T.C.
İNÖNÜ UNİVERSITY
INSTITUTE OF SCIENCES

PRODUCTION OF EPSILON TOXIN SECRETED BY *CLOSTRIDIUM PERFRINGENS TYPE D* UNDER DIFFERENT CULTURE CONDITIONS

MASTER THESIS

Fethiye SEVİMLİ
(36183623007)

Department of Molecular Biology and Genetic

Thesis Advisor: Prof. Dr. Hikmet GEÇKİL

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WORD OF HONOR

I declare this "**Production of Epsilon Toxin Secreted By *Clostridium Perfringens* Type D Under Different Culture Conditions**", which I submitted as my master thesis, was written by myself without resorting to any help that would otherwise contradict scientific morals and traditions, and that all the sources I benefited from were shown in accordance with the required methodology both in the text and in the bibliography and I proudly confirm this.

Fethiye Sevimli

TABLE OF CONTENTS

ACKNOWLEDGEMENTS i
WORD OF HONOR ii
TABLE OF CONTENTS iii
LIST OF TABLES iv
LIST OF FIGURES v
LIST OF SYMBOLS AND ABBREVIATIONS vi
ÖZET vii
ABSTRACT viii
1. INTRODUCTON 1
1.1. *Clostridium perfringens* 2
1.1.1. *Clostridium perfringens* types A-E 4
1.1.2. Classification of toxins according to general mechanisms of action 8
1.1.3. Cholesterol-dependent cytolysin 9
1.1.4. Intracellularly active clostridial toxins and pore formation 10
1.1.5. Clostridial heptameric β-pore-forming toxins 10
1.1.6. Aerolysin family 11
1.1.7. α-hemolysin family 12
1.2. *Clostridium perfringens* Toxins 14
1.2.1. *Clostridium perfringens* α Toxin (CPA) 14
1.2.2. *Clostridium perfringens* β Toxin (CPB) 15
1.2.3. Clostridium perfringens Beta2 Toxin (CPB2) 15
1.2.4. *Clostridium perfringens* Perfringolysin O Toxin 15
1.2.5. *Clostridium perfringens* Iota Toxin (CPI) 16
1.2.6. *Clostridium perfringens* Enterotoxin (CPE) 16
1.2.7. *Clostridium perfringens* Necrotic Enteritis B-like Toxin (NetB) 16
1.2.8. *Clostridium perfringens* Epsilon Toxin (ETX) 17
1.2.8.1. Pathogenesis of Epsilon Toxin 17
1.2.8.2. Characterization of Epsilon Toxin 18
1.2.8.3. Structure of Epsilon Toxin 18
1.2.8.4. General Mechanism of Action of Epsilon Toxin 19
1.3. Control of disease by *C. perfringens* Type D 20
1.4. Production of vaccines from *C. perfringens* Type D 20
1.4.1. Optimum Conditions for Production of Epsilon Toxin by *C. perfringens* Type D 22
2. MATERIALS AND METHODS 24
2.1. Reagents 24
2.2. Bacterial strain 24
2.3. Identification of the strain 24
2.3.1. DNA extraction 24
2.3.2. Real-time PCR 25
2.4. Growth Media and Bacterial Cultures 26
2.4.1. Bacterial Frozen and Working Stocks 26
2.4.2. Reinforced Clostridial Media 27
2.4.3. Clostridial Agar 27
2.4.4. Blood Agar 27
2.4.5. Tryptic Soy Broth and Fluid Thioglycollate Media 28

2.4.6. Adjusting pH and Temperature of Growth Media 28
2.4.7. Effect of Zn^{2+} 28
2.5. Identification of *C. perfringens* Type D 28
2.6. Measuring the cell mass (OD_{600}) of bacterial cultures 29
2.7. Analysis of epsilon toxin by SDS-PAGE 29
3. RESULTS…….. 31
3.1. Bacterial Cultures 31
3.2. Identification of Bacteria with RT-PCR 32
3.3. Growth at Different pH and Temperatures 33
3.4. Bacterial Growth in $ZnSO_4$ Supplemented RCM 35
3.5. Analysis of Epsilon toxin by SDS-PAGE 36
4. DISCUSSION 37
REFERENCES 39
CURRICULUM VITAE 44

LIST OF TABLES

Table 1.1 Typing of *C. perfringens* according to toxins 4
Table 1.2 *C. perfingens* toxins Types 14
Table 1.3 Different types of vaccines for clostridial disease 22
Table 2.1 Epsilon Toxin Primer sequence 26
Table 2.2 RT-PCR thermocycling conditions 26
Table 2.3 SDS-PAGE Gel Preparation… 29

LIST OF FIGURES

Figure 1.1 Pore formation by cholesterol-dependent cytolysins.....9
Figure 1.2 Soluble pore-forming toxins (PFTs) are recruited to the host membrane by specific interactions with protein receptors or lipids 11
Figure 1.3 Structure of aerolysin and related clostridial β-PFTs 12
Figure 1.4 Structure of *Staphylococcus aureus* alpha hemolysin and related clostridial β-PFTs (*C. perfringens* delta toxin and NetB)….....13
Figure 1.5 Structure of epsilon toxin and aerolysin 19
Figure 1.6 Cellular mechanism of action of epsilon toxin.....20
Figure 1.7 The production steps in a clostridial vaccine production process 22
Figure 3.1 *C. perfringens* type D bacterium under aerobic conditions.....31
Figure 3.2 *C. perfringens* type D purity test 32
Figure 3.3 The RT-PCR Panel of *C. perfringens* type D bacteria 33
Figure 3.4 *C. perfringens* type D bacteria culture growth at 37 ºC 34
Figure 3.5 *C. perfringens* type D culture at 35 ºC 34
Figure 3.6 Culture densities of *C. perfringens* type D at different pH and temperature of incubation 35
Figure 3.7 Total biomass (OD_{600}) of bacterial cultures supplemented with varying concentrations of $ZnSO_4$..... 36
Figure 3.8 Epsilon toxin on SDS-PAGE.....36

LIST OF SYMBOLS AND ABBREVIATIONS

C. perfringens: *Clostridium perfringens*

CPE : *Clostridium perfringens* enterotoxin

CPB : *Clostridium perfringens* β-toxin

CPB2 : *Clostridium perfringens* β-2 toxin

PFO : *Clostridium perfringens* perphringolisin O

CPA : *Clostridium perfringens* alpha toxin

PFT : Pore-Forming Toxin

CDC : Cholesterol-dependent cytolysins

ETX : *C. perfringens* epsilon toxin gene

ε Toxin : Epsilon toxin

kDa : Kilodalton

RCM : Reinfroced Clostridial Media

TSB : Tryptic Soy Broth

FTM : Fluid Thioglycollate media

OD : Optical density
ABS : Absorbance
PCR : Polymerase chain reaction
Ca++ : Calcium ion
K+ : Potassium ion
ATP : Adenosine Triphosphate
HAVCR1 : Hepatitis A virus cellular receptor 1
MAL : Myelin and lymphocyte
SDS-PAGE : Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
RPM : Radius per minute
NAOH : Sodium Hydroxide
HCI : Hydrochloric acid
APS : Ammonıum persulfate

ÖZET

Yüksek Lisans Tezi

FARKLI KÜLTÜR ŞARTLARINDA *Clostrıduum perfingens* TİP D TARAFINDAN SALGILANAN EPSİLON TOKSİN ÜRETİMİ

Fethiye SEVİMLİ

İnönü Üniversitesi
Fen Bilimleri Enstitüsü
Moleküler Biyoloji ve Genetik Anabilim Dalı

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Danışman: Prof. Dr. Hikmet GEÇKİL

Clostridium perfringens hayvanlarda ve insanlarda dizanteri, gazlı kangren gibi çeşitli hastalıklara neden olan Gram pozitif, çubuk şeklinde, anaerobik, spor oluşturan patojenik bir bakteridir. *C. perfringens* tip B ve tip D tarafından salgılanan epsilon (ε) toksini, koyun, keçi ve sığırlarda bağırsaklardan emilmesiyle ortaya çıkan ve dünya çapında enterotoksemi olarak bilinen hastalığa neden olur. Hastalığın hızlı ve şiddetli seyri nedeniyle aşılama, hastalıkla mücadelede önemlidir. Enterotoksemik hastalıkları kontrol etmenin bir yolu, "toksoidler" adı verilen inaktive edilmiş toksinler veya inaktive edilmiş bakteri hücreleri kullanılarak aşılamadır. *C. perfringens* tip D toksoid preparatları ile aşılamalar, bu suşun neden olduğu enterotoksemik hastalıkların bir dereceye kadar kontrol altına alınmasını mümkün kılmıştır. Bu toksoid preparasyonunun potensini artırmak için, yüksek derecede antikor yanıtı sağlayan toksini elde etmek gerekir. Daha önceki çalışmalarda epsilon toksini elde edilmesine rağmen toksin seviyeleri düşük olmuştur. Bu çalışmada, *C. perfringens* tip D tarafından salgılanan epsilon toksini üretimi üzerine sıcaklık, pH, çinkonun ($ZnSO_4$) etkisi araştırıldı. Kültür ortamındaki bakteri yoğunluğu ile epsilon toksin üretimi arasında herhangi bir ilişki olup olmadığını anlamak için bu tür değişken kültür koşulları altında bakterilerin büyüme dinamikleri incelendi. Toksinin kantitatif ve kalitatif tespiti SDS-PAGE analizi ile belirlendi. Bulgularımız optimum ε-toksin üretiminin 37 ^{o}C, pH 6.8'de olduğunu ve bakteri biyokütlesi ile toksin üretimi arasında bir korelasyon olmadığını gösterdi.

Anahtar Kelimeler: Clostridium perfingens, Enterotoksinler, Epsilon toksini

ABSTRACT

Master Thesis

PRODUCTION OF EPSILON TOXIN SECRETED BY *Clostridium perfringens TYPE D* UNDER DIFFERENT CULTURE CONDITIONS

Fethiye Sevimli

Inonu University
Graduate School of Natural and Applied Sciences
Department of Molecular Biology and Genetics

2022

Supervisor: Prof. Hikmet GEÇKİL

Clostridium perfringens is a Gram-positive, rod-shaped, anaerobic, spore-forming pathogenic bacterium that causes various diseases such as dysentery and gas gangrene in animals and humans. Epsilon (ε) toxin secreted by *C. perfringens* type B and type D causes a worldwide disease known as enterotoxemia, which occurs when the toxin is absorbed from the intestines in sheep, goats and cattle. Owing to the rapid and severe course of the disease, vaccination is important to fight the disease. One way to control enterotoxemic diseases is vaccination using inactivated toxins called "toxoids" or inactivated bacterial cells. Immunization with *C. perfringens* type D toxoid preparations has made it possible to control the enterotoxemic diseases caused by this strain to a certain extent. To increase the potency of this toxoid preparation, it is necessary to obtain the toxin that provides a high degree of antibody response. In previous studies, although ε-toxin was obtained, the toxin levels were low. In this study, the effect of temperature, pH, zinc ($ZnSO_4$) on epsilon toxin production by *C. perfringens* type D were investigated. The growth dynamics of bacteria under such varying culture conditions were studied to understand whether any relationship exists between the density of bacteria in the culture medium and epsilon toxin production. Quantitative and qualitative determination of the toxin was determined by SDS-PAGE analysis. Our findings showed optimum production of ε-toxin was observed at 37 ^{o}C, pH 6.8 and there was no correlation between bacterial biomass and toxin production.

Keywords: *Clostridium perfingens*, Enterotoxins, Epsilon toxin

1. INTRODUCTION

Toxins are substances that can be naturally produced by living cells and organisms or synthetically produced for various purposes. The latter are not within the context of the study carried here and thus will not be elaborated further. Structurally most toxins are either small molecules or proteins of various sizes. Protein toxins can naturally be produced by bacteria, fungi, algae, plants, and even animals. However, the most potent toxins are of microbial origin particularly produced by bacteria. Bacterial toxins are of cellular origin, have large molecular weights, are highly antigenic, and a significant number of these antigens appear to be enzymes.

Bacterial toxins are typically classified under two major categories: exotoxins and endotoxins. Exotoxins, including the subject of this thesis are normally released into the surrounding environment, whereas endotoxins (e.g., lipopolysaccharide (LPS) produced by gram-negative bacteria) are released when bacteria are lysed by the host immune system. Toxins help bacteria for manipulating host cell functions during infection or disease and they directly target innate immunity, shattering a major branch of the host immune defense. Such manipulation includes damaging cell membranes, disrupting protein synthesis and immunosuppression.

Bacterial toxins may also serve as biotechnological agents for a plethora of applications. In this context, toxins produced by *Clostridium* species have been shown to have a wide variety of applications, ranging from drug and vaccine development to their use as cancer

therapeutics. *Clostridium* is an important genus used in production of toxin. There are more than 200 species of *Clostridium* genus, of which 14 species have been identified as important animal pathogens (Uzal et al., 2016). In this regard, epsilon toxin, abbreviated as ETX, is one of the most potent clostridial toxins after botulinum and tetanus toxins. In this thesis, our aim was to determine how culture conditions, whether physical (temperature, pH) or chemical (varying Zn^{2+} concentrations), affected the production of epsilon toxin in *Clostridium perfringens* type D strain.

1.1 Clostridium perfringens

Clostridium perfringens was identified from human autopsies by William H. Welch in 1891 and originally was as *Bacillus perfringens*. The bacterium is also known as *Clostridium welchii* (Lucey & Hutchins, 2004). The term *perfringens* comes from the Latin and it means "breaking through" (Bergey et al., 1984). *C. perfringens* is associated with a variety of microbial environments, such as soil, food, sewage, and gastrointestinal (GI) system. The identification of *C. perfringens* from the GI tract of a 5000 years old mummy named Ötzi, found in the Alpine glacier in 1991, has clearly showed that this bacterium was present even in humans of Neolithic age (Kiu & Hall, 2018). While some members of the *Clostridium* genus are responsible for the initiation of the disease process through local accumulation or active invasion in tissues, others act through toxins. *C. perfringens* is a common bacterium responsible for many diseases such as gas gangrene (also known as Clostridial myonecrosis), hemorrhagic enteritis, and enterotoxemias, called as such given the absorption of toxins in the intestine and their release into the general circulation. Absorption of toxins causes a series of infections leading to systemic intoxication, cardiovascular shock and death (Khiav & Zahmatkesh, 2021; Markey, 2013). Most pathogenic species produce one or more exotoxins that have varying degrees of effects in different mammalian species. *C. perfringens* is one of the most pathogenic species of the *Clostridium* genus and capable of producing at least 17 toxins (Bokori-Brown et al., 2011).

C. perfringens is a Gram-positive, anaerobic, non-motile, rod-shaped spore-forming bacterium, widely distributed in the environment. It also lives in the GI tract of humans and animals through contamination and food poisoning (Cook et al., 2001; Lund, 1990). Depending on the type toxins produced, *C. perfringens* is responsible for diverse pathologies not only in animals but also in humans. The bacterium produces a variety of toxins, namely alpha, beta, epsilon, and iota (Nagahama, Oda, et al., 2015). These toxins are generally formed under extreme conditions such as heat, drought, radiation and poor nutritional conditions, which cause spore formation in this bacterium. Spore germination is also an important factor for *C. perfringens* food-borne disease transmission (Li et al., 2016). *C. perfringens* spores can remain in dormancy for extended periods of time and survive extreme environmental conditions. In the presence of favorable conditions spores can germinate into vegetative cells in less than 20 min and are ingested in foods.

Clostridial spores germinate in the GI tract of animals to form the vegetative cells that initiate *C. perfringens* infections (CPI). During CPI, the bacterium induces a sporulation pathway that produces more spores and these spores are responsible for the persistence of bacterial infection. Thus, there is a link between sporulation and toxin production by *C. perfringens* (Li et al., 2016).

C. perfringens strains are classified into five toxinotypes (A-E) based on their production of 4 main toxins; *alpha*, *beta*, *epsilon*, and *iota* (Nagahama, Oda, et al., 2015). However, recent studies classified *C. Perfringens* into 7 types (A to G) according to the combination of typing toxins they produce (Gohari et al., 2021; Kiu & Hall, 2018; Rood et al., 2018). Most diseases caused by *C. perfringens* isolates are mediated by one or more of these toxins acting synergistically (Kiu & Hall, 2018).

C. perfringens genome contains a chromosome of approximately 3 Mbp and plasmids of various sizes (Bruggemann, 2005; Gohari et al., 2021; Gurjar et al., 2010; Li et al., 2013; Popoff & Bouvet, 2013; Sayeed et al., 2010). The alpha toxin gene (*plc*), a phospholipase, is located on the chromosome close to the origin of replication. Therefore, alpha toxin (CPA) is produced by all strains of *C. perfringens* (Justin et al., 2002). Other toxins such as beta, epsilon and iota exist together or separately on plasmids of different sizes. For example, *etx*

gene can be found on plasmids ranging from 48 to 110 kb (Kiu & Hall, 2018; Sayeed et al., 2007).

Table 1.1 Typing of *C. perfringens* according to toxins (Uzal et al., 2014)

Type	Alfa (α)	Beta (β)	Epsilon (ε)	Iota (ι)
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

1.1.1 *Clostridium perfringens* types A-E

Clostridium perfringens is an enteric bacterium in animals including humans and is ubiquitous in the environment. The bacterium is able to form endospores which allows it to survive harsh environmental conditions. *C. perfringens* produce at least 15-17 toxins involved in pathogenesis (Li et al., 2016). However, the strains of *C. perfringens* are divided into five types (A-E), based on their ability to produce four major lethal toxins: *alpha* (*CPA*), *beta* (*CPB*), *epsilon* (*ETX*), and *iota* (*CPI*). *C. perfringens* produce two other toxins with pathological significance; β2 (*CPB2*), enterotoxin (*CPE*), and NetB. Only types A, C, and F have been determined to cause disease in humans, whereas all of the types have been demonstrated to cause disease in animals (Khiav & Zahmatkesh, 2021). The classification of *C. perfringens* based on the formation of lethal toxins (*α, β, ε,* and *ι*) was introduced by Wilsdon (Wilsdon, 1931) and later was accepted, given the role of the toxins in pathogenesis, rapid typing of strains based on the lethality of their culture filtrates to animals in the presence of type-specific antisera. Other toxins produced in *C. perfringens* cultures usually occur at sub lethal concentrations and therefore rarely interfere with the typing procedure. Each toxin is associated with a particular disease, which explains the wide spectrum of diseases associated with different toxinotypes.

***C. perfringens* type A** is ubiquitous in nature and is the most prevalent strain. It is commonly associated with enteritis and enterocolitis in poultry, ruminants, horses, pigs, dogs, and other species (Anju et al., 2021). Some of the enterotoxin producing strains (CPE) of *C.*

perfringens type A are responsible for acute food poisoning in humans (Uzal et al., 2016). *C. perfringens* type A food poisoning, is ranked the second most prevalent bacterial food poisoning after *Salmonella* (Scallan et al., 2011; Uzal et al., 2014). The enterotoxin (*cpe*) gene can be either chromosomal or plasmid-borne. Although only 1–5% of *C. perfringens* type A strains carry the *cpe* gene (McClane et al., 2013), about 75% of all *C. perfringens* food poisoning cases are caused by type A strains carrying a chromosomal *cpe* gene, whereas non-food poisoning type A isolates typically carry a plasmid-encoded *cpe* gene (Li et al., 2016). Type A strains carrying a chromosomal *cpe* gene reside within a distinct sub lineage of *C. perfringens* that appears to be well-adapted for food-borne transmission, due in part to their production of spores with exceptional resistance against heating, low temperatures and certain food preservatives (Uzal et al., 2014). The food poisoning is characterized by intense abdominal cramps, nausea and diarrhea. Although the illness is usually over within a day, it can be fatal in the elderly or in patients receiving constipation producing medication (McClane, 2014). The bacterium is also associated with gas gangrene with symptoms including fever, pain, edema, myonecrosis, and gas accumulation (Gohari et al., 2021; Kiu & Hall, 2018). Later stages of disease include necrotizing fasciitis, also known as "flesh-eating disease" which is a serious infection of the skin, subcutaneous tissue, and the tissue that covers internal organs (Shimizu et al., 2002). The primary toxin involved in gas gangrene is α-toxin produced by *C. perfringens* type A, which produces the highest amounts of this toxin. The toxin has phospholipase C and sphingomyelinase activities and functions to disrupt the membranes of host cells through the degradation of phospholipids and sphingolipids (Urbina et al., 2009).

***C. perfringens* type B** mainly affect lambs by causing dysentery (Zaragoza et al., 2019), is the etiologic agent of hemorrhagic enteritis in calves, goats and sheep (Rood et al., 1997), and it has been used for producing toxins used as veterinary vaccines (Brandi et al., 2014). While alpha toxins (CPAs) are produced by all strains of *C. perfringens*, beta toxins (CPBs), which are encoded by genes on large plasmids, are produced by *C. perfringens* type B and C (Brandi et al., 2014). CPBs forms an oligomeric complex that creates a channel in the cell membrane into the bloodstream (Nagahama, Ochi, et al., 2015), and causes necrosis of the small intestine (Uzal et al., 2014). Type B also carries genes encoding ε (ETX) toxins. Both CPB and ETX in the intestine of infected animals causes extensive enteritis and neurological damage, respectively (Zaragoza et al., 2019). ETX is the third most potent clostridial toxin

after botulinum and tetanus toxins. ETX is a causative agent of enterotoxemia, a highly lethal disease with major impacts on the farming of domestic ruminants, particularly sheep (Alves et al., 2014). ETX belongs to the aerolysin-like pore-forming toxin family and its structure shows a similar fold to aerolysin, produced by some Gram-negative bacteria, parasporin-2 produced by *Bacillus thuringiensis* and pore-forming lectin produced by *Laetiporus sulphureus*. Although the primary structures of these proteins have less than 20% sequence similarity, the proteins have high structural similarity in terms of their shapes and β-sheet arrangements. Despite the structural similarities of these toxins, ETX is far more potent than the others, as its lethal activity in mice is approximately two orders of magnitude greater than that of aerolysin (Alves et al., 2014). The *ETX* gene is located on plasmids of various sizes ranging from 48 kb to 110 kb. Even a single bacterium can harbor multiple plasmids, each of which may carry up to three different toxin genes or other accessory virulence factors (Alves et al., 2014). Some of the plasmids are conjugative in nature and they enable intra-species horizontal transfer of toxin genes and the consequent acquisition or loss of virulence factors, which contribute to changes in toxigenic types observed in some *C. perfringens* strains (Petit et al., 1999). In the GI, epsilon toxin (ETX) is produced as an inactive 32.981 kDa prototoxin which is activated by photolytic cleavage resulting from removal of 10-13 amino acids from N-terminal and 22-29 amino acids from C-terminal, depending on the protease that catalyzes the cleavage (Alves et al., 2014). Activated ETX is nearly three orders of magnitude more toxic than the prototoxin and is absorbed in the intestine and then transported to target organs such as lungs, brain and kidneys. Only a few cell lines are sensitive to the harmful effects of ETX. This restricted susceptibility is most likely due to the presence of specific receptors on these cells. Although the exact nature of the cellular receptors for ETX remains to be elucidated, it binds to receptors present on the outer surface of the vascular endothelial cell membranes of particular organs, including the brain, kidneys and liver. Endothelial cells of the brain are affected by this toxin, producing perivascular edema and resulting in cerebral necrosis (Shehzadi et al., 2021). CPB is very sensitive to proteases and it has to be processed by one or more proteases to be fully active (Uzal et al., 2016).

***C. perfringens* type C** strains are capable of causing severe disease in both humans and animals. Type C infections in humans cause enteritis necroticans, an intestinal infection resulting in vomiting, bloody stool, abdominal pain and, in severe cases, toxemia leading to

rapid death (Petit et al., 1999; Songer, 1996; Uzal et al., 2014). In humans, necrotizing enteritis is a serious GI problem as it inflames intestinal tissue perforation. Thus, bacteria can leak into the abdomen or bloodstream through the holes. Besides *C. perfringens* type A, type C is also of particular interest to the poultry industry because it is associated with diseases in avian species (Kasab-Bachi, 2017). *C. perfringens* type C produces alpha (CPA) and beta (CPB) toxins. However, certain strains of type C also produce other toxins, including enterotoxin (CPE), β 2 toxin (CPB2), perphringolisin O (PFO), and the large clostridial toxin TpeL (Uzal et al., 2016). Toxin production by *C. perfringens* type C depends on pH control, the strain used, an amino acid source, and the presence of fermentable carbohydrates, such as dextrin, glucose, or fructose, which have been demonstrated to increase toxin production and growth (Sakurai et al., 2009).

***C. perfringens* type D** causes enterotoxemia, a common clostridial diseases in sheep and goats worldwide. This bacterium that cause enterotoxemia is considered a non-communicable disease, although it can sometimes cause epidemics. The disease is almost always fatal and it can cause significant economic losses in livestock industry when herds are unvaccinated. (Uzal et al., 2016). *C. perfringens* type D produces two types of toxins alpha (CPA) and epsilon (ETX). Because of its potential as a biological weapon, ETX is classified as a select agent like some bacterial diseases (brucellosis, glanders and typhus) plus other protein toxins such as ricin and staphylococcal enterotoxin B (SEB) by the United States Department of Agriculture and Centers for Disease Control and Prevention (Uzal et al., 2016). Sialidases produced by the *C. perfringens* type D may play a key role during these stages by promoting the adhesion of the bacterium to enteric cells, thereby allowing *C. perfringens* type D to colonize the intestine. Sialidases also increase the sensitivity of cells to ETX through increased binding and oligomerization of the toxin. The sialidases may also expose additional receptors for ETX on the cell surface and they may modify non-receptor components of the cell surface and transform them into receptors for the toxin (Alves et al., 2014). Plasmids including an epsilon toxin gene from type D are conjugative and can be transferred into other *C. perfringens* strains such as *C. perfringens* type A (Hughes et al., 2007). Thus, the horizontal transfer of plasmids carrying the toxin gene contributes to the genetic complexity of *C. perfringens* toxinotypes. As in *C. perfringens* type C, the importance of glucose or dextrin in combination with pH control has also been demonstrated for toxin production by *C. perfringens* type D (Hauschild, 1965).

***C. perfringens* type E** causes rare enteric infections and it was first detected in domestic animals in the 1940s. The role of type E in human and animal disease has not been fully elucidated. Because *C. perfringens* type E can be found as a normal inhabitant in the intestine of healthy individuals of many animal species, isolation of this bacterium does not fulfill the diagnostic criteria for type E disease. *C. perfringens* type E produces alpha (α or CPA)- and plasmid-encoded binary iota (ι or ITX) toxins (Sakurai et al., 2009) and it causes antibiotic-associated enterotoxaemia in calves and lambs. ITX is as an inactive toxin produced only by *C. perfringens* type E strains and it consists of two non-covalently linked proteins (enzyme component ιa and binding component ιb). ιb is initially synthesized as an inactive toxin of ~100 kDa and proteolytic removal (by pepsin, trypsin, chymotrypsin, proteinase K, subtilisin, or thermolysin) of a ~ 20 kDa N-terminal fragment is required to produce the active form of ιb. The proteases also cleave off small peptides (9 to 13 amino acid residues) from the N-terminus of the ιa precursor, producing its active form (Gibert et al., 2000; Sakurai et al., 2009).

1.1.2 Classification of toxins according to general mechanisms of action

Pore formation in a host cell is a common mechanism of action for many bacterial toxins including clostridial toxins. Because, disruption of the membrane integrity of cell host is a direct and an efficient way for a pathogen to have access to indispensable nutrients in the host cell and it is conserved mode of action of most of bacterial toxins. Many of the clostridial toxins are pore-forming toxins belonging to the pore-forming toxin (PFT) class. They are made of monomers containing soluble β-strands that recognize a specific receptor on target cells. However, these toxins form oligomers when bound to receptors on the cell membrane, causing an indent in the lipid bilayer and disrupting the membrane structure (Popoff, 2014). Structural analysis show that these toxins retain a similar global mode of insertion into lipid membranes and binding components share a common evolutionary origin. Their interaction with the membrane drives refolding of the transmembrane helices into β-strands and insertion of the two β-strands into the membrane forms an amphipathic β-barrel pore with a diameter of 25–30 nm (Thapa et al., 2020). However, certain PFTs have evolved to acquire additional specialized functions to address specific requirements of

pathogens as is the case in heptameric PFTs which target receptors specific to only some cell types. Thus, according to their structure, clostridial β-PFTs are divided into several families: cholesterol-dependent cytolysins which form large pores, and disrupt the plasma membrane integrity, heptameric β-PFTs (aerolysin family and staphylococcal α-hemolysin family) which induce small pores and trigger signaling cascades, and intracellularly active toxins which cause pore formation through the endosomal membranes (Popoff, 2011).

1.1.3 Cholesterol-dependent cytolysins

Cholesterol-dependent cytolysins (CDCs) are a subset of pore-forming toxins that serve as key virulence factors produced by Gram-positive bacterial pathogens. CDCs are involved in many bacterial infections contributing to the loss of tissue integrity with wide variety of manifestations ranging from transient with no sequelae to fatal. While CDCs are produced by a wide range of Gram-positive bacteria, studies on CDCs have mostly carried in pathogenic *Streptococci*, *Clostridia*, and *Listeria* (Thapa et al., 2020). *C. perfringens* is the cause of myonecrosis (gas gangrene) through necrotizing soft tissue infection (NSTI), a type of necrotizing fasciitis, which is exacerbated by CDC perfringolysin O (PFO) (Stevens & Bryant, 2002). The schematic of CDC protein structure and pore-formation is given in Figure 1.

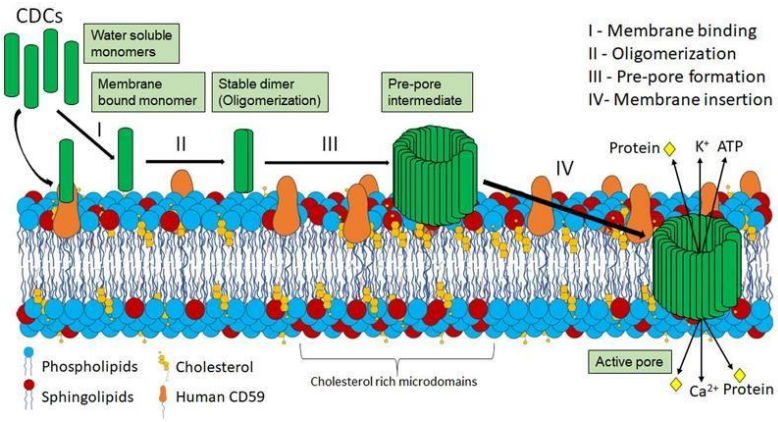

Figure 1.1 Pore formation by cholesterol-dependent cytolysins (CDCs). (I) CDCs are secreted as water-soluble monomers that bind their specific receptors on the plasma membrane. (II) Monomers dimerizes upon binding and form stable dimers. (III) CDCs then begin to oligomerize to form a pore made of ~35–50 monomers. (IV) After prepore formation, each monomer undergoes a coordinated conformational change to refold the transmembrane helices into membrane-spanning β-strands. These β-strands form an amphipathic β-barrel pore with a diameter of 25–30 nm. The formation of these pores causes ion influx (Ca^{2+}) or efflux (K^{+}) as well as loss of cellular ATP and proteins (Thapa et al., 2020).

1.1.4 Intracellularly active clostridial toxins and pore formation

Intracellularly active toxins are divided into two main groups according to their structure: single-chain protein toxins which form α-pores and binary or multicomponent toxins which form β-pores (Popoff, 2011). As in hydrophobic α-helices of botulinum and tetanus toxins to form pores, single chain protein toxins retain a different mode of insertion into the lipid bilayers. Intracellularly active toxins do not cause membrane pore formation directly. However, pore formation is an essential step for translocation of the enzymatic domain from the endosome to the cytosol. Clostridial intracellularly active toxins enter target cells through a receptor-mediated endocytosis and exploit pore formation through the endosomal membrane and deliver the enzymatic domain of toxin into the cytosol, where they recognize and modify a specific intracellular targets.

1.1.5 Clostridial heptameric β-pore-forming toxins

An important group of clostridial β-PFTs are the most studied heptameric β-PFTs (Iacovache et al., 2010) In contrast to CDCs, they associate in the membrane as oligomers (mostly to heptamers and a few extent to hexamers or octamers) leading to the formation of small pores which trigger signaling cascades that lead to different cell responses (Figure 1.2). Thereby, these toxins affect different cell types and are responsible for specific diseases. Clostridial heptameric β-PFTs are mainly involved in intestinal (enteric) diseases rather than in myonecrosis like clostridial CDCs. Binding to different receptors and activating at different sites, heptameric β-PFTs are divided into two families according to their structures: the aerolysin family and the *Staphylococcus aureus* α hemolysin family (Popoff, 2014).

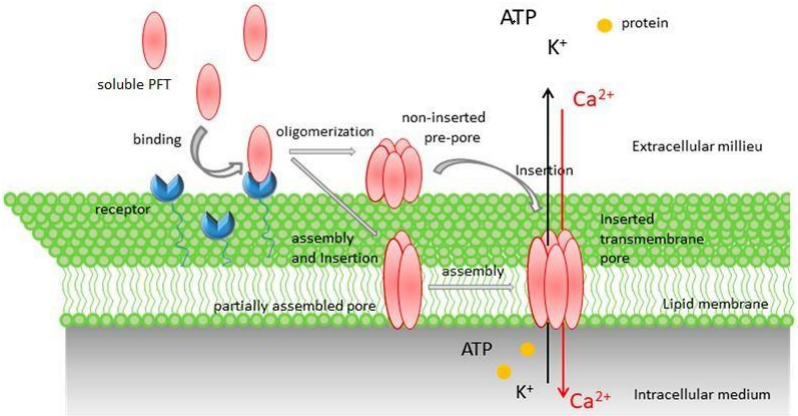

Figure 1.2 Soluble pore-forming toxins (PFTs) are recruited to the host membrane by specific interactions with protein receptors or lipids. Binding to the membrane, the toxins concentrate and initiate the oligomerization process. PFTs attachment to the membrane occurs via a sequential oligomerization mechanism to form a pore in which each monomer provides only one β-hairpin, forming the transmembrane β-barrel which causes influx or efflux of ions, small molecules, and proteins through the host cell membrane (Ostolaza et al., 2019).

1.1.6 Aerolysin family

C. perfringens epsilon (ETX), *C. perfringens* enterotoxin (CPE), and *C. septicum* alpha (CSA) toxins are structurally related to aerolysin produced by Gram-negative bacteria of *Aeromonas* sp. (Nagahama, Oda, et al., 2015). However, ETX and CPE proteins do not have much homology both structurally and at the amino acid level with aerolysin (Figure 1.3). All these toxins forms oligomeric (generally heptameric) pores in the host cell membrane and difference in their toxicity is likely due to their distinct amino acid sequence and protein structure, but is mainly due to their interaction with distinct receptor-binding sites on the cell membrane.

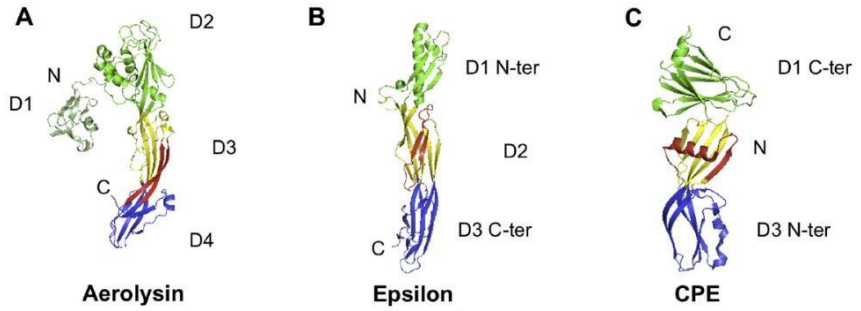

Figure 1.3 Structure of aerolysin and related clostridial β-PFTs: *C. perfringens* epsilon toxin and *C. perfringens* enterotoxin. (A) aerolysin monomer with receptor binding sites in domains 1 and 2. When bound to their receptor, these monomers heptamerize and form a prepore with an inverted mushroom shape. The domains 1 and 2 constitute the cap, while the domains 3 and 4 constitute stalk which rotates and completely collapses. The β-barrel extends in the opposite orientation to the stalk in the prepore conformation. Two monomers (red and blue) are shown in the heptameric structure. (B) *C. perfringens* epsilon (ETX) monomer and (C) *C. perfringens* enterotoxin (CPE) monomer. The receptor binding domains for all three toxins are in green, the domains containing the pre-stem loop (red) are in yellow, and the domains, which contain the propeptide and which are involved in the control of oligomerization, are in blue (Popoff, 2014).

1.1.7 α-hemolysin family

The α-hemolysin adopts a mushroom shape similar to that of aerolysin heptamer. However, in contrast to α-aerolysin, hemolysin associates with the membrane in an inverse orientation, the mushroom cap in the extracellular milieu and the stalk facing the membrane. Also as with CDCs, a-hemolysin show no drastic conformational change during the prepore to pore conversion. The α-hemolysin contains three domains, with a packed pore-forming domain showing a more spherical structure than β-PFTs of the aerolysin family. α-hemolysin family β-PFTs are not activated by trypsin or other proteases. Rather, they are susceptible to proteolytic degradation (Popoff, 2014) The clostridial heptameric β-PFTs of the α-hemolysin family are mainly involved in intestinal diseases such as necrotic enteritis. The main effect of β-PFTs forming small pores on cell membrane is the disruption its permeability and causing an efflux (K^+, ATP) and influx (Ca^{2+}) small molecules which subsequently cause mitochondrial dysfunction and release of inflammatory proteins. High concentrations of β-PFTs generally kill cells by necrosis, whereas at lower concentrations they induce cell death via programmed necrosis or apoptosis (Popoff, 2014).

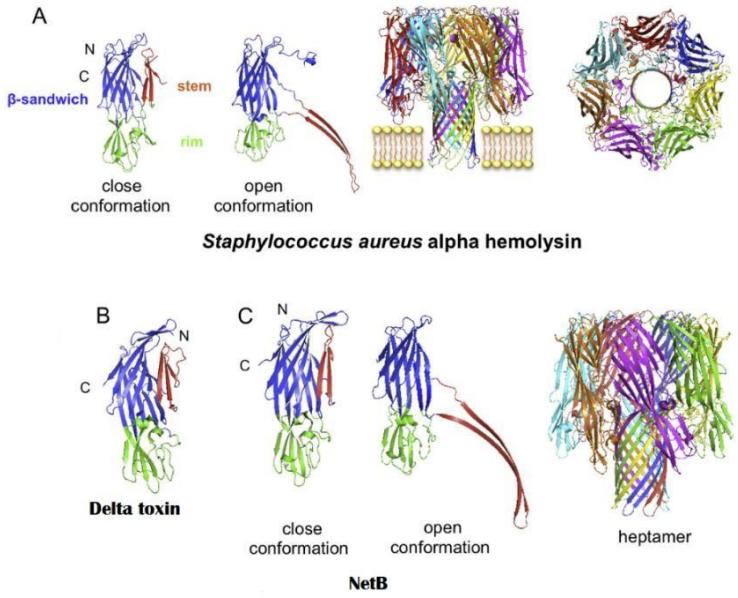

Figure 1.4 Structure of *Staphylococcus aureus* alpha hemolysin and related clostridial β-PFTs (*C. perfringens* delta toxin and NetB). The receptor binding domain (rim) is in green, the domain (stem) which unfolds in amphipathic β-hairpin in the open conformation and forms the β-barrel is in red, and the domain forming the cap of the mushroom shaped oligomer is in blue (Popoff, 2014).

C. perfringens delta toxin and relatively new identified NetB (necrotic enteritis toxin B-like) toxin constitute a β-PFT family structurally related to staphylococcal β-PFTs, and their heptameric assembly shares structural homology to the staphylococcal α-hemolysin (Figure 1.4). *C. perfringens* delta toxin and NetB secreted monomers have similar size and structure compared to α-hemolysin. Like other members of the α-hemolysin PFT family, the NetB monomer has four domains: the domain forming the cap of the mushroom (β-sandwich), the receptor binding domain (rim), and β-barrel forming domain (stem) (Figure 1.4). NetB forms heptameric pores with an internal diameter of ~26 Å on susceptible cell membranes and the β-barrel channel of this pore has a strong preference for cations to enter cells and causing cell lysis (Savva et al., 2013; Yan et al., 2013). The NetB was discovered in 2008 (Keyburn et al., 2008) and its receptor has not yet been identified (Gohari et al., 2021). This plasmid-borne toxin plays a major role in the pathogenesis of necrotic enteritis in poultry causing economically significant loss. It is suggested that the disease has emerged due to the removal of antibiotics in animal feedstuffs (Kasab-Bachi, 2017; Keyburn et al., 2010; Keyburn et al., 2008; Manich et al., 2008; Savva et al., 2013; Yan et al., 2013).

1.2 *Clostridium perfringens* Toxins

C. perfringens toxins has been under study for almost a century and new discoveries have been made gradually. Modern methods for the separation and purification have permitted to elucidate the structure of toxins, their biochemistry in terms of pharmacological and pathological effects and the treatment of disease. The composition of the nutrient medium has strong influence on the growth of bacterium and toxin production. It is well known that *C. perfringens* requires not only many amino acids, carbohydrates, salts, and vitamins for growth and toxin production, but also it is sensitive to physical conditions such as pH and temperature of the culture medium. *C. perfringens* toxins are either encoded by the genes on the ~3 Mbp chromosomal DNA or on native plasmids of various sizes in the bacterium. (Bruggemann, 2005; Gohari et al., 2021; Gurjar et al., 2010; Li et al., 2013; Popoff & Bouvet, 2013; Sayeed et al., 2010) (Table 1.2). The alpha toxin gene (*plc*), a phospholipase, is located on the chromosome close to the origin of replication. Therefore, alpha toxin (CPA) is produced by all strains of *C. perfringens* (Justin et al., 2002). Other toxins such as beta, epsilon and iota exist together or separately on plasmids of different sizes. For example, *etx* gene can be found on plasmids ranging from 48 to 110 kb (Kiu & Hall, 2018; Sayeed et al., 2007).

Table 1.2 *C. perfringens* toxins

Gene	Toxin	Mechanism of action	Toxin encoding genes
cpa or *plc*	α (CPA)	Phospholipase/sphingomyelinase C	Chromosome
cpb	β (CPB)	Pore-forming	Plasmid
etx	ε (ETX)	Pore-forming	Plasmid
itx	ι (ITX)	ıa ADP-ribosylation of actin	Plasmid
cpe	Enterotoxin (CPE)	Pore-forming	Chromosome-Plasmid
cpb2	β2 (CPB2)	Pore-forming	Plasmid

1.2.1 *Clostridium perfringens* α Toxin (CPA)

CPA, a zinc-containing metallo phospholipase C enzyme, is produced by all strains of *C. perfringens*, while type A strain produces the highest amounts of the toxin. It is the most conserved and well-known toxin and has both phospholipase C and sphingomyelinase

activities, which hydrolyse cell membrane phospholipids leading to cell necrosis, a key characteristic of pathology in gas gangrene and hemolysis. The α toxin gene (named *cpa* or *plc*), located on chromosome, encodes a 43 kDa protein with 370 amino acids. The α-helical N-terminal domain contains the phospholipase C active site and the α-sandwich C-terminal domain contains the membrane binding surface area (Oda et al., 2015).

1.2.2 *Clostridium perfringens* β Toxin (CPB)

Beta-toxin encoding gene (*cpb*) is located on plasmids and encodes a 35 kDa, 330 amino acids pore-forming toxin (CPB) causing intestinal necrosis and systemic enterotoxaemia in animals including humans. CPB is produced by Type B and Type C bacteria and is the main virulence factor of type C strains (Nagahama, Oda, et al., 2015). Beta toxin is thermolabile and highly sensitive to oxidizing and thiol group reagents (Nagahama, Oda, et al., 2015).

1.2.3 *Clostridium perfringens* Beta2 Toxin (CPB2)

CPB2 is encoded by *cpb2* gene on a plasmid and is a 28 kDa pore-forming cytotoxic toxin with less than 15% sequence homology to β-toxin (Kiu & Hall, 2018). First identified in pigs, the toxin was later detected in isolates from a wide variety of animals and humans with enteric disease. However, the mode of action still remains to be elucidated (Gohari et al., 2021).

1.2.4 *Clostridium perfringens* Perfringolysin O Toxin

Perfringolysin O (PFO or theta (θ)-toxin is encoded by the *pfo* gene is located on the chromosomal DNA near the origin of replication. PFO is a prototype of the CDC family and is produced by almost all *C. perfringens* strains. It is a pore-forming 53 kDa (472 amino acids) toxin with a 27 amino acid signal peptide. The toxin acts on cholesterol-containing cell membranes where PFO pore formation involves the binding of water-soluble PFO monomers to cholesterol, altering the membrane integrity. The toxin has been shown to play a role in the pathogenesis of gas gangrene and hemorrhagic enteritis in calves and is also known to induce tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in host cells activating apoptosis through mitogen-activated protein kinase p38 (MAPK) pathway and (Kiu & Hall, 2018; Popoff, 2014).

1.2.5 *Clostridium perfringens* Iota Toxin (CPI)

The *C. perfringens* iota (ι) toxin (CPI) consists of two parts; enzyme (ιa, ~48 kDa) and binder (ιb, ~74 kDa). CPI has cytotoxic lethal effects (Sakurai et al., 2009). It is a cytoskeleton-damaging toxin, exerting enzymatic activity on the ADP-ribosylating actin. Through disassembling the cytoskeleton, it causes apoptosis (Hilger et al., 2009; Kiu & Hall, 2018)

1.2.6 *Clostridium perfringens* Enterotoxin (CPE)

CPE is a 35 kDa single polypeptide pore-forming toxin and is responsible for diarrhea-related illness in dogs, pigs, horses, and humans. Its gene (*cpe*) can be either chromosomal or plasmid-borne, while ∼75% of all *C. perfringens* food poisoning is caused by type A strains carrying a chromosomal *cpe*. Food poisoning typically occurs when *C. perfringens* spores present in food are ingested and then sporulate in the small intestine, where they produce CPE. The *cpe* gene located on plasmids are responsible for 2-15% of all cases of non-food-borne human gastrointestinal diseases (Li et al., 2016). CPE is the main toxin to cause food poisoning and non-food-borne diarrhoea disease, although it has also been shown to disrupt intercellular tight junctions in intestinal epithelial cells, changing the cell permeability and causing lysis (Eichner et al., 2018; Uzal et al., 2016). The receptor-binding domain is on C-terminal and the cytotoxicity domain that mediates oligomerization and membrane insertion during pore formation is on N-terminal. The action mode of CPE on cells starts with its binding to receptors, which include certain claudins, a large family of proteins important for maintaining the structure and function of tight junctions. The toxin has been shown to bind and necrotize human ileal and colonic epithelium in vitro and induce apoptosis by the caspase-3 pathway (Chakrabarti & McClane, 2005; Gohari et al., 2021; Kiu & Hall, 2018).

1.2.7 *Clostridium perfringens* Necrotic Enteritis B-like Toxin (NetB)

The relatively newly discovered NetB (Keyburn et al., 2010; Keyburn et al., 2008) is a plasmid encoded gene that encodes a 323 amino acid (including a 30 amino acid secretion signal sequence) protein with a 33 kDa size, and is a member of the β-barrel pore-forming

toxin family encoded by *netB* (Figure 1.4). The toxin is mainly produced by C. *perfringens* type A strains and is associated with avian necrotic enteritis (Kasab-Bachi, 2017).

1.2.8 *Clostridium perfringens* Epsilon Toxin (ETX)

The epsilon (ε) toxin (ETX) is produced by *C. perfringens* Types B and D and is the deadliest of all toxins produced by *C. perfringens*, ranked only behind botulinum and tetanus neurotoxins produced by other members of *Clostridium* genus (Tamai et al., 2003). The epsilon toxin gene (*etx*) is located on plasmids of varying sizes and ETX is secreted as a ~33 kDa protoxin, which is later converted into an active form nearly 1000 times more toxic than the proprotein. ETX creates a pore in the plasma membrane and also it enters systemic circulation and affects almost all organs, causing enterotoxaemia in animals (particularly goat and sheep). Given its use as a potential biological weapon, ε-toxin producing *C. perfringens* strains are on the export control list in a number of countries (Bokori-Brown et al., 2011; Petit et al., 1999; Popoff, 2011). Because of its potential use as a biological weapon category B agent, ε-toxin-producing *C. perfringens* strains are on export control lists in many countries (Kiu & Hall, 2018).

1.2.8.1 Pathogenesis of Epsilon Toxin

The ETX toxin is the virulence factor of *C. perfringens* types B and D. *C. perfringens* type D is found in natural habitats such as soil, dust, sediment, cadavers, litter, as well as the digestive tract of healthy animals. ETX is a highly potent toxin, responsible for enterotoxemia in animals mainly in sheep. Enterotoxemia usually affects livestock, but can also affect humans. The disease is caused by the presence of large amounts of toxin in the gut, which then crosses the intestinal barrier and spreads through the circulation to various organs (Songer, 1996). It induces perivascular edema in various tissues and accumulates particularly in the kidneys and in the brain. The toxin passes the blood-brain barrier and stimulates the release of glutamate, the amino acid that accounts for the nervous excitation symptoms observed in animal enterotoxemia. At the cellular level, ETX causes a rapid swelling followed by a cell death involving necrosis. Recently, ETX has been found to induce demyelination and could be involved in demyelinating diseases like multiple sclerosis (Wioland et al., 2015).

1.2.8.2 Characterization of Epsilon Toxin

The ETX toxin produced by *C. perfringens* types B and D is the third deadliest of all clostridial toxins. Epsilon toxin is synthesized as a signal peptide-containing protein and secreted as an inactive prototoxin during the exponential growth phase of organisms. The prototoxin is converted into an active mature toxin by removal of 13 residues from its N-terminus and 22 residues from its C-terminus. This proteolytic activation can be catalyzed by proteases such as trypsin and α-chymotrypsin (Nagahama, Oda, et al., 2015). ETX synthesis is a highly regulated process involving the quorum sensing system. In Gram-positive bacteria, quorum sensing relies on the secretion of a signaling molecule activated by the proteases.

1.2.8.3 Structure of Epsilon Toxin

The three-dimensional structure of ETX toxin indicates that it is a very long molecule and consists mainly of β-layers (Bokori-Brown et al., 2011). It retains an elongated form and contains three domains (Cole et al., 2004). Compared to other pore-forming toxins, it has a weak sequence identity but 100 times more lethality. Epsilon loop is surrounded by two charged residues, Lys-162 and Glu-169, and contains a central proline like the corresponding aerolysin loop. Binding components share a similar structure organization with that of β-PFTs and notably contain an amphipathic elastic loop which forms a β-hairpin (Geny & Popoff, 2006).

Epsilon toxin contains three domains (Figure 1.5): Domain 1 contains a large α-helix, followed by a ring and three short α-helices (Cole et al., 2004). This domain is responsible for interaction with the host cell receptor. Domain 2 is a β-sandwich and contains a two-stranded sheet with an amphipathic sequence predicted to be the channel-forming domain (Cole et al., 2004).

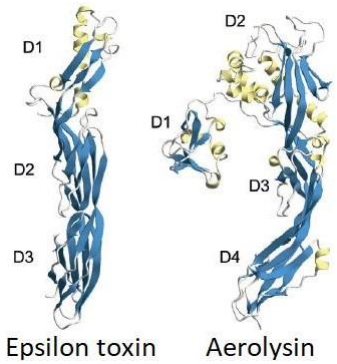

Figure 1.5 Structure of epsilon toxin and aerolysin. β-sheets are in blue and helices are in yellow (Nagahama M, 2015).

The pore-forming domain of ETX has been identified in domain 2. This domain stabilizes the interaction of the toxin with its receptor and triggers heptamerization (Ferreira et al., 2016). Domain 3 is a β-sandwich, analogous to domain 4 of aerolysin (Figure 1.5), which contains the cleavage site for toxin activation. After removal of its C-terminus sequence, domain 3 is likely involved in monomer-monomer interaction required for oligomerization (Cole et al., 2004) and responsible for the interaction of monomers for pore formation on the cell membrane (Ferreira et al., 2016).

1.2.8.4 General Mechanism of Action of Epsilon Toxin

Epsilon is produced in the gut and although present in cases of enterotoxemia the toxin mainly targets distant organs such as the central nervous system, lungs and heart (Navarro et al., 2018). The binding and cytotoxic activities of ETX have been extensively studied using kidney-derived cell lines of different species with no reported cases of spontaneous type D enterotoxemia, including dogs (Madin-Darby canine kidney cells, MDCK), mice and humans (Payne et al., 1994). Epsilon toxin action first settles on the plasma membrane by binding to the cell surface receptor and forming an active pore by heptamerization into a prepore on the membrane surface (Pawaiya et al., 2020; Robertson et al., 2011) (Figure 1.6).

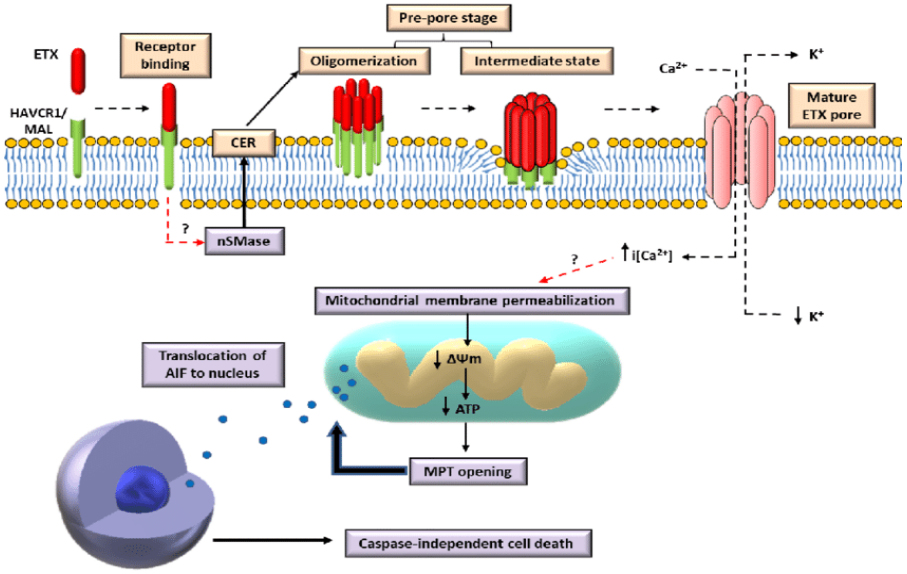

Figure 1.6 Cellular mechanism of action of epsilon toxin. The ETX binding to its putative receptors (Hepatitis A virus cellular receptor 1 (HAVCR1) and myelin and lymphocyte (MAL) protein) on cell membrane causes oligomerization of the toxin and subsequent membrane insertion. This constitutes the pre-pore stage and upon maturation, the pore results in efflux of K^+ ions and influx in Ca^{2+} ions. Increased intracellular calcium ion concentration is suggested to increase mitochondrial membrane permeability. This reduces mitochondrial membrane potential, causing ATP depletion and opening of membrane permeability transition. This results in translocation of apoptosis-inducing factor from mitochondria to nucleus which results in caspase-independent cell death. Red dashed arrows are used to depict proposed cellular effects of ETX (Pawaiya et al., 2020)

1.3. Control of disease by *C. perfringens* Type D

Enterotoxemic disease causes significant losses in sheep, lambs and calves. Since the *C. perfringens* is widespread in nature, it is not possible to completely erradicate it. Because the infection progresses rapidly and severely and prophylactic applications are more important than therapeutic applications for combating the disease. Vaccination is important in the control of infection. Vaccines have been developed using inactivated culture supernatants to prevent enterotoxemias caused by *C. perfringens* type D (McClane, 2014).

1.4 Production of vaccines from *C. perfringens* Type D

The most effective clostridial vaccines contain both the cellular “inactivated vaccine” and the “toxoids” prepared from toxins. Toxins recovered from *Clostridium* cultures are inactivated to form “toxoids”, which remain immunogenic but do not cause disease. Then toxoids are formulated into multivalent vaccines. Safe vaccine-toxoids are manufactured

using processes designed to completely inactivate all the toxins that were produced during culture of the vaccine seed. Clostridial vaccine production begins with culture of the desired organism using a fermentation process designed to maximize toxin production (Zaragoza et al., 2019). Most effective clostridial vaccines include "cellularly inactivated" vaccines prepared by formaldehyde and heat inactivated toxins to form toxoids. The first safe and effective inactivated vaccine was developed in 1924 by Gaston Ramon for the prevention of diphtheria. He used formaldehyde in combination with heat to inactivate the toxin, rendering it into a still immunogenic but to a non disease agent (vaccine) in humans. The procedure is still widely used for preparation of inactivated vaccines (Bonistalli, 2013). Immunization with toxoid vaccines is the only protective method against infection.

Clostridial species are strict anaerobes and will only produce toxin under appropriate conditions. For example, *C. perfringens* Type D actively produces toxin when it enters the acidic environment of the stomach and proximal small intestine. In vaccine manufacture, this process is simulated when strains are moved from culture to fermentation vats where the pH is then lowered allowing the bacteria to remain in a logarithmic growth and producing high levels of toxin (Bonistalli, 2013).

Vaccine potency is usually tested using live animal models. For clostridial vaccines this includes antitoxin (vaccine-induced antibody level) response in rabbits, paired with toxin neutralization tests. The toxin neutralization test is conducted by collecting serum from rabbits that have been immunized with vaccine (toxoid) then measuring the titer of antitoxin antibodies by comparing it to the number of units of standard antitoxin that is required to elicit the same level of neutralization activity (Mowat & Rweyemamu, 1991). After verifying potency, non-toxicity, and non-viability, the toxoids are combined with an adjuvant to formulate the final clostridial vaccine preparation. The vaccine can then be packaged for distribution and use *in vivo* after passing various additional quality assurance tests, including potency tests (Mowat & Rweyemamu, 1991; O'Hara & Bauer, 2008).

Different types of toxoid vaccines are widely available and have been used extensively over the past decades for use in animal husbandry (Table 1.3). However, the production and purification of toxins at industrial scale have been challenging (Aziminia et al., 2016). Moreover, the potential use of bacterial toxins (particularly ETX) as a biological weapon and the lack of effective treatments emphasize the need to develop safe and effective

vaccines for humans. Thus, heterologous or recombinant expression systems are good alternative tools for production of toxins for making vaccines (Nijland et al., 2007).

Table 1.3 Different types of vaccines for clostridial disease

Whole formalin–inactivated vaccines
Bacterin-toxoid vaccines
Toxoid vaccines
Genetically engineered vaccines
Nanovaccines
Polyvalent vaccines

Clostridial species are highly sensitive anaerobes and only produce toxins under suitable conditions. In vaccine production, environmental conditions are simulated so that the bacteria remain in logarithmic growth and produce high levels of toxin (Bonistalli, 2013). Modern clostridial vaccine production begins with culture using the desired organism and a fermentation process designed to maximize toxin production by the microorganism (Uzal et al., 2016). The current production process is briefly summarized in Figure 1.7.

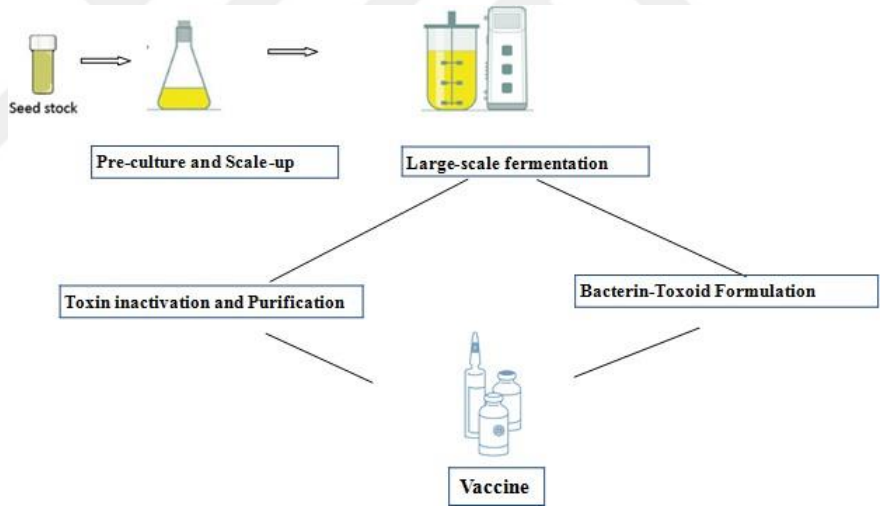

Figure 1.7 The production steps in a clostridial vaccine production process. Adapted from (Zaragoza et al., 2019)

1.4.1 Optimum conditions for production of epsilon toxin by *C. perfringens* Type D

The growth and epsilon production of *C. perfringens* are greatly affected by environmental conditions, such as acidity (pH), temperature, water activity, macro and micro nutrients, oxygen levels, and other toxins. Responding to cell-density cues and signalling molecules

by what is called “quarom sensing”, bacteria produce toxins only if they exceeded a certain threshold density. Thus, it is necessary to define the optimal culture conditions (whether physical or chemical) for toxin production by each strain. To do this, different culture parameters for the toxin (epsilon) production should be assessed to find the optimum conditions to scale up production of the toxin and its toxoid vaccines. Previous studies showed that high toxin production was achieved when synthetic, plant-based or animal-derived peptides were added to growth media. The concentrations of free amino acids in the medium also play important roles in toxin production, while high concentrations of free amino acids have inhibitory effect on toxin production. Futhermore, amino acids showed a varying degree effect (inhibitory or stimulatory) on toxin production, as glutamate, aspartate, glutamine, asparagine, histidine, and serine having the greatest inhibitory effect. Fermentable carbohydrates such as dextrin, glucose, fructose are other components important for toxin production, yet they are not fully consumed given the anaerobic conditions. Also, during the sterilization of the medium, glucose reacts with the amino acids and peptides, forming Maillard reaction products favorable for toxin production (Hauschild, 1965; Zaragoza et al., 2019). However, the effect of pH, temperature and mineral supplements on toxin production of today's media formulations is very limited. In this study, different culture parameters for epsilon toxin (ETX) production by *C. perfringens* type D are also evaluated.

2 MATERIALS AND METHODS

2.1 Reagents

The chemicals used cell growth, SDS-PAGE, and other tests were all reagent grade. Acrylamide, bisacrylamide, Ammonium persulphate (APS), methanol, and Brilliant Blue R-250 were from Merck Co.; TEMED, SDS, Isopropyl alcohol, Bromophenol blue, and glycerol were from Sigma Co.; Tris was from Ambresco Co.; Glacial acetic acid was from Carlo Erba; protein markers were from Abcam; $ZnSO_4 \cdot H_2O$ was from Sigma Chemical Co.

2.2 Bacterial strain

The strains of *C. perfringens* Type D Pendik were obtained from VETAL Animal Health Products Inc. (Adıyaman, Turkey).

2.3 Identification of the strain

2.3.1 DNA extraction

Bacterial colonies for identification were collected. DNA isolation was carried out with a commercial kit (QIAGEN DNA mini kit, Catalog number: 51304) using supplier instructions.

DNA Extraction Kit

Mini Spin Columns

Collection Tubes (2 ml)

Buffer AL

Buffer ATL

Buffer AW1 (concentrate)

Buffer AW2 (concentrate)

Buffer AE

Proteinase K

DNA extraction protocol

1. Collected bacterial colonies (10 colonies) were placed in a 1.5 ml microcentrifuge tube and 200 µl AL Buffer and 20 µl of Proteinase K were added. The mixture was gently vortexed and incubated at 56°C for 10 minutes.
2. 200 µl ethanol (96-100%) was added and gently vortexed until it was uniformly dispersed.
3. The mixture was pipetted into a Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x *g* for 1 min. The flow-through solution was discarded.
4. Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW1 was added and the mixture was centrifuged at 6000 x *g* for 1 min. Flow-through solution was discarded.
5. Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW2 was added. The mixture was centrifuged at 14,0000 rpm for 3 min. Flow-through solution was discarded.
6. The Mini spin column was placed in a new 1.5 ml microcentrifuge tube and 200 µl Buffer AE was added and centrifuged at 6000 x *g* for 1 min.
7. QIAamp was added to a new 1.5 ml microcentrifuge tube and 200 µl Buffer AE was added and the mixture was incubated at room temperature for 1 min. Then, the mixture was centrifuged at 6000 x *g* (8000 rpm) for 1 min to pellet the DNA.

2.3.2 Real-time PCR

Extracted DNA samples were identified by Real-Time PCR. The primer sequences, obtained from SenteBiolab, were used for identification is shown in Table 2.1. The specificity of the primers was determined using the BLAST service tool (Shehzadi et al., 2021). Identification was performed on a Roche Lightcycler 480 instrument using a commercial kit (Applied Biosystems BlastTaq 2X qPCR MasterMix, Catalog number: G891).

Table 2.1 Primers for epsilon toxin

Epsilon Toxin Primers
Forward primer: 5'-ATTAAAATCACAATCATTCACTTG-3'
Reverse primer: 5'-CTTGTGAAGGGACATTAGAGTAA-3'

Ten µl BlastTaq 2X qPCR MasterMix, 10 µM forward primer 0.5 µl, 10 µM reverse primer 0.5 µl, 5 µL DNase-free water and 4 µL DNA sample were added to each sample tube. Tubes were placed into the thermocycler and incubated at the conditions shown in Table 2.2.

Table 2.2 RT-PCR conditions

Step	Temperature	Time	Cycle
Enzyme activation	95 ºC	3 min	1
Denaturation	95 ºC	15 sec	40
Annealing	60 ºC	1 min	

2.4 Growth Media and Bacterial Cultures

2.4.1 Bacterial Frozen and Working Stocks

The main seed strain was inoculated into RCM medium and was confirmed with PCR. Then, to cultures grown in RCM for 12 h glycerol was added at 1:1 ratio to prepare working bacterial stocks which were either used or stored at -20 ºC.

2.4.2 Reinforced Clostridial Media (RCM) (L^{-1})

10 g of meat peptone (Merck- 107214)
10 g peptone (Conda-1616.00 Spain)
3 g of yeast extract (Merck-103753)
5 g of D-Glucose (Merck-346351)
1 g of starch (Merck-S4126)
5 g of NACI (Sigma- 9005-25-8)
3 g of $C_2H_3NaO_2$ (Sigma- 127-09-3)
0.5 g L-Cysteine chloride (Sigma- 7048-04-6)

Above content was dispensed into test tubes and autoclaved (15 min at 121 °C). The appearance of the medium in the tubes were yellowish clear. The pH value (unadjusted) of medium in room temperature was in the range of 6.6-7.0.

2.4.3 Clostridial Agar (L^{-1})

17 g Tryptone (Merck-91079-40-2)
3 g Peptone (Condalab-1616)
6 g Dextrose (Sigma-50-99-7)
2.5 g NACI (Sigma- 9005-25-8)
0.25 g L-Cysteine Chloride (Sigma- 7048-04-6)
12 g Agar (Merck-101614)

The medium was autoclaved at 121 °C for 15 min and poured into petri dishes, and was kept at room temperature to solidify.

2.4.4 Blood Agar (Merck-70133)

Commercial 40 g of blood agar base (10 g meat extract, 10 g Peptone, 5 g NaCl, 15 g agar) was dissolved in 1 liter ddH_2O. Final pH was adjusted to 7.3 at room temperature. Then, the medium was autoclaved at 121 ^oC for 15 min and cooled to a degree (50 -60 ^oC) before defibriniated sheep blood was added at 5% final volume. Then, the medium was poured into petri plates and left to cool.

2.4.5 Tryptic Soy Broth (TSB) and Fluid Thioglycollate Media (FTM*)*

TSB (Merck- 105459) was used for sterility tests of bacterial cultures. TSB was dissolved in ddH_2O to a final volume 30 g L^{-1} and the mixture was autoclaved for 15 min at 121 °C. The final pH was adjusted to 7.3 at room temperature. FTM (Merck- 108191) was used for cultivation and isolation and sterility testing of obligate and facultative anaerobic or microaerophilic bacteria. TSB was dissolved in ddH_2O to a final volume 30 g L^{-1} and the mixture was autoclaved for 15 min at 121 °C. The final pH was adjusted to 7.3 at room temperature.

2.4.6 Adjusting pH and Temperature of Growth Media

The acidity of bacterial growth media was adjusted with a pH-meter (Hanna USA) using either 1 M NaOH (Sigma-06203) or 1 M HCl (Merck-109973). The optic densities (OD_{600} values) were recorded after incubation of bacterial cultures under different temperature and pH conditions in a temperature controlled incubator (NUVE brand S120 and NUVE brand FN120).

2.4.7 Effect of Zn^{2+}

After the optimization of culture condition in terms of pHs (5.8, 6.8, 7.8) and temperatures (30 ºC, 35 ºC, 37 ºC), the effect of Zn was investigated at four different concentrations of $ZnSO_4$ (10^{-3} - 10^{-6} M) in 10 ml RCM medium.

2.5 Identification of *C. perfringens* Type D

C. perfringens type D was transferred to Fluid Thioglycollate broth, blood agar, Clostridial agar and incubated for 18-24 h at 35-37 °C under anaerobic conditions. Then the colonies were transferred back to the blood agar and incubated under anaerobic conditions for 18-24 h. Confirmation tests were carried out and the bacterium was identified as rod-shaped, Gram-positive bacteria which indicates that the bacterium was *Clostridium perfringens*. Putative *Clostridium* colonies were inoculated into blood agar and FTM medium and then they were identified as such by Real-time PCR.

2.6 Measuring the cell mass (OD_{600}) of bacterial cultures

The OD_{600} values of the cultures were measured with the Agilent Carry 60 UV/VIS spectrophotometer.

2.7 Analysis of epsilon toxin by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used to achieve analytical separation of protein mixtures. The SDS-PAGE procedure consists of electrophoresis through a porous polyacrylamide gel matrix that separates proteins according to molecular mass after initial denaturation with an anionic detergent (SDS) that imparts a negative charge to the proteins.

C. perfringens type D culture, which was produced under different culture conditions, was centrifuged at 3000 rpm for 30 min. Twenty µl of sample (supernatant plus dye) was loaded on SDS-PAGE gels (Sambrook & Smith, 2001) for toxin analysis (Table 2.3).

Table 2.3 SDS-PAGE Gel

5% stacking gel	**(ml)**
ddH_2O	5.5
30% acrylamide mix	1.3
Tris-Cl (1.0 M, pH 6.8)	1.0
SDS (10%)	0.08
Ammonium persulfate(10%)	0.08
TEMED	0.008
%12 resolving gel	**(ml)**
ddH_2O	3.3
30% acrylamide mix	4.0
Tris-Cl (1.5 M, pH 8.8)	2.5
SDS (10%)	0.1
Ammonium persulfate (10%)	0.1
TEMED	0.004

2X SDS gel-loading buffer

100 mM Tris-Cl (pH 6.8)

4% (w/v) SDS

0.2% (w/v) bromophenol blue

20% (v/v) glycerol

SDS (10%) stock solution

10 g of SDS was dissolved in 80 mL of ddH_2O and then ddH_2O added to 100 mL.

10% Ammonium persulfate

1 g of ammonium persulfate was dissolved in 10 ml of ddH_2O.

Tris-HCl

1 M Tris base solution: 12.11 g of Tris base was dissolved in 80 mL ddH_2O and total volume was completed to 100 ml. The pH of solution adjusted to 6.6 with 1M HCI.

1.5 M Tris base solution: 18.7 g of Tris base was dissolved in 80 mL ddH_2O andtotal volume was completed to 100 ml. The pH of solution was adjusted to 8.8 with 1M HCI.

Running Buffer (5X)

15.1 g Tris base

94 g glycine

50 ml of 10% SDS dissolved in ddH_2O to a final volume of 1-liter solution.

3 RESULTS

3.4 Bacterial Cultures

The *C. perfringens* type D, maintained in glycerol as a stock, was supplied to our laboratory by the VETAL Animal Health Products Inc. (Adıyaman, Turkey). A 50 µl of this stock was inoculated into 10 ml RCM medium in 25 ml glass tubes. The inoculated culture kept in an air-tight jar in which the air (oxygen) was removed by a burning candle and incubated at 37 °C under such anaerobic environment. After incubation, the growth was observable even by a naked eye after 8 h (Figure 3.1, right panel). These cultures were used both for experiments and also kept in glycerol (1:1 ratio) at -20 ºC as working stock solutions.

Also, for the purity and identification purposes, the bacterium was inoculated into blood agar as two replicates. As it is apparent no bacterial colonies were observable after an extended (72 h) period of time (Figure 3.1, left panel).

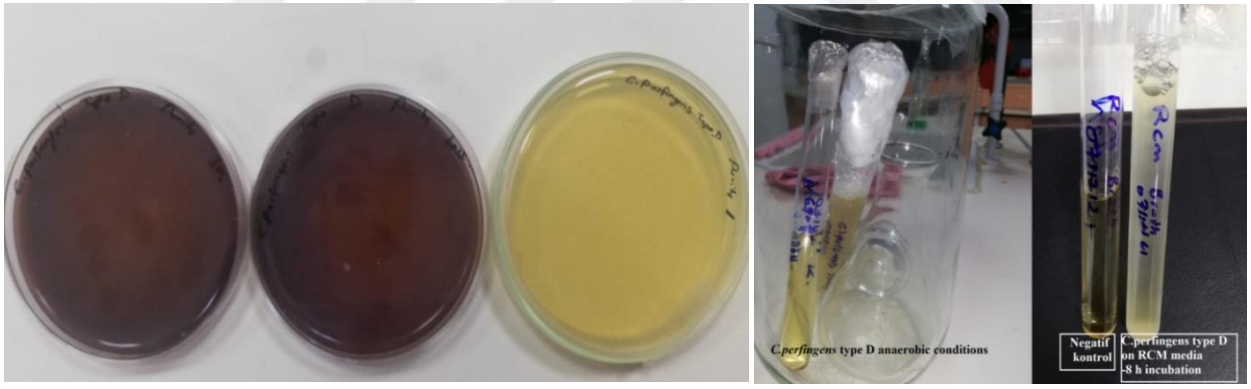

Figure 3.1 *C. perfringens* type D bacterium under aerobic conditions. No growth (colony formation) was determined under such growth condition as expected from a strictly anaerobic bacterium (left panel). Preparation of working seed from main stock cultures (right panel).

In addition, the stock samples were incubated in anaerobic Fluid Thioglycollate Medium (FTM, the bottle in Figure 3.2) and Tryptic Soy Broth (TSB, the tube with clear medium in Figure 3.2) media at 37 °C and 26 ºC to determine bacterial and yeast contamination, respectively. No contamination was observed in any of the bacterial cultures after 72 h of incubation (Figure 3.1, left panel). Then, the pure cultures at 8th hour of incubation were taken as working stocks of *C. perfringens* type D culture and they were kept at -20 ºC as stock cultures for the further experiments.

Figure 3.2 *C. perfringens* type D purity test. The bacterium was inoculated into the FTM medium at pH 7.0 and incubated under anaerobic conditions at 37 ºC.

3.5 Identification of Bacteria with RT-PCR

Bacteria grown on FTM medium used for DNA extraction and RT-PCR as given under Materials and Methods. PCR test was determined as positive for *C. perfringens* type D at 24.57 *ct* where a Sigmoidal Curve was observed. The negative control had a negative result even at 35 *ct* (Figure 3.3).

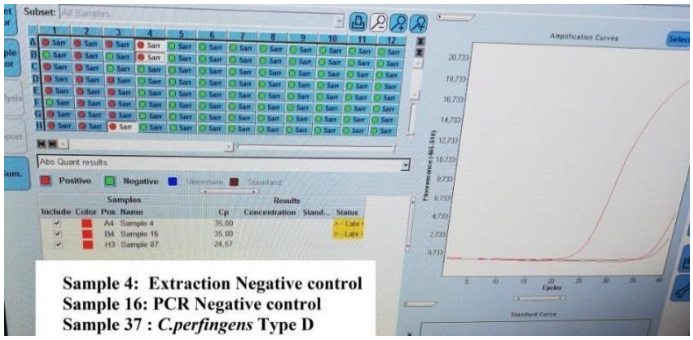

Figure 3.3 The RT-PCR Panel of *C. perfringens* type D bacteria.

3.3 Growth at Different pH and Temperatures

Initially the RCM medium was adjusted to three different pHs: pH 5.8, pH 6.8, pH 7.8. Ten ml of these media were dispensed into 20 ml volume tubes (Figures 3.4 and 3.5). To each tube 50 µl working stock of the bacteria was inoculated and incubated in anaerobic environment at 30 ^{o}C, 35 ^{o}C, 37 ^{o}C for 12 h. The experiment was repeated in triplicates at various time points. At the end of 12 h, the bacterial densities were measured as OD_{600} readings. Since bacteria appeared like a gooey suspension as it is apparent in Figures 3.4 and 3.5, they were homogenized by vortexing before recording their optic densities. The density of bacterial cultures as total mass was recorded at 600 nm with an UV-VIS spectrophotometer (Figure 3.6). The lowest cell densities ($A_{600,}$ ~0.750) were observed in cultures with pH 5.8 in both temperatures. Especially, there was almost no observable growth in pH 5.8 cultures at 37 ^{o}C. The highest growth ($A_{600,}$ ~4.1) was observed in cultures with pH 6.8 at 37 ^{o}C. The later condition (i.e., pH 6.8 at 37 ^{o}C) was selected for further experiments with $ZnSO_4$ supplemented RCM medium.

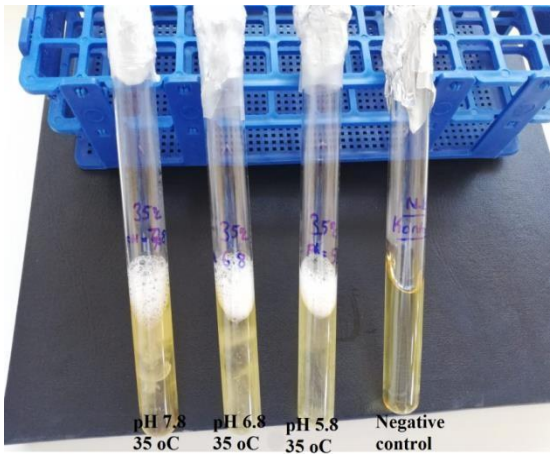

Figure 3.4 *C. perfringens* type D bacteria culture growth at 37 ºC. Bacterium was incubated a respective pHs at 35 ºC in RCM medium.

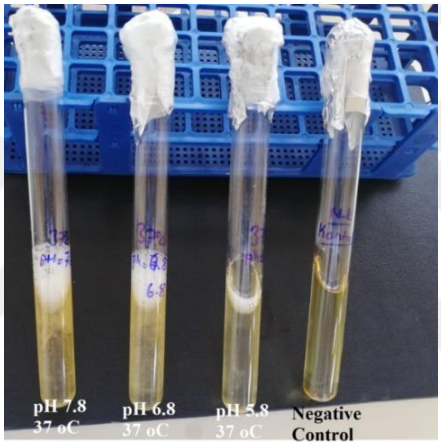

Figure 3.5 *C. perfringens* type D culture at 35 ºC. Bacterium was incubated a respective pHs at 35 ºC in RCM medium.

Increasing temperature and pH generally had a positive effect on cell growth (Figure 3.6). Thus, the highest growth (OD_{600}= 4.17) was observed at 37 ºC in cultures grown at pH 6.8. Low pH and temperature had a negative effect on cell growth. The lowest cell densities for all three pHs were recorded in cultures at 30 ºC. At this temperature, cell densities of pH 5.8 cultures were almost 3 times lower than the cell densities of 35 ºC and 37 ºC with the same pH. Conditions that supported the highest cell density (i.e., pH 6.8 and 37 ºC) were selected as optimal growth conditions for the experiment to be conducted with $ZnSO_4$ supplementation.

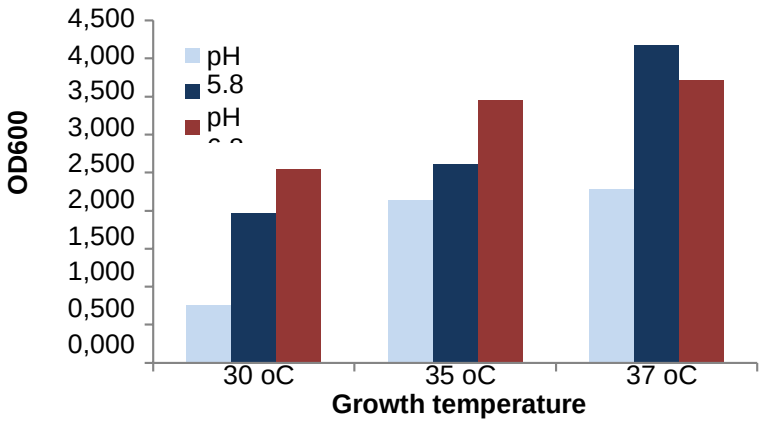

Figure 3.6 Culture densities of *C. perfringens* type D at different pH and temperature of incubation. The values are the averages of three experiments carried out at different times. For clarity, standard deviations (±) are not shown, but they were generally less than 10% of the recorded values.

3.4 Bacterial Growth in $ZnSO_4$ Supplemented RCM

The RCM medium at pH 6.8 was supplemented with $ZnSO_4$ at four different concentrations and cultures were incubated ate 37 ºC for 12 h. The highest bacterial density was observed in 10^{-4} M $ZnSO_4$ medium. Increased concentrations of $ZnSO_4$ caused a decrease in cell density. The lowest cell density as OD_{600} was about 0.650 with highest $ZnSO_4$ concentration of 0.01 M (Figure 3.7). The cell density at this concentration of Zn^{2+} was more than one order of magnitude lower than cultures with 10^{-4} M Zn^{2+}. Further decrease of $ZnSO_4$ concentration below 10^{-4} *M*, had not any effect (if not negative) on the total cell mass.

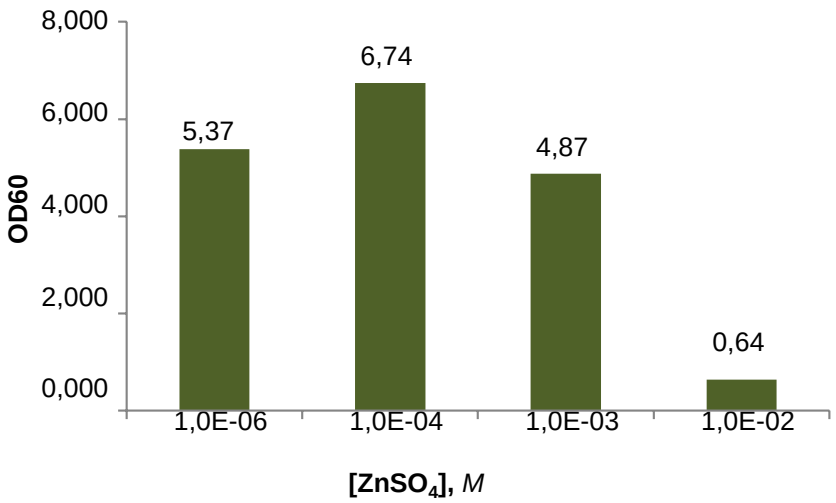

Figure 3.7 Total biomass (OD_{600}) of bacterial cultures supplemented with varying concentrations of $ZnSO_4$. Cells were grown at 37 ºC, pH 6.8 for 12 h. Each value is the average of three replicates made in different times. For clarity, standard deviations (±) are not shown, but they were generally less than 10% of the observed values.

3.5 Analysis of Epsilon toxin by SDS-PAGE

C. perfringens type D culture supernatant was loaded on SDS-PAGE gel for toxin analysis. The molecular weight of Epsilon toxin was determined to be around 35 kDa. Of the cultures studied, epsilon toxin expression was only observed at 37 ºC pH 6.8 cultures (Figure 3.8).

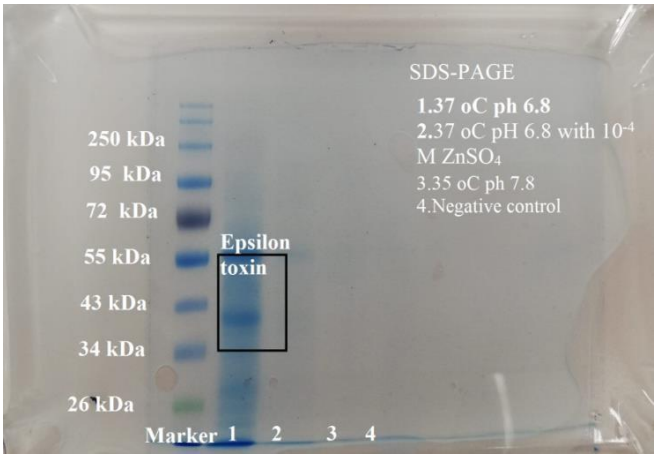

Figure 3.8 Epsilon toxin on SDS-PAGE

4 DISCUSSION

In toxoid vaccinations, a toxin is used as source of vaccines. A toxin made by a bacterium or fungus that causes a disease is used as an immunogen (antigen). Such vaccination creates an immunity to the parts of the pathogens (e.g., bacteria) that cause a disease instead of the whole pathogen itself. In other words, the immune response in vaccinated animals is targeted to the toxin instead of the whole microbe. Toxoid vaccines have been widely used for years in the fight against *C. perfringens*, a common cause of enterotoxemia (Khan et al., 2018; Zaragoza et al., 2019). The vaccines are obtained from various strains of *C. perfringens* which produces a large number of protein toxins with cytotoxic, histotoxic, neurologic effects during infections in humans and animals (Popoff, 2014). Epsilon toxin (ETX), the subject of our work here, is used also used as a vaccine after its inactivation. Inactivation is a required manipulation in order to prevent morbidity and mortality in vaccinated animals. However, since inactivation causes also damage to the antigenic structure of toxin, there is a high demand for large scale toxin production to prepare effective toxoid vaccines (Bonistalli, 2013). Thus, it is imperative to understand the physical and chemical conditions required for toxin production is of interest. Knowing that toxin expression is highly affected by culture conditions (Brandi et al., 2014) such as total cell density (Pivnick et al., 1965), pH, temperature and divalent metal cations (Murata et al., 1956), our goal in this study was to determine the optimal growth conditions for production of epsilon (ε) toxin (ETX) by *C. perfringens* Type D strain in these respects. The pH of medium on the production of this toxin was studied previously (Pivnick et al., 1965) and it was shown that optimum synthesis of the various toxins by the same strain was achieved at pH 7.0, while lower or higher pHs effected ETX production negatively. To the best of our knowledge, our study is the first to demonstrate a relationship between culture pH and temperature of growth and effect of a divalent cation ($ZnSO_4$) on the cell growth for *C. perfringens* type D. Considering all the

parameters studied, the highest bacterial densities, determined as OD_{600} values, were recorded at 37 ºC, pH 6.8 medium supplemented with 10^{-4} M $ZnSO_4$. As a result, $1.2x10^9$ cell/ml was obtained according to MC Farland calculation with a value of 0.675 obtained at OD_{600}. However, with the addition of 10^{-4} M $ZnSO_4$, the highest value obtained at about 6.75 OD and the absence of protein expression shows that there is no correlation between bacterial growth and toxin expression. Highest level of the toxin expression was observed at the at 37 ºC and pH 6.8. Our findings are in line previous studies (Brandi et al., 2014; Gohari et al., 2021; Li et al., 2016 ; Sakurai et al., 2009; Zaragoza et al., 2019), where it has been reported that toxin, including ETX, production does not necessarily require an active growth phase and high cell densities given that most toxins are produced under extreme (sporulation) conditions or post-logarithmic phase of growth and toxin production is highly affected by environmental conditions of both physical and chemical. Thus, an optimum level toxin production by a specific bacterial strain requires case by case study of these conditions. Clearly, there is much more to learn regarding optimal conditions for ETX production.

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CURRICULUM VITAE

Name Surname: Fethiye Sevimli

EDUCATION STATUS:

- **Bachelor's Degree:** 2017, İnönü University, Molecular Biology and Genetic
- **Master's Degree:** 2022, İnönü University, Molecular Biology and Genetic

EXPERIENCE:

1. Trainee at Erasmus Program (2017-2018)- University of Padova, Italy, Padova (Topic of the study: *Interaction between mitochondrial DNA variants and mitochondria/endoplasmic reticulum contact sites*)
2. Internship in Genetic Diagnosis Center at Akdeniz University - (2 months)
3. Vetal Serum and Biological Products Inc - 2018-2021 (Topic of research: *Vaccines and sera production*

PROJECTS:

1. Researcher, *Development of Inactive Rabies Vaccine for Use in Animals* (TÜBİTAK Project, 2019)
2. Researcher, *Preparation of Monovalent Hyperimmune Serum Against Foot and Mouth Disease* (TÜBİTAK Project, 2020)
3. Researcher, *Development of Viral Vaccines Using Microcarrier Systems for Cats and Dogs* (TÜBİTAK Project, 2021)
4. Researcher, *Development of a Quadrivalent Influenza Vaccine for Human Use* (TÜBİTAK Project, 2021)