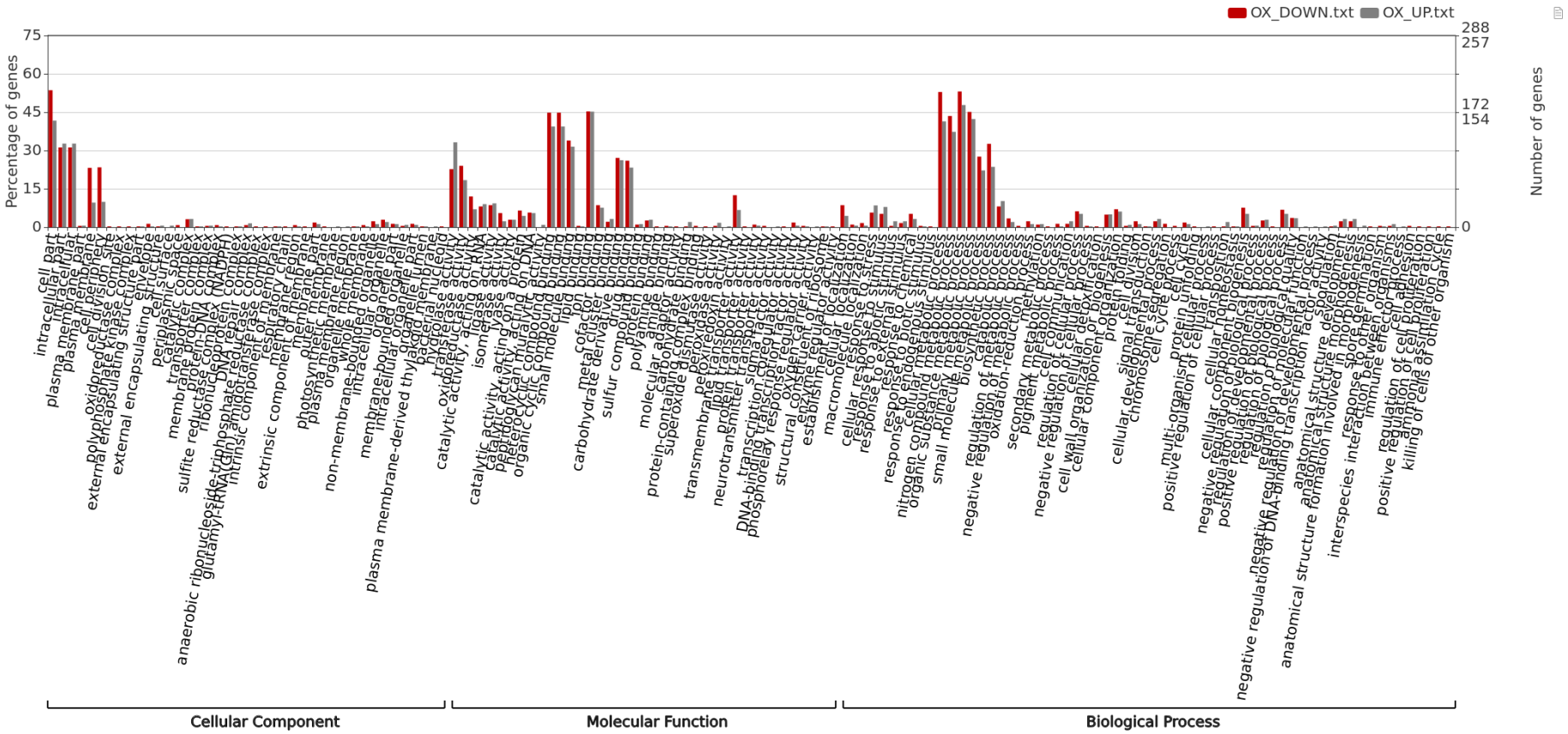


Figure S4.4: Gene Ontology (GO) analysis of differentially expressed transcripts identified in *Clostridium perfringens* when exposed to sublethal concentrations of Cefoxitin. The graphs show the GO terms in the three main categories: cellular component, molecular function and biological process. RED: up-regulated transcripts GREY: down-regulated transcripts.

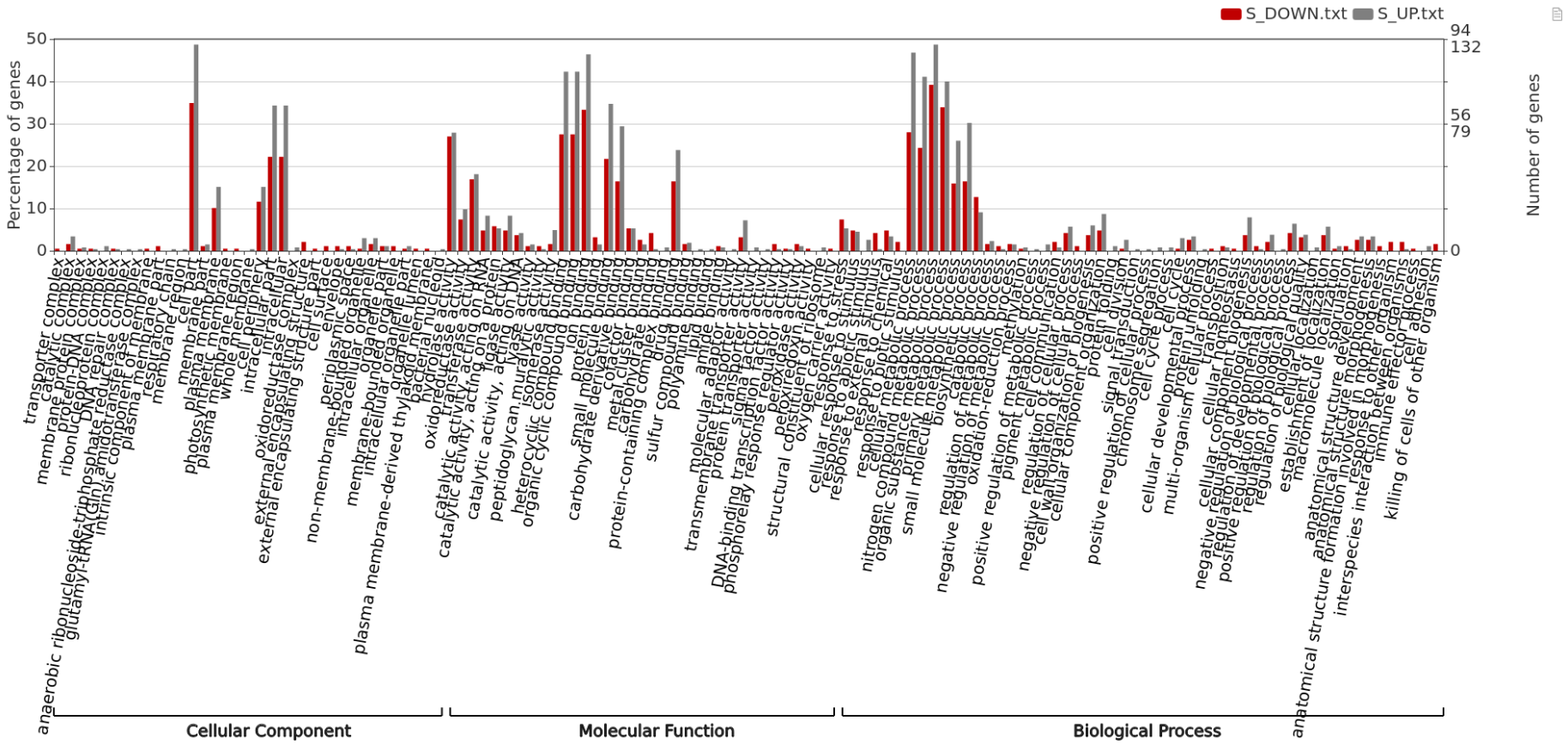


Figure S4.5: Gene Ontology (GO) analysis of differentially expressed transcripts identified in *Clostridium perfringens* when exposed to sublethal concentrations of Sulfamethoxazole. The graphs show the GO terms in the three main categories: cellular component, molecular function and biological process. RED: up-regulated transcripts GREY: down-regulated transcripts.

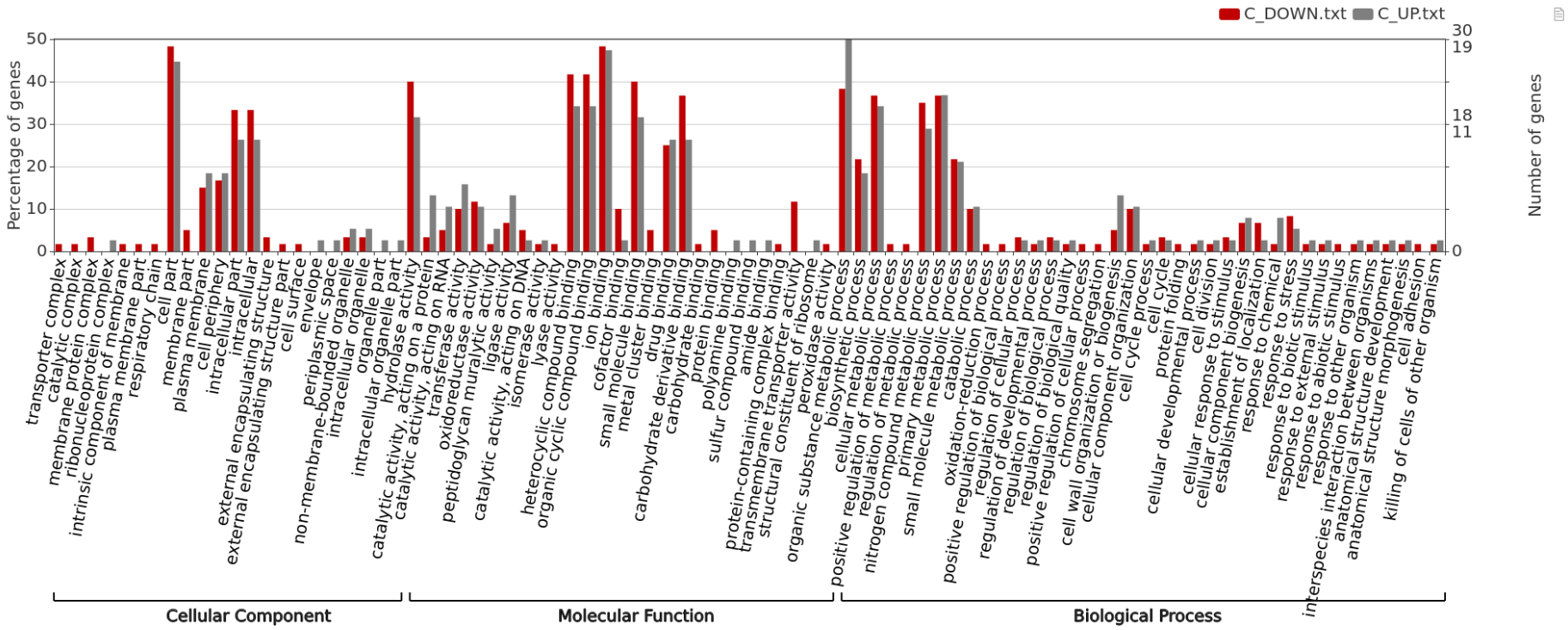


Figure S4.6: Gene Ontology (GO) analysis of differentially expressed transcripts identified in *Clostridium perfringens* when exposed to sublethal concentrations of Clindamycin. The graphs show the GO terms in the three main categories: cellular component, molecular function and biological process. RED: up-regulated transcripts GREY: down-regulated transcripts.

Table S4.3: The KEGG pathways of the differentially expressed transcripts in *Clostridium perfringens* treated with Erythromycin.

Query	e-value	Score	Description	Preferred name	KEGG
TRINITY_DN0_c0_g1_i3	0.0	1459.0	DNA-dependent ATPase and ATP-dependent 5'-3' DNA helicase. Has no activity on blunt DNA or DNA with 3'-overhangs, requires at least 10 bases of 5'-ssDNA for helicase activity	recD2	ko:K03581
TRINITY_DN10_c0_g1_i1	3.05e-297	812.0	The central subunit of the protein translocation channel SecYEG. Consists of two halves formed by TMs 1-5 and 6-10. These two domains form a lateral gate at the front which open onto the bilayer between TMs 2 and 7, and are clamped together by SecE at the back. The channel is closed by both a pore ring composed of hydrophobic SecY resides and a short helix (helix 2A) on the extracellular side of the membrane which forms a plug. The plug probably moves laterally to allow the channel to open. The ring and the pore may move independently	secY	ko:K03076
TRINITY_DN122_c0_g2_i1	1.39e-81	241.0	Domain of Unknown Function (DUF1540)	-	-
TRINITY_DN236_c0_g1_i1	0.0	1036.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN28_c0_g3_i1	0.0	1425.0	ABC transporter	-	ko:K06147
TRINITY_DN29_c0_g1_i1	0.0	969.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN31_c0_g1_i1	2.85e-154	454.0	cellulose binding	-	ko:K01179,ko:K07318
TRINITY_DN33_c0_g1_i3	0.0	1161.0	helicase	-	ko:K17675
TRINITY_DN33_c0_g3_i1	1.44e-259	716.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN35_c0_g1_i6	0.0	1024.0	PAS domain	-	-
TRINITY_DN42_c0_g1_i1	0.0	1529.0	penicillin-binding protein 1A	mrcB	ko:K05366
TRINITY_DN47_c0_g3_i1	0.0	1324.0	In eubacteria ppGpp (guanosine 3'-diphosphate 5-' diphosphate) is a mediator of the stringent response that coordinates a variety of cellular activities in response to changes in nutritional abundance	relA	ko:K00951
TRINITY_DN51_c0_g1_i2	0.0	1926.0	Peptidase M16	-	ko:K06972
TRINITY_DN55_c0_g1_i8	1.01e-133	390.0	Amino acid permease	-	-
TRINITY_DN63_c0_g1_i1	0.0	880.0	Involved in cell wall formation. Catalyzes the final step in the synthesis of UDP-N-acetylmuramoyl-pentapeptide, the precursor of murein	murF	ko:K01929
TRINITY_DN65_c0_g1_i1	0.0	922.0	Catalyzes the reduction of hydroxylamine to form NH(3) and H(2)O	hcp	ko:K05601
TRINITY_DN76_c0_g1_i3	0.0	1353.0	elongation factor G	fusA2	ko:K02355
TRINITY_DN89_c0_g1_i1	0.0	893.0	Catalyzes the reversible phosphatidyl group transfer from one phosphatidylglycerol molecule to another to form cardiolipin (CL) (diphosphatidylglycerol) and glycerol	cls	ko:K06131
TRINITY_DN55_c0_g2_i1	0.0	1257.0	transporter of a GTP-driven Fe(2) uptake system	feoB	ko:K04759
TRINITY_DN35_c0_g1_i2	3.41e-79	255.0	PAS domain	-	-
TRINITY_DN18_c0_g1_i3	0.0	937.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN33_c0_g2_i2	0.0	967.0	import. Responsible for energy coupling to the transport system	rbsA	ko:K10441,ko:K10542
TRINITY_DN43_c0_g2_i1	1.54e-29	111.0	YSIRK type signal peptide	-	ko:K13733,ko:K14201
TRINITY_DN70_c0_g1_i1	1.34e-130	379.0	Helix-turn-helix domain	-	ko:K07496
TRINITY_DN183_c0_g1_i2	0.0	1017.0	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions	groL	ko:K04077

TRINITY_DN28_c0_g3_i4	0.0	1425.0	ABC transporter	-	ko:K06147
TRINITY_DN44_c0_g1_i10	0.0	873.0	UPF0210 protein	-	ko:K09157
TRINITY_DN51_c0_g1_i1	0.0	2327.0	Oxidoreductase required for the transfer of electrons from pyruvate to flavodoxin	nifJ	ko:K03737
TRINITY_DN113_c0_g1_i1	1.22e-72	235.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN14744_c0_g1_i1	4.56e-128	382.0	COG3209 Rhs family protein	-	-
TRINITY_DN14_c0_g1_i1	0.0	998.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN34_c0_g1_i1	0.0	1182.0	Required for accurate and efficient protein synthesis under certain stress conditions. May act as a fidelity factor of the translation reaction, by catalyzing a one-codon backward translocation of tRNAs on improperly translocated ribosomes. Back- translocation proceeds from a post-translocation (POST) complex to a pre-translocation (PRE) complex, thus giving elongation factor G a second chance to translocate the tRNAs correctly. Binds to ribosomes in a GTP-dependent manner	lepA	ko:K03596
TRINITY_DN35_c0_g1_i4	6.41e-286	794.0	PAS domain	-	-
TRINITY_DN55_c0_g1_i2	0.0	1774.0	Permease	-	ko:K02004
TRINITY_DN56_c0_g1_i8	0.0	941.0	amino acid ABC transporter	-	ko:K16961,ko:K16962
TRINITY_DN84_c0_g1_i3	0.0	940.0	Amino acid permease family protein	-	-
TRINITY_DN113_c0_g2_i1	8.88e-124	372.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN1_c0_g1_i11	0.0	2130.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN55_c0_g3_i2	0.0	984.0	CotH protein	-	-
TRINITY_DN12385_c0_g1_i1	3.7e-237	654.0	Sulphur transport	yeeE	ko:K07112
TRINITY_DN104_c0_g1_i2	0.0	1108.0	iron hydrogenase, small subunit	hydA	ko:K00532,ko:K18332
TRINITY_DN12403_c0_g1_i1	2.64e-41	146.0	Belongs to the UPF0753 family	ybcC	ko:K09822
TRINITY_DN274_c0_g1_i1	1.5e-314	856.0	Glutamate-1-semialdehyde aminotransferase	hemL	ko:K01845
TRINITY_DN69_c0_g2_i1	1.97e-214	595.0	Relaxase/Mobilisation nuclease domain	-	-
TRINITY_DN73_c0_g1_i1	1.79e-83	248.0	IF-3 binds to the 30S ribosomal subunit and shifts the equilibrum between 70S ribosomes and their 50S and 30S subunits in favor of the free subunits, thus enhancing the availability of 30S subunits on which protein synthesis initiation begins	infC	ko:K02520
TRINITY_DN91_c0_g1_i1	0.0	963.0	Resolvase, N terminal domain	-	-
TRINITY_DN97_c0_g1_i1	0.0	2967.0	Psort location Cytoplasmic, score 7.50	-	ko:K15923
TRINITY_DN131_c0_g1_i3	4.46e-258	710.0	ABC-type antimicrobial peptide transport system, permease component	-	ko:K02004
TRINITY_DN100_c1_g1_i1	5.33e-124	369.0	Regulatory protein BlaR1	blaR1-1	ko:K02172,ko:K02547
TRINITY_DN119_c0_g1_i1	3.32e-185	528.0	Catalyzes the reversible conversion of 2- phosphoglycerate into phosphoenolpyruvate. It is essential for the degradation of carbohydrates via glycolysis	eno	ko:K01689
TRINITY_DN122_c0_g1_i1	0.0	1773.0	DNA polymerase	dnaE	ko:K02337,ko:K14162
TRINITY_DN12_c0_g1_i1	0.0	1918.0	Polysaccharide lyase family 8, C-terminal beta-sandwich domain	-	ko:K01727
TRINITY_DN14_c0_g2_i1	3.37e-108	335.0	E domain	-	ko:K13732,ko:K13733,ko:K14195

TRINITY_DN15_c0_g1_i4	0.0	1288.0	helicase	-	-
TRINITY_DN17_c0_g1_i1	0.0	5038.0	Belongs to the glycosyl hydrolase 13 family	pulA	ko:K01200
TRINITY_DN181_c0_g1_i2	0.0	902.0	Biotin carboxylase	accC	ko:K01961
TRINITY_DN18_c0_g1_i2	0.0	1791.0	amino acids such as threonine, to avoid such errors, it has a posttransfer editing activity that hydrolyzes mischarged Thr-tRNA(Val) in a tRNA-dependent manner	valS	ko:K01873
TRINITY_DN19_c0_g1_i1	1.07e-254	716.0	ABC transporter	-	ko:K18231
TRINITY_DN38_c0_g1_i1	5.89e-272	744.0	Belongs to the glycosyl hydrolase 8 (cellulase D) family	-	-
TRINITY_DN41_c0_g1_i6	0.0	1315.0	-	-	-
TRINITY_DN55_c0_g1_i4	0.0	984.0	CotH protein	-	-
TRINITY_DN5_c0_g1_i1	0.0	3411.0	Endo-alpha-N-acetylgalactosaminidase	-	ko:K17624
TRINITY_DN5_c0_g2_i1	0.0	2738.0	domain protein	-	-
TRINITY_DN66_c0_g1_i1	2.23e-99	297.0	-	-	-
TRINITY_DN72_c0_g1_i4	0.0	1429.0	beta-galactosidase	pbg	ko:K12308
TRINITY_DN74_c0_g1_i1	2.37e-105	306.0	-	-	-
TRINITY_DN75_c0_g1_i1	0.0	1436.0	Ribonucleoside-triphosphate reductase	nrdD	ko:K21636
TRINITY_DN7_c0_g2_i1	0.0	916.0	Histidine kinase	-	-
TRINITY_DN7_c0_g2_i2	0.0	1913.0	repeat protein	-	-
TRINITY_DN7_c1_g1_i1	0.0	974.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN8_c0_g1_i1	5.61e-186	522.0	Transposase	-	ko:K07496
TRINITY_DN10696_c0_g1_i1	1.86e-75	250.0	peptidoglycan catabolic process	-	-
TRINITY_DN15884_c0_g1_i1	2.47e-272	747.0	Nucleoside	nupC	ko:K03317,ko:K11535
TRINITY_DN233_c0_g1_i1	1.44e-159	448.0	Poly-gamma-glutamate hydrolase	ymaC	-
TRINITY_DN9186_c0_g1_i1	5.66e-168	469.0	Purine nucleoside phosphorylase	deoD	ko:K03784
TRINITY_DN28_c0_g3_i3	3.09e-97	283.0	PFAM regulatory protein, MarR	-	-
TRINITY_DN9305_c0_g1_i1	9.49e-117	341.0	Belongs to the bacterial solute-binding protein 9 family	adcA	ko:K09815,ko:K09818
TRINITY_DN13960_c0_g1_i1	6.88e-110	322.0	Ornithine cyclodeaminase	rapL	ko:K01750
TRINITY_DN13976_c0_g1_i1	0.0	1550.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN15830_c0_g1_i1	0.0	947.0	Polyphosphate kinase 2 (PPK2)	-	-
TRINITY_DN15906_c0_g1_i1	1.93e-141	421.0	Lysin motif	ebpS	-
TRINITY_DN18_c0_g1_i5	0.0	885.0	Domain of unknown function (DUF3502)	-	ko:K17318

TRINITY_DN241_c0_g1_i1	2.28e-71	228.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN81_c0_g1_i1	0.0	892.0	Na Pi-cotransporter II-like protein	-	ko:K03324
TRINITY_DN9221_c0_g1_i1	0.0	1372.0	accessory protein	tex	ko:K06959
TRINITY_DN109_c0_g1_i1	8.35e-267	735.0	signal transduction protein with a C-terminal ATPase domain	comD	ko:K07706,ko:K12294
TRINITY_DN23_c0_g1_i1	0.0	1373.0	exonuclease	sbcC	ko:K03546

Table S4.4: The KEGG pathways of the differentially expressed transcripts in *Clostridium perfringens* treated with Chloramphenicol.

Query	e-value	Score	Description	Preferred name	KEGG
TRINITY_DN18_c0_g1_i3	0.0	937.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN33_c0_g2_i2	0.0	967.0	import. Responsible for energy coupling to the transport system	rbsA	ko:K10441,ko:K10542
TRINITY_DN43_c0_g2_i1	1.54e-29	111.0	YSIRK type signal peptide	-	ko:K13733,ko:K14201
TRINITY_DN70_c0_g1_i1	1.34e-130	379.0	Helix-turn-helix domain	-	ko:K07496
TRINITY_DN104_c0_g1_i2	0.0	1108.0	iron hydrogenase, small subunit	hydA	ko:K00532,ko:K18332
TRINITY_DN10696_c0_g1_i1	1.86e-75	250.0	peptidoglycan catabolic process	-	-
TRINITY_DN109_c0_g1_i1	8.35e-267	735.0	signal transduction protein with a C-terminal ATPase domain	comD	ko:K07706,ko:K12294
TRINITY_DN12385_c0_g1_i1	3.7e-237	654.0	Sulphur transport	yeeE	ko:K07112
TRINITY_DN12403_c0_g1_i1	2.64e-41	146.0	Belongs to the UPF0753 family	ybcC	ko:K09822
TRINITY_DN13960_c0_g1_i1	6.88e-110	322.0	Ornithine cyclodeaminase	rapL	ko:K01750
TRINITY_DN13976_c0_g1_i1	0.0	1550.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN15830_c0_g1_i1	0.0	947.0	Polyphosphate kinase 2 (PPK2)	-	-
TRINITY_DN15884_c0_g1_i1	2.47e-272	747.0	Nucleoside	nupC	ko:K03317,ko:K11535
TRINITY_DN15906_c0_g1_i1	1.93e-141	421.0	Lysin motif	ebpS	-
TRINITY_DN18_c0_g1_i5	0.0	885.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN1_c0_g1_i11	0.0	2130.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN233_c0_g1_i1	1.44e-159	448.0	Poly-gamma-glutamate hydrolase	ymaC	-
TRINITY_DN23_c0_g1_i1	0.0	1373.0	exonuclease	sbcC	ko:K03546
TRINITY_DN241_c0_g1_i1	2.28e-71	228.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN274_c0_g1_i1	1.5e-314	856.0	Glutamate-1-semialdehyde aminotransferase	hemL	ko:K01845
TRINITY_DN35_c0_g1_i2	3.41e-79	255.0	PAS domain	-	-
TRINITY_DN55_c0_g3_i2	0.0	984.0	CotH protein	-	-
TRINITY_DN69_c0_g2_i1	1.97e-214	595.0	Relaxase/Mobilisation nuclease domain	-	-
TRINITY_DN73_c0_g1_i1	1.79e-83	248.0	IF-3 binds to the 30S ribosomal subunit and shifts the equilibrum between 70S ribosomes and their 50S and 30S subunits in favor of the free subunits, thus enhancing the availability of 30S subunits on which protein synthesis initiation begins	infC	ko:K02520
TRINITY_DN81_c0_g1_i1	0.0	892.0	Na Pi-cotransporter II-like protein	-	ko:K03324
TRINITY_DN9186_c0_g1_i1	5.66e-168	469.0	Purine nucleoside phosphorylase	deoD	ko:K03784

TRINITY_DN91_c0_g1_i1	0.0	963.0	Resolvase, N terminal domain	-	-
TRINITY_DN9221_c0_g1_i1	0.0	1372.0	accessory protein	tex	ko:K06959
TRINITY_DN97_c0_g1_i1	0.0	2967.0	Psort location Cytoplasmic, score 7.50	-	ko:K15923
TRINITY_DN131_c0_g1_i3	4.46e-258	710.0	ABC-type antimicrobial peptide transport system, permease component	-	ko:K02004
TRINITY_DN0_c0_g1_i3	0.0	1459.0	DNA-dependent ATPase and ATP-dependent 5'-3' DNA helicase. Has no activity on blunt DNA or DNA with 3'-overhangs, requires at least 10 bases of 5'-ssDNA for helicase activity	recD2	ko:K03581
TRINITY_DN100_c1_g1_i1	5.33e-124	369.0	Regulatory protein BlaR1	blaR1-1	ko:K02172,ko:K02547
TRINITY_DN10_c0_g1_i1	3.05e-297	812.0	The central subunit of the protein translocation channel SecYEG. Consists of two halves formed by TMs 1-5 and 6-10. These two domains form a lateral gate at the front which open onto the bilayer between TMs 2 and 7, and are clamped together by SecE at the back. The channel is closed by both a pore ring composed of hydrophobic SecY resides and a short helix (helix 2A) on the extracellular side of the membrane which forms a plug. The plug probably moves laterally to allow the channel to open. The ring and the pore may move independently	secY	ko:K03076
TRINITY_DN113_c0_g1_i1	1.22e-72	235.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN119_c0_g1_i1	3.32e-185	528.0	Catalyzes the reversible conversion of 2- phosphoglycerate into phosphoenolpyruvate. It is essential for the degradation of carbohydrates via glycolysis	eno	ko:K01689
TRINITY_DN122_c0_g1_i1	0.0	1773.0	DNA polymerase	dnaE	ko:K02337,ko:K14162
TRINITY_DN122_c0_g2_i1	1.39e-81	241.0	Domain of Unknown Function (DUF1540)	-	-
TRINITY_DN12_c0_g1_i1	0.0	1918.0	Polysaccharide lyase family 8, C-terminal beta-sandwich domain	-	ko:K01727
TRINITY_DN14744_c0_g1_i1	4.56e-128	382.0	COG3209 Rhs family protein	-	-
TRINITY_DN14_c0_g1_i1	0.0	998.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN14_c0_g2_i1	3.37e-108	335.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN15_c0_g1_i4	0.0	1288.0	helicase	-	-
TRINITY_DN17_c0_g1_i1	0.0	5038.0	Belongs to the glycosyl hydrolase 13 family	pulA	ko:K01200
TRINITY_DN181_c0_g1_i2	0.0	902.0	Biotin carboxylase	accC	ko:K01961
TRINITY_DN18_c0_g1_i2	0.0	1791.0	amino acids such as threonine, to avoid such errors, it has a posttransfer editing activity that hydrolyzes mischarged Thr-tRNA(Val) in a tRNA-dependent manner	valS	ko:K01873
TRINITY_DN19_c0_g1_i1	1.07e-254	716.0	ABC transporter	-	ko:K18231
TRINITY_DN236_c0_g1_i1	0.0	1036.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN28_c0_g3_i1	0.0	1425.0	ABC transporter	-	ko:K06147
TRINITY_DN29_c0_g1_i1	0.0	969.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN31_c0_g1_i1	2.85e-154	454.0	cellulose binding	-	ko:K01179,ko:K07318
TRINITY_DN33_c0_g1_i3	0.0	1161.0	helicase	-	ko:K17675
TRINITY_DN33_c0_g3_i1	1.44e-259	716.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN34_c0_g1_i1	0.0	1182.0	Required for accurate and efficient protein synthesis under certain stress conditions. May act as a fidelity factor of the translation reaction, by catalyzing a one-codon backward translocation of tRNAs on improperly translocated ribosomes. Back- translocation proceeds from a post-translocation (POST) complex to a pre-translocation (PRE)	lepA	ko:K03596

			complex, thus giving elongation factor G a second chance to translocate the tRNAs correctly. Binds to ribosomes in a GTP-dependent manner		
TRINITY_DN35_c0_g1_i4	6.41e-286	794.0	PAS domain	-	-
TRINITY_DN35_c0_g1_i6	0.0	1024.0	PAS domain	-	-
TRINITY_DN38_c0_g1_i1	5.89e-272	744.0	Belongs to the glycosyl hydrolase 8 (cellulase D) family	-	-
TRINITY_DN41_c0_g1_i6	0.0	1315.0	-	-	-
TRINITY_DN42_c0_g1_i1	0.0	1529.0	penicillin-binding protein 1A	mrcB	ko:K05366
TRINITY_DN47_c0_g3_i1	0.0	1324.0	In eubacteria ppGpp (guanosine 3'-diphosphate 5-' diphosphate) is a mediator of the stringent response that coordinates a variety of cellular activities in response to changes in nutritional abundance	relA	ko:K00951
TRINITY_DN51_c0_g1_i2	0.0	1926.0	Peptidase M16	-	ko:K06972
TRINITY_DN55_c0_g1_i2	0.0	1774.0	Permease	-	ko:K02004
TRINITY_DN55_c0_g1_i4	0.0	984.0	CotH protein	-	-
TRINITY_DN55_c0_g1_i8	1.01e-133	390.0	Amino acid permease	-	-
TRINITY_DN56_c0_g1_i8	0.0	941.0	amino acid ABC transporter	-	ko:K16961,ko:K16962
TRINITY_DN5_c0_g1_i1	0.0	3411.0	Endo-alpha-N-acetylgalactosaminidase	-	ko:K17624
TRINITY_DN5_c0_g2_i1	0.0	2738.0	domain protein	-	-
TRINITY_DN63_c0_g1_i1	0.0	880.0	Involved in cell wall formation. Catalyzes the final step in the synthesis of UDP-N-acetylmuramoyl-pentapeptide, the precursor of murein	murF	ko:K01929
TRINITY_DN65_c0_g1_i1	0.0	922.0	Catalyzes the reduction of hydroxylamine to form NH(3) and H(2)O	hcp	ko:K05601
TRINITY_DN66_c0_g1_i1	2.23e-99	297.0	-	-	-
TRINITY_DN72_c0_g1_i4	0.0	1429.0	beta-galactosidase	pbg	ko:K12308
TRINITY_DN74_c0_g1_i1	2.37e-105	306.0	-	-	-
TRINITY_DN75_c0_g1_i1	0.0	1436.0	Ribonucleoside-triphosphate reductase	nrdD	ko:K21636
TRINITY_DN76_c0_g1_i3	0.0	1353.0	elongation factor G	fusA2	ko:K02355
TRINITY_DN7_c0_g2_i1	0.0	916.0	Histidine kinase	-	-
TRINITY_DN7_c0_g2_i2	0.0	1913.0	repeat protein	-	-
TRINITY_DN7_c1_g1_i1	0.0	974.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN84_c0_g1_i3	0.0	940.0	Amino acid permease family protein	-	-
TRINITY_DN89_c0_g1_i1	0.0	893.0	Catalyzes the reversible phosphatidyl group transfer from one phosphatidylglycerol molecule to another to form cardiolipin (CL) (diphosphatidylglycerol) and glycerol	cls	ko:K06131
TRINITY_DN8_c0_g1_i1	5.61e-186	522.0	Transposase	-	ko:K07496
TRINITY_DN113_c0_g2_i1	8.88e-124	372.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN55_c0_g2_i1	0.0	1257.0	transporter of a GTP-driven Fe(2) uptake system	feoB	ko:K04759

Table S4.5: The KEGG pathways of the differentially expressed transcripts in *Clostridium perfringens* treated with Clindamycin.

Query	e-value	Score	Description	Preferred name	KEGG
TRINITY_DN109_c0_g1_i1	8.35e-267	735.0	signal transduction protein with a C-terminal ATPase domain	comD	ko:K07706,ko:K12294
TRINITY_DN23_c0_g1_i1	0.0	1373.0	exonuclease	sbcC	ko:K03546
TRINITY_DN55_c0_g2_i1	0.0	1257.0	transporter of a GTP-driven Fe(2) uptake system	feoB	ko:K04759
TRINITY_DN12385_c0_g1_i1	3.7e-237	654.0	Sulphur transport	yeeE	ko:K07112
TRINITY_DN113_c0_g2_i1	8.88e-124	372.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN104_c0_g1_i2	0.0	1108.0	iron hydrogenase, small subunit	hydA	ko:K00532,ko:K18332
TRINITY_DN10696_c0_g1_i1	1.86e-75	250.0	peptidoglycan catabolic process	-	-
TRINITY_DN12403_c0_g1_i1	2.64e-41	146.0	Belongs to the UPF0753 family	ybcC	ko:K09822
TRINITY_DN13960_c0_g1_i1	6.88e-110	322.0	Ornithine cyclodeaminase	rapL	ko:K01750
TRINITY_DN13976_c0_g1_i1	0.0	1550.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN15830_c0_g1_i1	0.0	947.0	Polyphosphate kinase 2 (PPK2)	-	-
TRINITY_DN15884_c0_g1_i1	2.47e-272	747.0	Nucleoside	nupC	ko:K03317,ko:K11535
TRINITY_DN15906_c0_g1_i1	1.93e-141	421.0	Lysin motif	ebpS	-
TRINITY_DN18_c0_g1_i5	0.0	885.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN1_c0_g1_i11	0.0	2130.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN233_c0_g1_i1	1.44e-159	448.0	Poly-gamma-glutamate hydrolase	ymaC	-
TRINITY_DN241_c0_g1_i1	2.28e-71	228.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN274_c0_g1_i1	1.5e-314	856.0	Glutamate-1-semialdehyde aminotransferase	hemL	ko:K01845
TRINITY_DN35_c0_g1_i2	3.41e-79	255.0	PAS domain	-	-
TRINITY_DN55_c0_g3_i2	0.0	984.0	CotH protein	-	-
TRINITY_DN69_c0_g2_i1	1.97e-214	595.0	Relaxase/Mobilisation nuclease domain	-	-
TRINITY_DN73_c0_g1_i1	1.79e-83	248.0	IF-3 binds to the 30S ribosomal subunit and shifts the equilibrum between 70S ribosomes and their 50S and 30S subunits in favor of the free subunits, thus enhancing the availability of 30S subunits on which protein synthesis initiation begins	infC	ko:K02520
TRINITY_DN81_c0_g1_i1	0.0	892.0	Na Pi-cotransporter II-like protein	-	ko:K03324
TRINITY_DN9186_c0_g1_i1	5.66e-168	469.0	Purine nucleoside phosphorylase	deoD	ko:K03784
TRINITY_DN91_c0_g1_i1	0.0	963.0	Resolvase, N terminal domain	-	-
TRINITY_DN9221_c0_g1_i1	0.0	1372.0	accessory protein	tex	ko:K06959

TRINITY_DN97_c0_g1_i1	0.0	2967.0	Psort location Cytoplasmic, score 7.50	-	ko:K15923
TRINITY_DN18_c0_g1_i3	0.0	937.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN33_c0_g2_i2	0.0	967.0	import. Responsible for energy coupling to the transport system	rbsA	ko:K10441,ko:K10542
TRINITY_DN43_c0_g2_i1	1.54e-29	111.0	YSIRK type signal peptide	-	ko:K13733,ko:K14201
TRINITY_DN70_c0_g1_i1	1.34e-130	379.0	Helix-turn-helix domain	-	ko:K07496
TRINITY_DN0_c0_g1_i3	0.0	1459.0	DNA-dependent ATPase and ATP-dependent 5'-3' DNA helicase. Has no activity on blunt DNA or DNA with 3'-overhangs, requires at least 10 bases of 5'-ssDNA for helicase activity	recD2	ko:K03581
TRINITY_DN100_c1_g1_i1	5.33e-124	369.0	Regulatory protein BlaR1	blaR1-1	ko:K02172,ko:K02547
TRINITY_DN10_c0_g1_i1	3.05e-297	812.0	The central subunit of the protein translocation channel SecYEG. Consists of two halves formed by TMs 1-5 and 6-10. These two domains form a lateral gate at the front which open onto the bilayer between TMs 2 and 7, and are clamped together by SecE at the back. The channel is closed by both a pore ring composed of hydrophobic SecY resides and a short helix (helix 2A) on the extracellular side of the membrane which forms a plug. The plug probably moves laterally to allow the channel to open. The ring and the pore may move independently	secY	ko:K03076
TRINITY_DN113_c0_g1_i1	1.22e-72	235.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN119_c0_g1_i1	3.32e-185	528.0	Catalyzes the reversible conversion of 2- phosphoglycerate into phosphoenolpyruvate. It is essential for the degradation of carbohydrates via glycolysis	eno	ko:K01689
TRINITY_DN121_c0_g3_i1	1.94e-231	640.0	alcohol dehydrogenase	4hbD	ko:K18120
TRINITY_DN122_c0_g1_i1	0.0	1773.0	DNA polymerase	dnaE	ko:K02337,ko:K14162
TRINITY_DN122_c0_g2_i1	1.39e-81	241.0	Domain of Unknown Function (DUF1540)	-	-
TRINITY_DN12_c0_g1_i1	0.0	1918.0	Polysaccharide lyase family 8, C-terminal beta-sandwich domain	-	ko:K01727
TRINITY_DN131_c0_g1_i3	4.46e-258	710.0	ABC-type antimicrobial peptide transport system, permease component	-	ko:K02004
TRINITY_DN14396_c0_g1_i1	1.12e-169	487.0	Histidine kinase	phoR	ko:K07636
TRINITY_DN14744_c0_g1_i1	4.56e-128	382.0	COG3209 Rhs family protein	-	-
TRINITY_DN14_c0_g1_i1	0.0	998.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN14_c0_g2_i1	3.37e-108	335.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN15618_c0_g1_i1	1.07e-72	221.0	acetyltransferase, isoleucine patch superfamily	vioB	ko:K21379
TRINITY_DN15_c0_g1_i4	0.0	1288.0	helicase	-	-
TRINITY_DN17_c0_g1_i1	0.0	5038.0	Belongs to the glycosyl hydrolase 13 family	pulA	ko:K01200
TRINITY_DN181_c0_g1_i2	0.0	902.0	Biotin carboxylase	accC	ko:K01961
TRINITY_DN18_c0_g1_i2	0.0	1791.0	amino acids such as threonine, to avoid such errors, it has a posttransfer editing activity that hydrolyzes mischarged Thr-tRNA(Val) in a tRNA-dependent manner	valS	ko:K01873
TRINITY_DN19_c0_g1_i1	1.07e-254	716.0	ABC transporter	-	ko:K18231
TRINITY_DN234_c0_g1_i1	0.0	2156.0	Dynamin family	-	-
TRINITY_DN236_c0_g1_i1	0.0	1036.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714

TRINITY_DN254_c0_g1_i1	1.06e-114	339.0	Belongs to the folylpolyglutamate synthase family	folC	ko:K11754
TRINITY_DN28_c0_g3_i1	0.0	1425.0	ABC transporter	-	ko:K06147
TRINITY_DN28_c0_g3_i3	3.09e-97	283.0	PFAM regulatory protein, MarR	-	-
TRINITY_DN29_c0_g1_i1	0.0	969.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN31_c0_g1_i1	2.85e-154	454.0	cellulose binding	-	ko:K01179,ko:K07318
TRINITY_DN33_c0_g1_i3	0.0	1161.0	helicase	-	ko:K17675
TRINITY_DN33_c0_g3_i1	1.44e-259	716.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN34_c0_g1_i1	0.0	1182.0	Required for accurate and efficient protein synthesis under certain stress conditions. May act as a fidelity factor of the translation reaction, by catalyzing a one-codon backward translocation of tRNAs on improperly translocated ribosomes. Back- translocation proceeds from a post-translocation (POST) complex to a pre-translocation (PRE) complex, thus giving elongation factor G a second chance to translocate the tRNAs correctly. Binds to ribosomes in a GTP-dependent manner	lepA	ko:K03596
TRINITY_DN35_c0_g1_i4	6.41e-286	794.0	PAS domain	-	-
TRINITY_DN35_c0_g1_i6	0.0	1024.0	PAS domain	-	-
TRINITY_DN38_c0_g1_i1	5.89e-272	744.0	Belongs to the glycosyl hydrolase 8 (cellulase D) family	-	-
TRINITY_DN41_c0_g1_i6	0.0	1315.0	-	-	-
TRINITY_DN42_c0_g1_i1	0.0	1529.0	penicillin-binding protein 1A	mrcB	ko:K05366
TRINITY_DN47_c0_g3_i1	0.0	1324.0	In eubacteria ppGpp (guanosine 3'-diphosphate 5-' diphosphate) is a mediator of the stringent response that coordinates a variety of cellular activities in response to changes in nutritional abundance	relA	ko:K00951
TRINITY_DN51_c0_g1_i2	0.0	1926.0	Peptidase M16	-	ko:K06972
TRINITY_DN55_c0_g1_i2	0.0	1774.0	Permease	-	ko:K02004
TRINITY_DN55_c0_g1_i4	0.0	984.0	CotH protein	-	-
TRINITY_DN55_c0_g1_i8	1.01e-133	390.0	Amino acid permease	-	-
TRINITY_DN56_c0_g1_i8	0.0	941.0	amino acid ABC transporter	-	ko:K16961,ko:K16962
TRINITY_DN5_c0_g1_i1	0.0	3411.0	Endo-alpha-N-acetylgalactosaminidase	-	ko:K17624
TRINITY_DN5_c0_g2_i1	0.0	2738.0	domain protein	-	-
TRINITY_DN63_c0_g1_i1	0.0	880.0	Involved in cell wall formation. Catalyzes the final step in the synthesis of UDP-N-acetylmuramoyl-pentapeptide, the precursor of murein	murF	ko:K01929
TRINITY_DN65_c0_g1_i1	0.0	922.0	Catalyzes the reduction of hydroxylamine to form NH(3) and H(2)O	hcp	ko:K05601
TRINITY_DN66_c0_g1_i1	2.23e-99	297.0	-	-	-
TRINITY_DN72_c0_g1_i4	0.0	1429.0	beta-galactosidase	pbg	ko:K12308
TRINITY_DN74_c0_g1_i1	2.37e-105	306.0	-	-	-
TRINITY_DN75_c0_g1_i1	0.0	1436.0	Ribonucleoside-triphosphate reductase	nrdD	ko:K21636
TRINITY_DN76_c0_g1_i3	0.0	1353.0	elongation factor G	fusA2	ko:K02355

TRINITY_DN7_c0_g2_i1	0.0	916.0	Histidine kinase	-	-
TRINITY_DN7_c0_g2_i2	0.0	1913.0	repeat protein	-	-
TRINITY_DN7_c1_g1_i1	0.0	974.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN84_c0_g1_i3	0.0	940.0	Amino acid permease family protein	-	-
TRINITY_DN89_c0_g1_i1	0.0	893.0	Catalyzes the reversible phosphatidyl group transfer from one phosphatidylglycerol molecule to another to form cardiolipin (CL) (diphosphatidylglycerol) and glycerol	cls	ko:K06131
TRINITY_DN8_c0_g1_i1	5.61e-186	522.0	Transposase	-	ko:K07496
TRINITY_DN9305_c0_g1_i1	9.49e-117	341.0	Belongs to the bacterial solute-binding protein 9 family	adcA	ko:K09815,ko:K09818

Table S4.5: The KEGG pathways of the differentially expressed transcripts in *Clostridium perfringens* treated with Sulfamethoxazole.

Query	e-value	Score	Description	Preferred name	KEGG
TRINITY_DN131_c0_g1_i3	4.46e-258	710.0	ABC-type antimicrobial peptide transport system, permease component	-	ko:K02004
TRINITY_DN122_c0_g1_i1	0.0	1773.0	DNA polymerase	dnaE	ko:K02337,ko:K14162
TRINITY_DN55_c0_g1_i4	0.0	984.0	CotH protein	-	-
TRINITY_DN7_c0_g2_i2	0.0	1913.0	repeat protein	-	-
TRINITY_DN10696_c0_g1_i1	1.86e-75	250.0	peptidoglycan catabolic process	-	-
TRINITY_DN15884_c0_g1_i1	2.47e-272	747.0	Nucleoside	nupC	ko:K03317,ko:K11535
TRINITY_DN233_c0_g1_i1	1.44e-159	448.0	Poly-gamma-glutamate hydrolase	ymaC	-
TRINITY_DN9186_c0_g1_i1	5.66e-168	469.0	Purine nucleoside phosphorylase	deoD	ko:K03784
TRINITY_DN13960_c0_g1_i1	6.88e-110	322.0	Ornithine cyclodeaminase	rapL	ko:K01750
TRINITY_DN13976_c0_g1_i1	0.0	1550.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN15830_c0_g1_i1	0.0	947.0	Polyphosphate kinase 2 (PPK2)	-	-
TRINITY_DN15906_c0_g1_i1	1.93e-141	421.0	Lysin motif	ebpS	-
TRINITY_DN241_c0_g1_i1	2.28e-71	228.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN81_c0_g1_i1	0.0	892.0	Na Pi-cotransporter II-like protein	-	ko:K03324
TRINITY_DN9221_c0_g1_i1	0.0	1372.0	accessory protein	tex	ko:K06959
TRINITY_DN109_c0_g1_i1	8.35e-267	735.0	signal transduction protein with a C-terminal ATPase domain	comD	ko:K07706,ko:K12294
TRINITY_DN23_c0_g1_i1	0.0	1373.0	exonuclease	sbcC	ko:K03546
TRINITY_DN113_c0_g1_i1	1.22e-72	235.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN14744_c0_g1_i1	4.56e-128	382.0	COG3209 Rhs family protein	-	-
TRINITY_DN14_c0_g1_i1	0.0	998.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN34_c0_g1_i1	0.0	1182.0	Required for accurate and efficient protein synthesis under certain stress conditions. May act as a fidelity factor of the translation reaction, by catalyzing a one-codon backward translocation of tRNAs on improperly translocated ribosomes. Back- translocation proceeds from a post-translocation (POST) complex to a pre-translocation (PRE) complex, thus giving elongation factor G a second chance to translocate the tRNAs correctly. Binds to ribosomes in a GTP-dependent manner	lepA	ko:K03596
TRINITY_DN35_c0_g1_i4	6.41e-286	794.0	PAS domain	-	-

TRINITY_DN55_c0_g1_i2	0.0	1774.0	Permease	-	ko:K02004
TRINITY_DN56_c0_g1_i8	0.0	941.0	amino acid ABC transporter	-	ko:K16961,ko:K16962
TRINITY_DN84_c0_g1_i3	0.0	940.0	Amino acid permease family protein	-	-
TRINITY_DN113_c0_g2_i1	8.88e-124	372.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN1_c0_g1_i11	0.0	2130.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN55_c0_g3_i2	0.0	984.0	CotH protein	-	-
TRINITY_DN12385_c0_g1_i1	3.7e-237	654.0	Sulphur transport	yeeE	ko:K07112
TRINITY_DN104_c0_g1_i2	0.0	1108.0	iron hydrogenase, small subunit	hydA	ko:K00532,ko:K18332
TRINITY_DN12403_c0_g1_i1	2.64e-41	146.0	Belongs to the UPF0753 family	ybcC	ko:K09822
TRINITY_DN274_c0_g1_i1	1.5e-314	856.0	Glutamate-1-semialdehyde aminotransferase	hemL	ko:K01845
TRINITY_DN69_c0_g2_i1	1.97e-214	595.0	Relaxase/Mobilisation nuclease domain	-	-
TRINITY_DN73_c0_g1_i1	1.79e-83	248.0	IF-3 binds to the 30S ribosomal subunit and shifts the equilibrum between 70S ribosomes and their 50S and 30S subunits in favor of the free subunits, thus enhancing the availability of 30S subunits on which protein synthesis initiation begins	infC	ko:K02520
TRINITY_DN91_c0_g1_i1	0.0	963.0	Resolvase, N terminal domain	-	-
TRINITY_DN97_c0_g1_i1	0.0	2967.0	Psort location Cytoplasmic, score 7.50	-	ko:K15923
TRINITY_DN18_c0_g1_i2	0.0	1791.0	amino acids such as threonine, to avoid such errors, it has a posttransfer editing activity that hydrolyzes mischarged Thr-tRNA(Val) in a tRNA-dependent manner	valS	ko:K01873
TRINITY_DN0_c0_g1_i3	0.0	1459.0	DNA-dependent ATPase and ATP-dependent 5'-3' DNA helicase. Has no activity on blunt DNA or DNA with 3'-overhangs, requires at least 10 bases of 5'-ssDNA for helicase activity	recD2	ko:K03581
TRINITY_DN10_c0_g1_i1	3.05e-297	812.0	The central subunit of the protein translocation channel SecYEG. Consists of two halves formed by TMs 1-5 and 6-10. These two domains form a lateral gate at the front which open onto the bilayer between TMs 2 and 7, and are clamped together by SecE at the back. The channel is closed by both a pore ring composed of hydrophobic SecY resides and a short helix (helix 2A) on the extracellular side of the membrane which forms a plug. The plug probably moves laterally to allow the channel to open. The ring and the pore may move independently	secY	ko:K03076
TRINITY_DN122_c0_g2_i1	1.39e-81	241.0	Domain of Unknown Function (DUF1540)	-	-
TRINITY_DN236_c0_g1_i1	0.0	1036.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN29_c0_g1_i1	0.0	969.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN31_c0_g1_i1	2.85e-154	454.0	cellulose binding	-	ko:K01179,ko:K07318
TRINITY_DN33_c0_g1_i3	0.0	1161.0	helicase	-	ko:K17675
TRINITY_DN33_c0_g3_i1	1.44e-259	716.0	Domain of unknown function (DUF3502)	-	ko:K17318

TRINITY_DN47_c0_g3_i1	0.0	1324.0	In eubacteria ppGpp (guanosine 3'-diphosphate 5-' diphosphate) is a mediator of the stringent response that coordinates a variety of cellular activities in response to changes in nutritional abundance	relA	ko:K00951
TRINITY_DN63_c0_g1_i1	0.0	880.0	Involved in cell wall formation. Catalyzes the final step in the synthesis of UDP-N-acetylmuramoyl-pentapeptide, the precursor of murein	murF	ko:K01929
TRINITY_DN76_c0_g1_i3	0.0	1353.0	elongation factor G	fusA2	ko:K02355
TRINITY_DN55_c0_g2_i1	0.0	1257.0	transporter of a GTP-driven Fe(2) uptake system	feoB	ko:K04759
TRINITY_DN35_c0_g1_i2	3.41e-79	255.0	PAS domain	-	-
TRINITY_DN43_c0_g2_i1	1.54e-29	111.0	YSIRK type signal peptide	-	ko:K13733,ko:K14201
TRINITY_DN70_c0_g1_i1	1.34e-130	379.0	Helix-turn-helix domain	-	ko:K07496
TRINITY_DN44_c0_g1_i10	0.0	873.0	UPF0210 protein	-	ko:K09157
TRINITY_DN51_c0_g1_i1	0.0	2327.0	Oxidoreductase required for the transfer of electrons from pyruvate to flavodoxin	nifJ	ko:K03737
TRINITY_DN100_c1_g1_i1	5.33e-124	369.0	Regulatory protein BlaR1	blaR1-1	ko:K02172,ko:K02547
TRINITY_DN12_c0_g1_i1	0.0	1918.0	Polysaccharide lyase family 8, C-terminal beta-sandwich domain	-	ko:K01727
TRINITY_DN14_c0_g2_i1	3.37e-108	335.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN15_c0_g1_i4	0.0	1288.0	helicase	-	-
TRINITY_DN17_c0_g1_i1	0.0	5038.0	Belongs to the glycosyl hydrolase 13 family	pulA	ko:K01200
TRINITY_DN181_c0_g1_i2	0.0	902.0	Biotin carboxylase	accC	ko:K01961
TRINITY_DN19_c0_g1_i1	1.07e-254	716.0	ABC transporter	-	ko:K18231
TRINITY_DN38_c0_g1_i1	5.89e-272	744.0	Belongs to the glycosyl hydrolase 8 (cellulase D) family	-	-
TRINITY_DN41_c0_g1_i6	0.0	1315.0	-	-	-
TRINITY_DN5_c0_g1_i1	0.0	3411.0	Endo-alpha-N-acetylgalactosaminidase	-	ko:K17624
TRINITY_DN5_c0_g2_i1	0.0	2738.0	domain protein	-	-
TRINITY_DN66_c0_g1_i1	2.23e-99	297.0	-	-	-
TRINITY_DN72_c0_g1_i4	0.0	1429.0	beta-galactosidase	pbg	ko:K12308
TRINITY_DN74_c0_g1_i1	2.37e-105	306.0	-	-	-
TRINITY_DN75_c0_g1_i1	0.0	1436.0	Ribonucleoside-triphosphate reductase	nrdD	ko:K21636
TRINITY_DN7_c0_g2_i1	0.0	916.0	Histidine kinase	-	-
TRINITY_DN7_c1_g1_i1	0.0	974.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN8_c0_g1_i1	5.61e-186	522.0	Transposase	-	ko:K07496

TRINITY_DN18_c0_g1_i5	0.0	885.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN28_c0_g3_i1	0.0	1425.0	ABC transporter	-	ko:K06147
TRINITY_DN35_c0_g1_i6	0.0	1024.0	PAS domain	-	-
TRINITY_DN42_c0_g1_i1	0.0	1529.0	penicillin-binding protein 1A	mrcB	ko:K05366
TRINITY_DN51_c0_g1_i2	0.0	1926.0	Peptidase M16	-	ko:K06972
TRINITY_DN55_c0_g1_i8	1.01e-133	390.0	Amino acid permease	-	-
TRINITY_DN65_c0_g1_i1	0.0	922.0	Catalyzes the reduction of hydroxylamine to form NH(3) and H(2)O	hcp	ko:K05601
TRINITY_DN18_c0_g1_i3	0.0	937.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN33_c0_g2_i2	0.0	967.0	import. Responsible for energy coupling to the transport system	rbsA	ko:K10441,ko:K10542

Table S4.6: The KEGG pathways of the differentially expressed transcripts in *Clostridium perfringens* treated with Cefoxitin.

Query	e-value	Score	Description	Preferred name	KEGG
TRINITY_DN104_c0_g1_i2	0.0	1108.0	iron hydrogenase, small subunit	hydA	ko:K00532,ko:K18332
TRINITY_DN121_c0_g3_i1	1.94e-231	640.0	alcohol dehydrogenase	4hbD	ko:K18120
TRINITY_DN341_c0_g1_i1	4.88e-155	452.0	Belongs to the heme-copper respiratory oxidase family	qoxB	ko:K02827
TRINITY_DN7786_c0_g1_i1	9.46e-260	712.0	Iron-containing alcohol dehydrogenase	gldA	ko:K00005
TRINITY_DN69_c0_g2_i1	1.97e-214	595.0	Relaxase/Mobilisation nuclease domain	-	-
TRINITY_DN13960_c0_g1_i1	6.88e-110	322.0	Ornithine cyclodeaminase	rapL	ko:K01750
TRINITY_DN9131_c0_g1_i1	3.89e-305	831.0	COG2309 Leucyl aminopeptidase (aminopeptidase T)	pepS	ko:K19689
TRINITY_DN2205_c0_g2_i1	4.02e-185	513.0	Belongs to the PAPS reductase family. CysH subfamily	cysH	ko:K00390
TRINITY_DN7778_c0_g1_i1	5.11e-302	825.0	phosphorylase	deoA	ko:K00756
TRINITY_DN97_c0_g1_i1	0.0	2967.0	Psort location Cytoplasmic, score 7.50	-	ko:K15923
TRINITY_DN274_c0_g1_i1	1.5e-314	856.0	Glutamate-1-semialdehyde aminotransferase	hemL	ko:K01845
TRINITY_DN128_c0_g1_i2	0.0	1732.0	cyanophycin synthetase	cphA	ko:K03802
TRINITY_DN12489_c0_g1_i1	1.55e-175	490.0	Enoyl- acyl-carrier-protein reductase	fabI	ko:K00208
TRINITY_DN147_c0_g1_i1	3.33e-192	534.0	Lipid kinase	dagK	ko:K07029
TRINITY_DN130_c0_g1_i1	6.48e-152	434.0	Catalyzes the conversion of 1-hydroxy-2-methyl-2-(E)- butenyl 4-diphosphate (HMBPP) into a mixture of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Acts in the terminal step of the DOXP MEP pathway for isoprenoid precursor biosynthesis	ispH	ko:K02945,ko:K03527
TRINITY_DN166_c0_g1_i1	4.25e-139	411.0	Catalyzes the GTP-dependent ribosomal translocation step during translation elongation. During this step, the ribosome changes from the pre-translocational (PRE) to the post-translocational (POST) state as the newly formed A-site-bound peptidyl-tRNA and P-site-bound deacylated tRNA move to the P and E sites, respectively. Catalyzes the coordinated movement of the two tRNA molecules, the mRNA and conformational changes in the ribosome	fusA	ko:K02355
TRINITY_DN73_c0_g1_i1	1.79e-83	248.0	IF-3 binds to the 30S ribosomal subunit and shifts the equilibrum between 70S ribosomes and their 50S and 30S subunits in favor of the free subunits, thus enhancing the availability of 30S subunits on which protein synthesis initiation begins	infC	ko:K02520
TRINITY_DN241_c0_g1_i1	2.28e-71	228.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046
TRINITY_DN9221_c0_g1_i1	0.0	1372.0	accessory protein	tex	ko:K06959
TRINITY_DN823_c0_g1_i1	0.0	1135.0	Serine threonine protein kinase	prkC	ko:K08884,ko:K12132
TRINITY_DN141_c0_g1_i1	6.44e-128	378.0	Probable transposase	-	ko:K07496

TRINITY_DN23_c0_g1_i1	0.0	1373.0	exonuclease	sbcC	ko:K03546
TRINITY_DN91_c0_g1_i1	0.0	963.0	Resolvase, N terminal domain	-	-
TRINITY_DN13954_c0_g1_i1	6.45e-209	578.0	penicillin-binding protein	pbp1B	ko:K03693,ko:K12551
TRINITY_DN13976_c0_g1_i1	0.0	1550.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN14014_c0_g1_i1	5.22e-124	361.0	Cysteine protease able to cleave elastin, insulin, myoglobin, fibronectin, fibrinogen, HMW-kininogen, alpha-1- protease inhibitor and alpha-1-antitrypsin. Along with other extracellular proteases may contribute to the colonization and infection of human tissues (By similarity)	-	ko:K08258
TRINITY_DN15906_c0_g1_i1	1.93e-141	421.0	Lysin motif	ebpS	-
TRINITY_DN216_c0_g1_i1	4.16e-78	244.0	Catalyzes the last two sequential reactions in the de novo biosynthetic pathway for UDP-N-acetylglucosamine (UDP- GlcNAc). The C-terminal domain catalyzes the transfer of acetyl group from acetyl coenzyme A to glucosamine-1-phosphate (GlcN-1-P) to produce N-acetylglucosamine-1-phosphate (GlcNAc-1-P), which is converted into UDP-GlcNAc by the transfer of uridine 5- monophosphate (from uridine 5-triphosphate), a reaction catalyzed by the N-terminal domain	glmU	ko:K04042
TRINITY_DN9401_c0_g1_i1	1.11e-191	532.0	Glycosyl hydrolases family 25	-	ko:K07273
TRINITY_DN183_c0_g1_i2	0.0	1017.0	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions	groL	ko:K04077
TRINITY_DN10764_c0_g1_i1	9.09e-62	195.0	ABC transporter permease	metI	ko:K02072
TRINITY_DN529_c0_g1_i1	2.32e-201	558.0	ATP-binding (A) component of a common energy-coupling factor (ECF) ABC-transporter complex. Unlike classic ABC transporters this ECF transporter provides the energy necessary to transport a number of different substrates	ecfA2	ko:K16787
TRINITY_DN81_c0_g1_i1	0.0	892.0	Na Pi-cotransporter II-like protein	-	ko:K03324
TRINITY_DN12403_c0_g1_i1	2.64e-41	146.0	Belongs to the UPF0753 family	ybcC	ko:K09822
TRINITY_DN14016_c0_g1_i1	9.47e-70	211.0	Domain of unknown function (DUF4889)	-	-
TRINITY_DN14294_c0_g1_i1	9.03e-108	310.0	COG3942 Surface antigen	p40	ko:K21471
TRINITY_DN15830_c0_g1_i1	0.0	947.0	Polyphosphate kinase 2 (PPK2)	-	-
TRINITY_DN234_c0_g1_i1	0.0	2156.0	Dynamin family	-	-
TRINITY_DN87_c0_g1_i1	0.0	888.0	DUF1237	-	ko:K09704
TRINITY_DN9194_c0_g1_i1	0.0	1503.0	Catalyzes the transfer of a lysyl group from L-lysyl- tRNA(Lys) to membrane-bound phosphatidylglycerol (PG), which produces lysylphosphatidylglycerol (LPG), a major component of the bacterial membrane with a positive net charge. LPG synthesis contributes to bacterial virulence as it is involved in the resistance mechanism against cationic antimicrobial peptides (CAMP) produces by the host's immune system (defensins, cathelicidins) and by the competing microorganisms	mprF	ko:K14205
TRINITY_DN9247_c0_g1_i1	5.47e-196	543.0	NAD synthase	larE	ko:K06864
TRINITY_DN9366_c0_g1_i1	2.99e-162	456.0	CAAX amino terminal protease family protein	ydiL	ko:K07052

TRINITY_DN109_c0_g1_i1	8.35e-267	735.0	signal transduction protein with a C-terminal ATPase domain	comD	ko:K07706,ko:K12294
TRINITY_DN14396_c0_g1_i1	1.12e-169	487.0	Histidine kinase	phoR	ko:K07636
TRINITY_DN35_c0_g1_i2	3.41e-79	255.0	PAS domain	-	-
TRINITY_DN124_c0_g1_i1	0.0	913.0	type I restriction-modification system	hsdM	ko:K03427
TRINITY_DN29_c0_g1_i1	0.0	969.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN65_c0_g1_i1	0.0	922.0	Catalyzes the reduction of hydroxylamine to form NH(3) and H(2)O	hcp	ko:K05601
TRINITY_DN7_c1_g1_i1	0.0	974.0	Produces ATP from ADP in the presence of a proton gradient across the membrane. The alpha chain is a regulatory subunit	atpA	ko:K02111
TRINITY_DN43_c0_g2_i1	1.54e-29	111.0	YSIRK type signal peptide	-	ko:K13733,ko:K14201
TRINITY_DN14_c0_g1_i1	0.0	998.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN14_c0_g2_i1	3.37e-108	335.0	E domain	-	ko:K13732,ko:K13733,ko:K14195
TRINITY_DN55_c0_g1_i8	1.01e-133	390.0	Amino acid permease	-	-
TRINITY_DN84_c0_g1_i3	0.0	940.0	Amino acid permease family protein	-	-
TRINITY_DN75_c0_g1_i1	0.0	1436.0	Ribonucleoside-triphosphate reductase	nrdD	ko:K21636
TRINITY_DN17_c0_g1_i1	0.0	5038.0	Belongs to the glycosyl hydrolase 13 family	pulA	ko:K01200
TRINITY_DN18_c0_g1_i3	0.0	937.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN18_c0_g1_i5	0.0	885.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN33_c0_g3_i1	1.44e-259	716.0	Domain of unknown function (DUF3502)	-	ko:K17318
TRINITY_DN38_c0_g1_i1	5.89e-272	744.0	Belongs to the glycosyl hydrolase 8 (cellulase D) family	-	-
TRINITY_DN5_c0_g1_i1	0.0	3411.0	Endo-alpha-N-acetylgalactosaminidase	-	ko:K17624
TRINITY_DN5_c0_g2_i1	0.0	2738.0	domain protein	-	-
TRINITY_DN72_c0_g1_i4	0.0	1429.0	beta-galactosidase	pbg	ko:K12308
TRINITY_DN113_c0_g1_i1	1.22e-72	235.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN113_c0_g2_i1	8.88e-124	372.0	One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex	infB	ko:K02519
TRINITY_DN18_c0_g1_i2	0.0	1791.0	amino acids such as threonine, to avoid such errors, it has a posttransfer editing activity that hydrolyzes mischarged Thr-tRNA(Val) in a tRNA-dependent manner	valS	ko:K01873
TRINITY_DN76_c0_g1_i3	0.0	1353.0	elongation factor G	fusA2	ko:K02355

TRINITY_DN47_c0_g3_i1	0.0	1324.0	In eubacteria ppGpp (guanosine 3'-diphosphate 5-' diphosphate) is a mediator of the stringent response that coordinates a variety of cellular activities in response to changes in nutritional abundance	relA	ko:K00951
TRINITY_DN100_c1_g1_i1	5.33e-124	369.0	Regulatory protein BlaR1	blaR1-1	ko:K02172,ko:K02547
TRINITY_DN0_c0_g1_i3	0.0	1459.0	DNA-dependent ATPase and ATP-dependent 5'-3' DNA helicase. Has no activity on blunt DNA or DNA with 3'-overhangs, requires at least 10 bases of 5'-ssDNA for helicase activity	recD2	ko:K03581
TRINITY_DN122_c0_g1_i1	0.0	1773.0	DNA polymerase	dnaE	ko:K02337,ko:K14162
TRINITY_DN15_c0_g1_i4	0.0	1288.0	helicase	-	-
TRINITY_DN33_c0_g1_i3	0.0	1161.0	helicase	-	ko:K17675
TRINITY_DN70_c0_g1_i1	1.34e-130	379.0	Helix-turn-helix domain	-	ko:K07496
TRINITY_DN8_c0_g1_i1	5.61e-186	522.0	Transposase	-	ko:K07496
TRINITY_DN14744_c0_g1_i1	4.56e-128	382.0	COG3209 Rhs family protein	-	-
TRINITY_DN236_c0_g1_i1	0.0	1036.0	the rest of the oligosaccharide is released intact. Cleaves the peptidoglycan connecting the daughter cells at the end of the cell division cycle, resulting in the separation of the two newly divided cells. Acts as an autolysin in penicillin-induced lysis (By similarity)	atl	ko:K13714
TRINITY_DN34_c0_g1_i1	0.0	1182.0	Required for accurate and efficient protein synthesis under certain stress conditions. May act as a fidelity factor of the translation reaction, by catalyzing a one-codon backward translocation of tRNAs on improperly translocated ribosomes. Back- translocation proceeds from a post-translocation (POST) complex to a pre-translocation (PRE) complex, thus giving elongation factor G a second chance to translocate the tRNAs correctly. Binds to ribosomes in a GTP-dependent manner	lepA	ko:K03596
TRINITY_DN42_c0_g1_i1	0.0	1529.0	penicillin-binding protein 1A	mrcB	ko:K05366
TRINITY_DN55_c0_g1_i4	0.0	984.0	CotH protein	-	-
TRINITY_DN63_c0_g1_i1	0.0	880.0	Involved in cell wall formation. Catalyzes the final step in the synthesis of UDP-N-acetylmuramoyl-pentapeptide, the precursor of murein	murF	ko:K01929
TRINITY_DN12_c0_g1_i1	0.0	1918.0	Polysaccharide lyase family 8, C-terminal beta-sandwich domain	-	ko:K01727
TRINITY_DN33_c0_g2_i2	0.0	967.0	import. Responsible for energy coupling to the transport system	rbsA	ko:K10441,ko:K10542
TRINITY_DN55_c0_g2_i1	0.0	1257.0	transporter of a GTP-driven Fe(2) uptake system	feoB	ko:K04759
TRINITY_DN56_c0_g1_i8	0.0	941.0	amino acid ABC transporter	-	ko:K16961,ko:K16962
TRINITY_DN122_c0_g2_i1	1.39e-81	241.0	Domain of Unknown Function (DUF1540)	-	-
TRINITY_DN19_c0_g1_i1	1.07e-254	716.0	ABC transporter	-	ko:K18231
TRINITY_DN31_c0_g1_i1	2.85e-154	454.0	cellulose binding	-	ko:K01179,ko:K07318
TRINITY_DN51_c0_g1_i2	0.0	1926.0	Peptidase M16	-	ko:K06972
TRINITY_DN7_c0_g2_i2	0.0	1913.0	repeat protein	-	-
TRINITY_DN35_c0_g1_i4	6.41e-286	794.0	PAS domain	-	-

TRINITY_DN35_c0_g1_i6	0.0	1024.0	PAS domain	-	-
TRINITY_DN7_c0_g2_i1	0.0	916.0	Histidine kinase	-	-
TRINITY_DN10_c0_g1_i1	3.05e-297	812.0	The central subunit of the protein translocation channel SecYEG. Consists of two halves formed by TMs 1-5 and 6-10. These two domains form a lateral gate at the front which open onto the bilayer between TMs 2 and 7, and are clamped together by SecE at the back. The channel is closed by both a pore ring composed of hydrophobic SecY resides and a short helix (helix 2A) on the extracellular side of the membrane which forms a plug. The plug probably moves laterally to allow the channel to open. The ring and the pore may move independently	secY	ko:K03076
TRINITY_DN28_c0_g3_i1	0.0	1425.0	ABC transporter	-	ko:K06147
TRINITY_DN55_c0_g1_i2	0.0	1774.0	Permease	-	ko:K02004
TRINITY_DN10820_c0_g1_i1	7.92e-88	265.0	Dehydrogenase E1 component	acoA	ko:K21416
TRINITY_DN169_c0_g1_i3	0.0	1123.0	alcohol dehydrogenase	adhE	ko:K04072
TRINITY_DN7377_c0_g1_i1	1.03e-48	157.0	Pyruvate flavodoxin/ferredoxin oxidoreductase, thiamine diP-bdg	-	-
TRINITY_DN14009_c0_g1_i1	1.99e-128	367.0	Prolyl oligopeptidase family	ytmA	-
TRINITY_DN661_c0_g1_i1	3.12e-135	395.0	Gamma-glutamyltranspeptidase	-	ko:K00681
TRINITY_DN7910_c0_g1_i1	9.6e-66	209.0	COG0626 Cystathionine beta-lyases cystathionine gamma-synthases	metB	ko:K01739
TRINITY_DN86_c0_g1_i1	0.0	1138.0	Psort location Cytoplasmic, score	-	-
TRINITY_DN15884_c0_g1_i1	2.47e-272	747.0	Nucleoside	nupC	ko:K03317,ko:K11535
TRINITY_DN9186_c0_g1_i1	5.66e-168	469.0	Purine nucleoside phosphorylase	deoD	ko:K03784
TRINITY_DN14096_c0_g1_i1	3.02e-220	607.0	Catalyzes the ATP-dependent phosphorylation of L- homoserine to L-homoserine phosphate	thrB	ko:K00872
TRINITY_DN175_c0_g1_i1	0.0	1020.0	Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate	gpmI	ko:K15633
TRINITY_DN254_c0_g1_i1	1.06e-114	339.0	Belongs to the folylpolyglutamate synthase family	folC	ko:K11754
TRINITY_DN138_c0_g1_i1	0.0	1275.0	Catalyzes the ATP-dependent amidation of deamido-NAD to form NAD. Uses L-glutamine as a nitrogen source	nadE	ko:K01950
TRINITY_DN7906_c0_g1_i1	2.83e-74	223.0	Involved in the synthesis of autoinducer 2 (AI-2) which is secreted by bacteria and is used to communicate both the cell density and the metabolic potential of the environment. The regulation of gene expression in response to changes in cell density is called quorum sensing. Catalyzes the transformation of S-ribosylhomocysteine (RHC) to homocysteine (HC) and 4,5- dihydroxy-2,3-pentadione (DPD)	luxS	ko:K07173
TRINITY_DN9144_c0_g1_i1	3.25e-109	324.0	Glutamate-1-semialdehyde aminotransferase	hemL	ko:K01845
TRINITY_DN243_c0_g1_i1	1.04e-75	229.0	One of the primary rRNA binding proteins, it binds directly to 16S rRNA where it nucleates assembly of the body of the 30S subunit	rpsD	ko:K02986
TRINITY_DN823_c0_g2_i1	0.0	872.0	Specifically methylates the cytosine at position 967 (m5C967) of 16S rRNA	sun	ko:K03500
TRINITY_DN1_c0_g1_i11	0.0	2130.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoC	ko:K03046

TRINITY_DN28_c0_g3_i3	3.09e-97	283.0	PFAM regulatory protein, MarR	-	-
TRINITY_DN1_c0_g1_i1	0.0	2426.0	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates	rpoB	ko:K03043
TRINITY_DN7804_c0_g1_i1	1.17e-140	400.0	Represses a number of genes involved in the response to DNA damage (SOS response), including recA and lexA. In the presence of single-stranded DNA, RecA interacts with LexA causing an autocatalytic cleavage which disrupts the DNA-binding part of LexA, leading to derepression of the SOS regulon and eventually DNA repair	lexA	ko:K01356
TRINITY_DN10740_c0_g1_i1	2.07e-173	511.0	Transposase Tn3 family protein	-	-
TRINITY_DN136_c0_g1_i1	0.0	2248.0	Couples transcription and DNA repair by recognizing RNA polymerase (RNAP) stalled at DNA lesions. Mediates ATP-dependent release of RNAP and its truncated transcript from the DNA, and recruitment of nucleotide excision repair machinery to the damaged site	mfd	ko:K03723
TRINITY_DN199_c0_g1_i1	0.0	962.0	RNA polymerase that catalyzes the synthesis of short RNA molecules used as primers for DNA polymerase during DNA replication	dnaG	ko:K02316
TRINITY_DN662_c0_g2_i1	0.0	1680.0	A type II topoisomerase that negatively supercoils closed circular double-stranded (ds) DNA in an ATP-dependent manner to modulate DNA topology and maintain chromosomes in an underwound state. Negative supercoiling favors strand separation, and DNA replication, transcription, recombination and repair, all of which involve strand separation. Also able to catalyze the interconversion of other topological isomers of dsDNA rings, including catenanes and knotted rings. Type II topoisomerases break and join 2 DNA strands simultaneously in an ATP-dependent manner	gyrA	ko:K02469
TRINITY_DN55_c0_g3_i2	0.0	984.0	CotH protein	-	-
TRINITY_DN137_c0_g1_i1	0.0	1443.0	penicillin-binding protein	pbpX	ko:K08724,ko:K12552,ko:K12556
TRINITY_DN9404_c0_g1_i1	1.2e-201	558.0	Belongs to the peptidase S1B family	sspA	ko:K01318
TRINITY_DN157_c0_g1_i1	0.0	1569.0	ATP-dependent Clp protease, ATP-binding subunit ClpC	clpC	ko:K03696
TRINITY_DN179_c0_g1_i1	0.0	1484.0	ATP-dependent serine protease that mediates the selective degradation of mutant and abnormal proteins as well as certain short-lived regulatory proteins. Required for cellular homeostasis and for survival from DNA damage and developmental changes induced by stress. Degrades polypeptides processively to yield small peptide fragments that are 5 to 10 amino acids long. Binds to DNA in a double-stranded, site-specific manner	lon	ko:K01338
TRINITY_DN9305_c0_g1_i1	9.49e-117	341.0	Belongs to the bacterial solute-binding protein 9 family	adcA	ko:K09815,ko:K09818
TRINITY_DN1796_c0_g1_i1	3.52e-93	276.0	Part of the ABC transporter complex PhnCDE involved in phosphonates import. Responsible for energy coupling to the transport system	phnC	ko:K02041
TRINITY_DN10696_c0_g1_i1	1.86e-75	250.0	peptidoglycan catabolic process	-	-
TRINITY_DN233_c0_g1_i1	1.44e-159	448.0	Poly-gamma-glutamate hydrolase	ymaC	-
TRINITY_DN15618_c0_g1_i1	1.07e-72	221.0	acetyltransferase, isoleucine patch superfamily	vioB	ko:K21379
TRINITY_DN12385_c0_g1_i1	3.7e-237	654.0	Sulphur transport	yeeE	ko:K07112
TRINITY_DN111_c0_g1_i1	2.05e-260	727.0	hmm pf06207	-	-
TRINITY_DN12529_c0_g1_i1	2.83e-112	322.0	DinB family	-	-
TRINITY_DN15822_c0_g1_i1	2.46e-156	453.0	MCRA family	mycA	ko:K10254

TRINITY_DN15908_c0_g1_i1	2.24e-183	510.0	Metal-dependent hydrolases of the beta-lactamase superfamily I	vicX	ko:K00784
TRINITY_DN176_c0_g1_i1	0.0	1111.0	Fusaric acid resistance protein-like	-	-
TRINITY_DN191_c0_g2_i1	4.56e-104	300.0	Belongs to the UPF0178 family	-	ko:K09768
TRINITY_DN15994_c0_g1_i1	1.55e-307	841.0	Belongs to the dicarboxylate amino acid cation symporter (DAACS) (TC 2.A.23) family	tcyP	ko:K06956
TRINITY_DN131_c0_g1_i3	4.46e-258	710.0	ABC-type antimicrobial peptide transport system, permease component	-	ko:K02004

Appendix III

CHAPTER 5: THE EFFECTS OF IRRIGATION ON THE SURVIVAL OF *CLOSTRIDIUM SPOROGENES* IN THE PHYLLOSPHERE AND SOIL ENVIRONMENTS OF LETTUCE

***Please see next page**

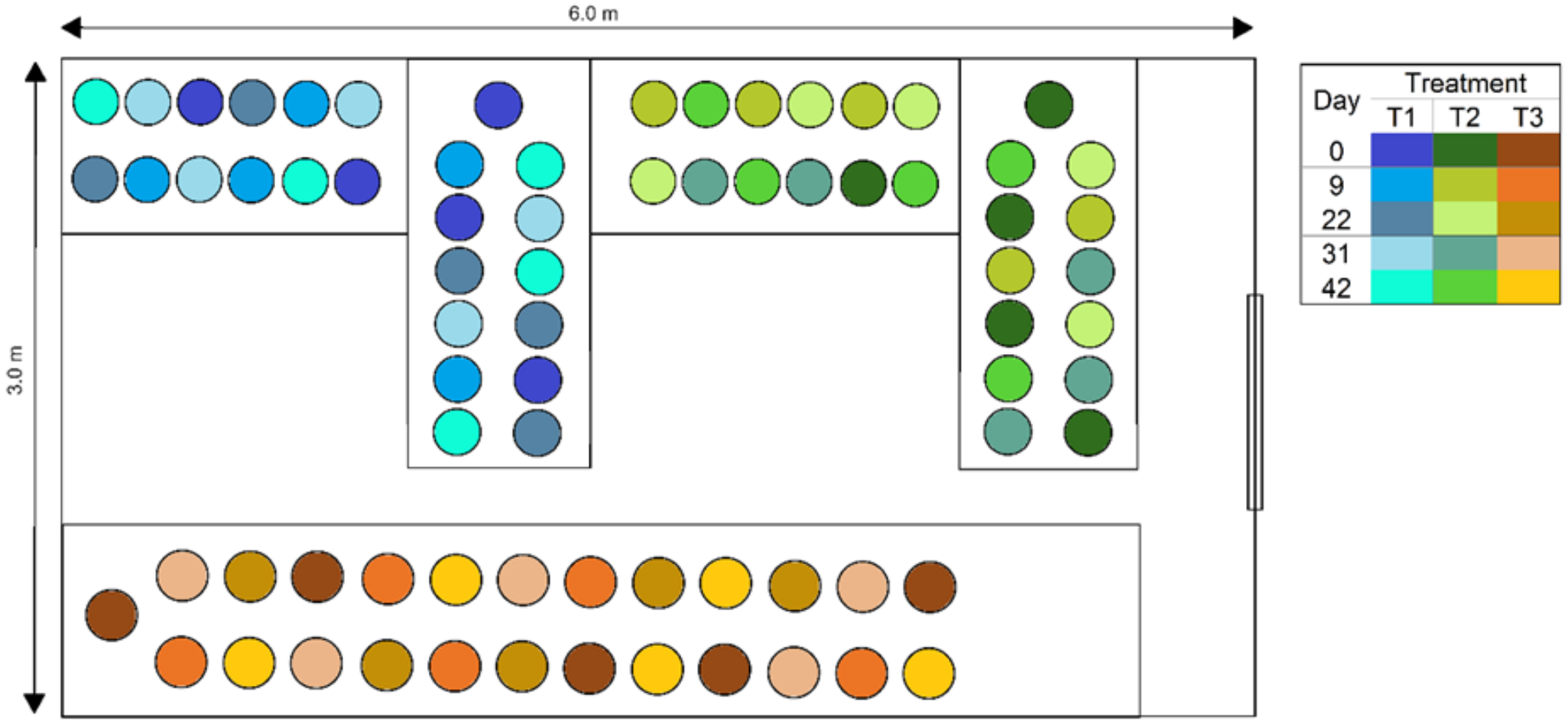


Figure S5.1: Layout of experimental design with different treatments (T1, T2 and T3) at Eco-Rehab greenhouse, where T1: surface irrigation, T2: spray irrigation, T3: control. Trail ran for 42 days, where sampling of rhizosphere, non-rhizosphere soil, and phyllosphere of lettuce was done on days 0, 9, 22, 31 and 42. Each sampling run consisted of 5 replicates for each lettuce environment.

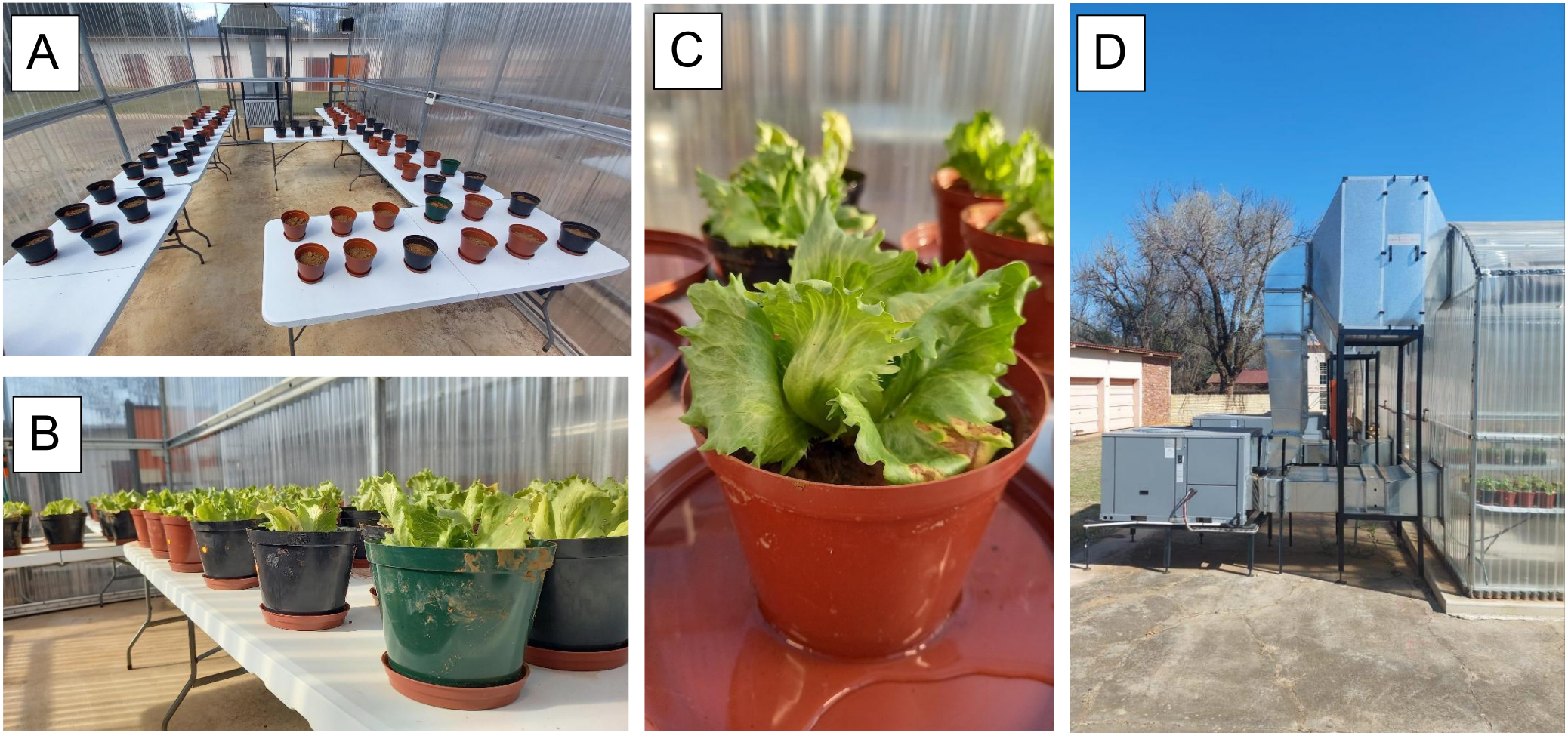


Figure S5.2: A: Layout of greenhouse design before lettuce transplant and inoculation via irrigation. B: Lettuce seedlings after inoculation with *C. sporogenes* via irrigation. C: Bottom watering of lettuce seedings. D: The biosafety level 1 (BSL-1) greenhouse that is equipped with temperature control and HEPA filters.

Table S5.1: Particle size measurements of soil used in this study via sieves with different mesh diameters.

Soil sample	Mass before shaking (g)	Sand		Silt		Clay		Total mass after shaking (g)	Loss (g)	% Sand	% Silt	% Clay
		Sieve 2,0 M (g)	Sieve 853' M (g)	Sieve 707' M (g)	Sieve 250 µm (g)	Sieve 50 µm (g)	Bottom (g)					
Rep 1	100,32	13,04	20,57	3,80	28,74	29,58	2,42	98,15	2,17	33,61	32,54	32,00
Rep 2	100,09	25,66	14,99	3,18	27,18	26,19	1,49	98,69	1,40	40,65	30,36	27,68
Rep 3	100,07	24,97	18,05	3,45	24,81	25,01	2,22	98,51	1,56	43,02	28,26	27,23
Mean	**100,16**	**21,22**	**17,87**	**3,48**	**26,91**	**26,93**	**2,04**	**98,45**	**1,71**	**39,09**	**30,39**	**28,97**

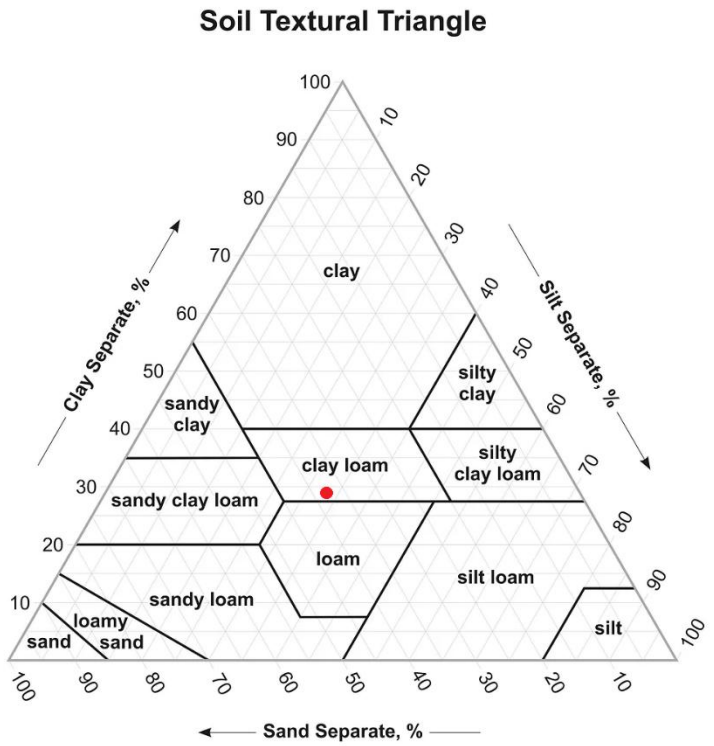
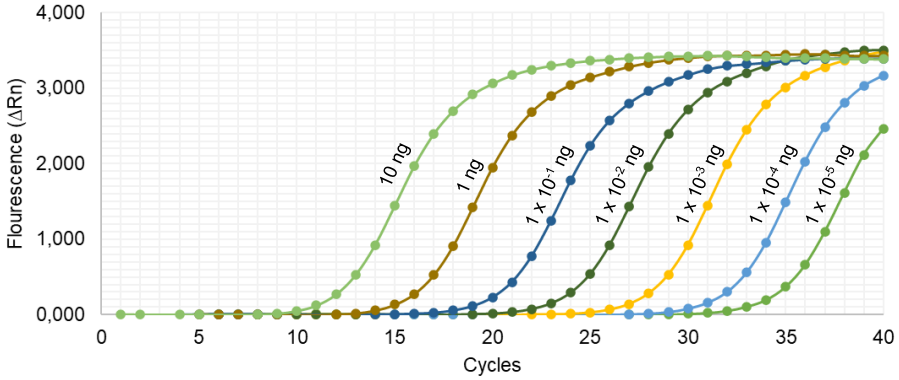
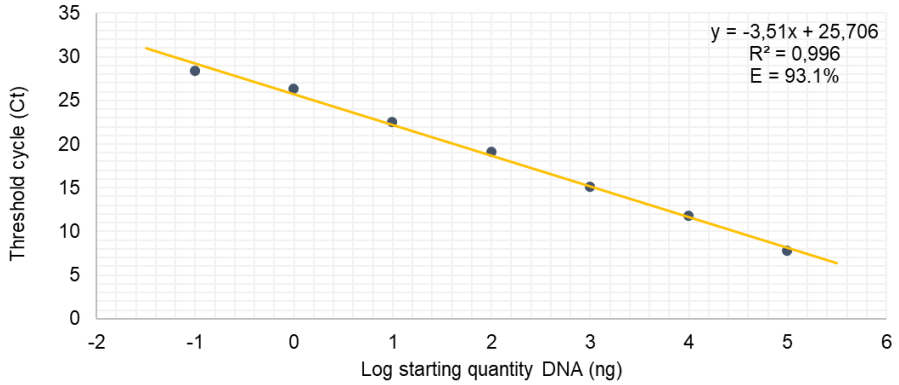


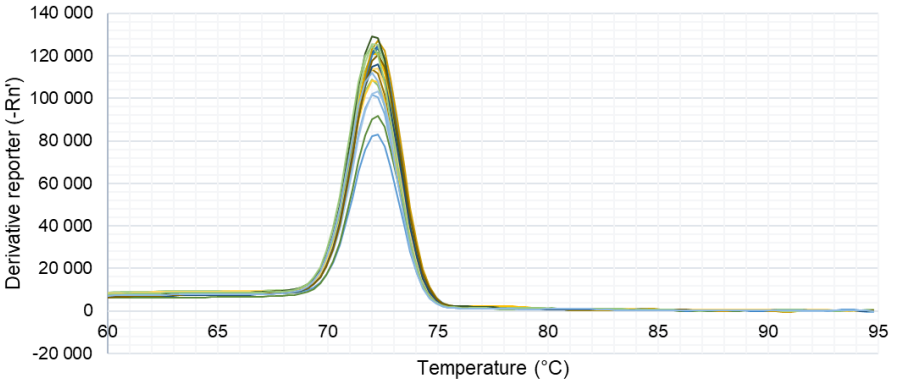
Figure S5.3: Graph illustrating soil texture and composition used in this study. Soil composition was determined by particle size distribution of three replicates: clay (28.97%), sand (39.09%) and silt (30.39%).



A



B



C

Figure S5.4: Performance and sensitivity of the qPCR assay. **(A)** Amplification curves, **(B)** standard curve, and **(C)** melting curves of the assay used for the detection and quantification of *C. sporogenes* based on the *ger*AA gene. **(A)** Standard amplification plot for the 10-fold serial dilution of gDNA, ranging from 10 ng to 1 x 10^{-5} ng. **(B)** Standard curve obtained by plotting the log quantity of pure *C. sporogenes* ATCC 3584 DNA (ng) against the Ct values detected by absolute qPCR. Ct values are the mean of three replicates. **(C)** Melting curves using SYBR Green fluorescence showing a single dissociation peak at 72.1°C.