INVESTIGATIONS INTO METABOLICALLY ACTIVE YET NON-CULTURABLE (MAYNC) C*LOSTRIDIUM PERFRINGENS* TO CONTROL NECROTIC ENTERITIS IN BROILER CHICKENS

A Dissertation

by

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ABSTRACT

Clostridium perfringens-based *Necrotic* Enteritis (NE) is a disease that can cause significant morbidity and mortality in poultry, causing economic losses of approximately $6 billion annually. This disease was previously controlled using antibiotic growth promoters, which have been phased-out due to concerns of the antibiotic resistance genes. Vaccines against infectious diseases have been proposed as a solution to the use of antibiotics in livestock animals. Electron Beam (eBeam) technology is a commercial technology that inactivates microorganisms by causing significant damage to the nucleic acid. While the cell is unable to replicate, membrane integrity and metabolic activity are maintained. This state is termed a Metabolically Active, yet Non-culturable (MAyNC) and possess characteristics of a potential vaccine: MAyNC cells are unable to replicate and cause disease within a host, but because they are intact and metabolically active, they may retain their antigenicity and immunogenicity.

In this study, the MAyNC state of eBeam inactivated *C. perfringens* (EBCP) was investigated as a potential control mechanism against NE in broiler chickens. The results demonstrated that when exposed to a target dose of 10 kGy, *C. perfringens* was irreversibly inactivated even when enriched *in vitro* and administered in a NE challenge model *in vivo*. The MAyNC state of EBCP was confirmed using metabolic activity and membrane integrity assays. Metabolomic analysis demonstrated that MAyNC *C. perfringens* had a distinct metabolomic

profile immediately after inactivation with increased production of many cell-signaling metabolites. The protective ability of two EBCP cocktails was assessed by vaccinating birds with adjuvanted or non-adjuvanted formulations on day-of-hatch, and then challenging them with homologous live *C. perfringens* in a NE challenge model. EBCP cocktails alone were not protective upon challenge and birds vaccinated with adjuvanted EBCP had significantly more signs of disease than even unvaccinated control birds. The results of these studies suggest that eBeam inactivation induces a MAyNC state in *C. perfringens* that has the potential to be used to protect against NE challenge, although further optimization of the vaccine formulation is required.

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1. INTRODUCTION

1.1. Relevance

In order to feed a growing global population estimated to reach 8.6 billion by 2030, there is a need for robust, safe, and efficiently produced animal protein (UN-DESA, 2017). Poultry meat consumption is increasing globally as it is perceived as a healthy and a low-cost protein source, with global poultry meat consumption approximated to be 100 million metric tons in 2020 (USDA-FAS, 2020). The need to meet this growing demand has put significant stress on the poultry industry.

Necrotic enteritis, an enteric poultry disease caused by the bacteria *C. perfringens* and its toxins, is one of the most economically significant diseases in poultry with up to 1% flock mortality/day in clinical outbreaks and with economic costs estimated at $5-6 billion annually (Wade and Keyburn, 2015). While acute clinical cases of NE cause up to 30-40% mortality, subclinical cases are of greater concern due to poor nutrient absorption, decreased weight gain, decreased feed conversion, and the increased risk for the contamination of poultry products leading to foodborne illness in humans (Timbermont et al., 2011; Wade and Keyburn, 2015; Moore, 2016; Prescott et al., 2016a).

Historically, Antibiotic Growth Promoters (AGPs) have protected poultry from enteric pathogens (Hargis, 2004; Dibner and Richards, 2005). Due to concerns of antibiotic resistance, recent legislation has severely limited the use of AGPs leading to a drastic increase in NE outbreaks (Immerseel et al., 2004; Hermans and Morgan, 2007; Van Immerseel et al., 2009). Currently, feed additives are used to partially control NE (Caly et al., 2015). Vaccination has been proposed as the most efficient method to control NE, but there is currently no vaccine against this disease on the market. Therefore, the there is a need for a vaccine against *C. perfringens*-based NE that offers increased protection in order to increase animal health and poultry production.

1.2. Significance

The resurgence of previously controlled diseases has led to the intensive investigation and commercialization of multiple methods to control and improve animal health, including pre- and probiotics, organic acids, nutrition-based feed additives, immunomodulators, and vaccines (Van Immerseel et al., 2009; Cheng et al., 2014; Caly et al., 2015; Marquardt and Li, 2018). Vaccination has been proposed as an efficient method to control NE, and while commercial *C. perfringens* toxoid vaccines have been available for use in the poultry industry previously, there are currently no commercial vaccines that protect against NE in poultry (Lovland et al., 2004; Crouch et al., 2010). Therefore, there is an urgent need for a vaccine against *C. perfringens*-based NE in order to protect animal health and subsequently human health. The significance of this research is that it not only addresses a singular disease in the poultry industry, but also establishes the ability of high energy electron beam technology to be used as a platform technology. Furthermore, a molecular understanding of the effect of high energy eBeam on pathogenic bacteria have applications in the food and medical device industries, two key arenas that already use this technology for microbial control.

The long-term goal of this project was to establish a foundation for a vaccine platform technology – a technology that could create a vaccine that protects against *C. perfringens* in poultry and then be tailored to create vaccines against pathogens in various species.

1.3. Overall objective

The overall objective of this research was to create a vaccine candidate using a cocktail of Metabolically Active, yet Non-Culturable (MAyNC), electron beam-inactivated *C. perfringens* that is protective against NE in poultry broilers. The central hypothesis was that eBeam technology would produce MAyNC *C. perfringens*, a state that could be broadly antigenic and immunogenic, serving as an effective vaccine that protect against NE in broiler chickens.

1.4. Specific objectives

The specific objectives of this study were to:

1. Generate the MAyNC state in *C. perfringens* and perform their molecular characterization.
2. Identify the molecular markers of exposure to high energy eBeam doses.
3. Establish a Necrotic Enteritis challenge model.
4. Perform challenge studies in broilers to evaluate the efficacy of the immunomodulators at protecting broiler chickens upon challenge.

2. LITERATURE REVIEW

2.1. Poultry industry

In order to feed a growing global population estimated to reach 8.6 billion by 2030, there is a need for robust, safe, and efficiently produced animal protein (UN-DESA, 2017). Poultry meat consumption is increasing globally as it is perceived as a healthy and a low-cost protein source, with global poultry meat consumption approximated to be 100 million metric tons in 2020 (USDA-FAS, 2020). The need to meet this growing demand has put significant stress on the poultry industry. In 2019, out of over 9.2 billion broiler chickens weighing 58.3 billion pounds produced, more than 42.1 billion pounds of chicken product made it to market, representing a staggering efficiency and resource utilization (NCC, 2019). With broiler sales representing 70% of the $40.4 billion US poultry industry, there are economic pressures for efficiency (USDA-NASS, 2019). This is most clearly demonstrated by the impressive increase in feed efficiency that has been achieved by the poultry industry. In 1925, it took on average 112 days for a 2.5 lb broiler to make it to market, with a feed conversion ratio (pounds of feed to one pound broiler weight – FCR) of 4.7. By 1990, it only took on average 48 days for a 4.37 lb broiler to make it to market, with a FCR of 2. In 2019, such advances have been made that in an average of 47 days, a 6.32 lb bird can make it to market with a FCR of 1.8 (NCC, 2020). Such advances in the poultry industry have been a product of extensive research into genetics and selective breeding, optimized nutrition regimes, and intensive rearing practices. A combination of these circumstances have subsequently led to an increase in poultry diseases (Havenstein et al., 2003; Lumpkins et al., 2010; Guardia et al., 2011; Mot et al., 2014).

2.2. Antibiotic growth promoters

With the growth of intensive livestock rearing practices in the 20th century, came the introduction of antibiotics that could control the increase in diseases. Historically, antibiotic growth promoters (AGPs) have been primarily used to improve broiler chicken performance but have had the additional benefit of protecting poultry from enteric pathogens. Used in sub-therapeutic doses in livestock, AGPs have been shown to contribute to the development of antibiotic resistance in bacteria located in the intestinal tract of animals, which can then be shed and spread to humans (Dibner and Richards, 2005; Asai et al., 2007). Globally, the prevalence of multi-drug tolerant microorganisms has become a serious risk to humans and animals alike. For example, while vancomycin-resistant enterococci are a common cause of nosocomial infection in humans, the prevalence of vancomycin-resistant enterococci in flocks of turkey has been reported to be up to 75% (Arias et al., 2010; Sting et al., 2013; Maasjost et al., 2015). Due to these concerns, in 2006 the European Union Commission banned the use of AGPs in feed, leading to drastic increases in previously controlled diseases (Wierup, 2001; Van Immerseel et al., 2009; Maron et al., 2013; McEwen et al., 2017). In 2017, the US Food and Drug Administration Veterinary Feed Directive severely limited the use of antibiotics in food animals by requiring veterinary oversight during administration, which will likely lead to an increase in disease in the United States as well.

2.3. Necrotic enteritis

Necrotic enteritis is an enteric disease of poultry that usually occurs in broiler chickens between two and six weeks of age as protective maternal antibodies are disappearing (Songer, 1996; Lovland et al., 2004; Cooper et al., 2010; Crouch et al., 2010). It is estimated that the disease causes up to $6 billion in global economic losses annually (Wade and Keyburn, 2015). While typically afflicting broiler chickens raised on floor pens, NE outbreaks have been reported in food egg layers (Dhillon et al., 2004). The disease has been found in all poultry-rearing countries of the world (Dahiya et al., 2006; Flores-Díaz et al., 2018). The disease presents in two forms: a clinical form and a subclinical form. The clinical form is characterized by a drastic increase in flock mortality with 1% mortality per day and up to 50% overall flock mortality being reported (Helmboldt and Bryant, 1971; Long et al., 1974; Mcdevitt et al., 2006). Occasionally, birds may also demonstrate signs of depression, ruffled feathers, and diarrhea (Hargis, 2004). The subclinical form is typically more prevalent (Wu et al., 2010). In this form, there is chronic intestinal damage leading to poor digestion and nutrient absorption, reduced weight gain, and ultimately an increased FCR and increased costs during production (Kaldhusdal et al., 2001; Allaart et al., 2012; Wade and Keyburn, 2015). Chronic intestinal damage allows the bacteria to travel to the bile duct and portal blood stream, leading to cholangiohepatitis (Løvland and

Kaldhusdal, 1999; Lovland and Kaldhusdal, 2001). Diseased livers are often enlarged and pale in color with white or red foci. Damaged livers are most often found during slaughter leading to carcass condemnation (Lovland and Kaldhusdal, 2001). Because the subclinical form often goes undiagnosed and untreated for the life of the bird and is only diagnosed at the time of slaughter, it is generally considered the more damaging and economically important form of the disease (Kaldhusdal et al., 2001; Dahiya et al., 2006; Wade and Keyburn, 2015). Upon necropsy, necrotic lesions are often found in all parts of the small intestine. Typically, the intestine is thin-walled and can be pale in color. Focal mucosal necrosis is often evident. Ulcers or lesions often form depressions in the mucosal surface. When swabbed, *Clostridium perfringens* is typically found in high concentrations of approximately 10^7-10^9 CFU/g compared to 10^2 – 10^4 CFU/g typically found in healthy birds. (Long and Truscott, 1976; Kondo, 1988).

2.3.1. ***Clostridium perfringens***

Clostridium perfringens is a spore-forming, anaerobic, Gram positive, rod-shaped bacterium, ubiquitous to the environment and the GI tract of humans and animals (Songer, 1996; Kiu and Hall, 2018). It was first isolated in 1891 from the autopsy of a man with gas bubbles present in infected blood vessels, by William H. Welch (Welch and Nuttall, 1892). At the time, it was named *Bacillus aerogenes capsulatus* with *Bacillus* indicating the shape of the cells and *aerogenes* literally translating to ‘air-producing’ in Latin. *C. perfringens* has been isolated from a variety of environments such as soils, wastewater, sewage, food, and in the GI tract of healthy and diseased humans and animals (Songer, 1996; Lisle et al., 2004; Kiu and Hall, 2018). Most notably, *C. perfringens* has been isolated from the over 5000 year-old frozen mummified GI tract of a human from the Copper Age (Lugli et al., 2017). Clinically, *C. perfringens* is the causative agent of numerous diseases in humans and animals with some examples shown in Table 1.

2.3.1.1. Toxins

C. perfringens strains can produce numerous toxins and as of 2018 are classified as types A-G based on the presence or absence of six toxin genes (Rood et al., 2018). Prior to 2018, *C. perfringens* were classified as types A-E with the new system published in 2018 further differentiating type A strains into Type A, F, or G. All strains produce alpha toxin, while the other toxins are variable depending on the presence of specific virulence plasmids. Thus, the typing scheme is a reflection of the plasmids possessed by an individual strain.

Table 1. *C. perfringens* causes different diseases in different hosts

	Major Toxins Produced						Disease	Host
Type	**Alpha (α)**	**Beta (β)**	**Epsilon (ε)**	**Iota (ι)**	**CPE[a]**	**netB**		
A	+	-	-	-	-	-	Myonecrosis (gas gangrene)	Human and most animals
							Food poisoning	Human
							Enterotoxemia	Cattle and lambs
							Necrotizing enterocolitis	Neonatal pigs
B	+	+	+	-	-	-	Necrohemorrhagic enterotoxemia	Sheep
							Dysentery	Neonatal sheep
							Chronic enteritis	Neonatal sheep
							Hemorrhagic enteritis	Neonatal cattle, horse
C	+	+	-	-	-	-	Necrotic enteritis (pigbel)	Human
							Necrotic enteritis	Neonatal horse, cattle, sheep, pigs
							Necrotic enteritis	Fowl
							Acute enterotoxemia	Sheeps and goats
D	+	-	+	-	-	-	Enterotoxemia	Sheep and neonatal cattle
E	+	-	-	+	-	-	Enterotoxemia	Neonatal cattle and sheep
							Enteritis	Rabbits
F[b]	+	-	-	-	+	-	Non-foodborne GI disease	Human, canine
G[b]	+	-	-	-	-	+	Necrotic Enteritis	Poultry

[a] *Clostridium perfringens* Enterotoxin

[b] Formerly included in Type A

Adapted from (Songer, 1996; Popoff, 2014; Uzal et al., 2014; Hassan et al., 2015; Kiu and Hall, 2018; Rood et al., 2018; Zaragoza et al., 2019)

Commonly referred to as a “flesh-eating” bacterium, the hallmark signs of a *C. perfringens* infection include necrotic tissue regardless of whether the infection is dermal, such as in gas gangrene in humans or gangrenous dermatitis in poultry, or enteric, as is the case in Necrotic Enteritis in poultry. The pathogenesis of *C. perfringens* is largely based on its ability to produce of a variety of virulent proteins. *C. perfringens* lacks biochemical pathways for the synthesis of 13 amino acids (Fuchs and Bonde, 1957; Shimizu et al., 2002). To compensate, *C. perfringens* secretes an assortment of degradative enzymes and toxins that act on host tissues. The species can create up to 23 exotoxins and virulent enzymes, although no individual strain can express all of the toxins (Kiu and Hall, 2018) (Table 2). A majority of these virulence factors, with some important exceptions, are encoded on conjugative plasmids. Therefore, the virulence and specific pathogenicity of a strain heavily depends on the presence or absence of specific plasmids.

Table 2. List of 23 toxins identified in *C. perfringens*

Toxin	Gene(s)	Location	Involved in NE in poultry?	Action	Source
Alpha	*plc or cpa*	Chromosome	Yes	Phospholipase C /sphingomyelinase; activates host cell signaling	(Titball et al., 1999; Sakurai et al., 2004)
Beta	*Cpb*	Plasmid	Yes	Pore forming toxin	(Shatursky et al., 2000; Nagahama et al., 2015)
Epsilon	*Etx*	Plasmid	No	Pore forming toxin	(Hughes et al., 2007; Chen et al., 2011; Li et al., 2011b; Alves et al., 2014)
Iota	*iap, ibp*	Plasmid	No	Actin-specifc ribosyltransferase; facilitates binding	(Stiles et al., 2002; Nagahama et al., 2011)
Enterotoxin	*Cpe*	Chromosome or Plasmid	Yes	Pore forming toxin and tight junction disintigration	(Li et al., 2011a; Freedman et al., 2016; Eichner et al., 2017)
Beta 2	*cpb2*	Plasmid	Yes	Pore forming toxin	(Gibert et al., 1997)
NetB	*Netb*	Plasmid	Yes	Pore-forming toxin	(Keyburn et al., 2008, 2010b)
Kappa	*colA*	Chromosome	Unknown	Collagenase	(Matsushita et al., 1994; Awad et al., 2000)
Perfringolysin (theta-toxin)	*pfoA*	Chromosome	Yes	Pore forming toxin;cholesterol-dependent cytolysin	(Ohno-Iwashita et al., 1986; Verherstraeten et al., 2015)
TpeL	*tpeL*	Plasmid	Yes	Ras-specific mono-glucosyltransferase	(Coursodon et al., 2012; Bailey et al., 2014; Chen and McClane, 2015)

Table 2. Continued

Toxin	Gene(s)	Location	Involved in NE in poultry?	Action	Source
Mu	*nagH, nagI, nagJ, nagK, nagL*	Chromosome	Unknown	Hyaluronidases	(Canard et al., 1994; Goossens et al., 2017)
Sialidase	*nanH, nanI, nanJ*	Chromosome	Unknown	Sialidase	(Roggentin et al., 1988; Chiarezza et al., 2009; Goossens et al., 2017)
Alpha clostripain	*ccp*	Chromosome	Unknown	Cysteine protease	(Chakravorty et al., 2011)
Delta	*cpd*	Plasmid	Unknown	Pore-forming toxin	(Manich et al., 2008)
NetE	*netE*	Plasmid	Unknown	Putative pore-forming toxin	(Mehdizadeh Gohari et al., 2015)
NetF	*netF*	Plasmid	Unknown	Pore-forming toxin	(Mehdizadeh Gohari et al., 2015; Gohari et al., 2016)
NetG	*netG*	Plasmid	Unknown	Putative pore-forming toxin	(Mehdizadeh Gohari et al., 2015)
BecA BecB	*becA, becB*	Plasmid	Unknown	Actin-specifc ADP-ribosyltransferase	(Yonogi et al., 2014)
Lamda	*lam*	Plasmid	Unknown	Metalloprotease	(Jin et al., 1996)
Zinc metalloprotease	*zmpA*	Plasmid	Yes	Metalloprotease	(Wade et al., 2020)
Zinc metalloprotease	*zmpB*	Chromosome	Yes	Metalloprotease	(Wade et al., 2020)

The understanding of the toxins produced by *C. perfringens* is constantly evolving. The production and regulation of *C. perfringens* toxins are controlled by gene regulatory systems, mainly two-component regulatory and quorum sensing systems. Two-component regulatory systems (TCSs) are widely used by bacteria to sense and respond to changes in their environment. The primary TCS utilized by *C. perfringens* is the VirS/VirR system, with the *virS* gene responsible for the histidine kinase that senses stimuli and the *virR* gene encoding the corresponding cytoplasmic response regulator (Ohtani and Shimizu, 2015). Phosphorylated VirR binds directly to a target promoter region, which activates the transcription of target toxin genes such as *netB* (netB toxin), *pfoA* (perfringolysin O) and ccp (alpha-clostripain), as well as the regulatory gene *vrr* which encodes VR-RNA. VR-RNA further regulates the expression of *plc* (alpha toxin) and *colA* (kappa toxin). The VirS/VirR system has been shown to play a role in the ability of *C. perfringens* to sense cells. For example, the production of toxins in type C isolates may be influenced by the presence of Caco-2 cells. Type C isolates, by definition produce alpha and beta toxins, but often also produce beta2 toxin, perfringolysin O, and TpeL (Vidal et al., 2009). In this study, *cpb, cpb2, pfoA, and plc* genes encoding beta toxin, beta2 toxin, perfringolysin O, and alpha toxin, respectively, were rapidly upregulated in the presence of Caco-2 cells, while transcription of *TpeL* was unaffected (Vidal et al., 2009; McClane, 2010).

C. perfringens also uses quorum sensing to regulate toxin production depending on both accessory gene regulators (agr) and *luxS* for intraspecies and interspecies communication. The *agr* system has been heavily linked to the production of *pfoA* in *C. perfringens*, although the exact mechanism of the relationship is unknown. For example, when *agrBD* mutant strains that normally produce weak zones of hemolysis on blood agar plates are co-streaked with a strain that is negative for *pfoA* but produces agr-related signaling substances, the *agrBD* strain recovers hemolytic ability (Ohtani et al., 2009). In isolates from foodborne illness outbreaks, *agr* systems have been shown to regulate the translation of *cpe* and *cpb2* and the subsequent production of *C. perfringens* enterotoxin and beta2 toxin, respectively (Li et al., 2011a). From genomic analysis, orthologs of *agrB* and *agrD* have been found to positively regulate genes responsible for the production of alpha toxin, perfringolysin O, beta toxin, epsilon toxin, beta2 toxin, and enterotoxin (Vidal et al., 2009, 2012; Chen et al., 2011; Li et al., 2011a; Chen and McClane, 2012). The production of *luxS* and the production of autoinducer 2 (AI-2) has been shown to regulate the production of *plc, colA, and pfoA* in specific *C. perfringens* strains.

Less is known about the regulation of toxins and virulence factors *in vivo*. While studies exposing *C. perfringens* to cultured cells are indicative to action within the body, they do not fully mimic pathogenicity *in vivo*. In broth cultures, amino acids are provided in abundance, but in a host, the bacteria must acquire these nutrients from the environment (host) by actively secreting degradative enzymes and toxins. The degradation of host tissue *in vivo* often benefits the pathogen by making conditions more suitable for proliferation. The regulation of virulence *in vitro* has been demonstrated to be complex and variable. However, patterns and relationships seen in *in vitro* assays have been mimicked using animal models. For example, *agrB*-null mutants were shown to have significantly reduced expression of NetB toxin in *in vitro*. When the mutant strains were used in a Necrotic Enteritis poultry model, virulence was severely attenuated, but was restored with complementation (Yu et al., 2017). This suggests that factors that govern the regulation of NetB, and other clostridial toxins in vitro, may also apply *in vivo.*

2.3.1.2. Pathogenesis

The pathogenesis of *C. perfringens* within chicken has not been fully elucidated although there has been significant research in this area in the past decade (Lee et al., 2011; Timbermont et al., 2011; Li et al., 2013; Prescott et al., 2016a; Rood et al., 2016; Wade et al., 2016). The process of bacterial infection can be broken down into multiple elements that occur with some overlap. In order to cause infection, *C. perfringens* must first colonize the small intestine. While there is a high amount of genetic diversity in isolates from healthy birds, birds suffering from NE typically possess only a single strain (Nauerby et al., 2003; Gholamiandekhordi et al., 2006).

Until recently, the generally accepted hypothesis was that NE developed after a disruption from specific risk-factors allowed preexisting low-levels of commensal *C. perfringens* to proliferate to levels that induced disease (Cooper and Songer, 2009). With recent understandings that most stains circulating at low-levels in healthy flocks are non-pathogenic, it is now recognized that pathogenic strains are likely introduced to a flock through external means (Barbara et al., 2008; Timbermont et al., 2009; Lacey et al., 2016). Figure 1 highlights the overall mechanism of infection.

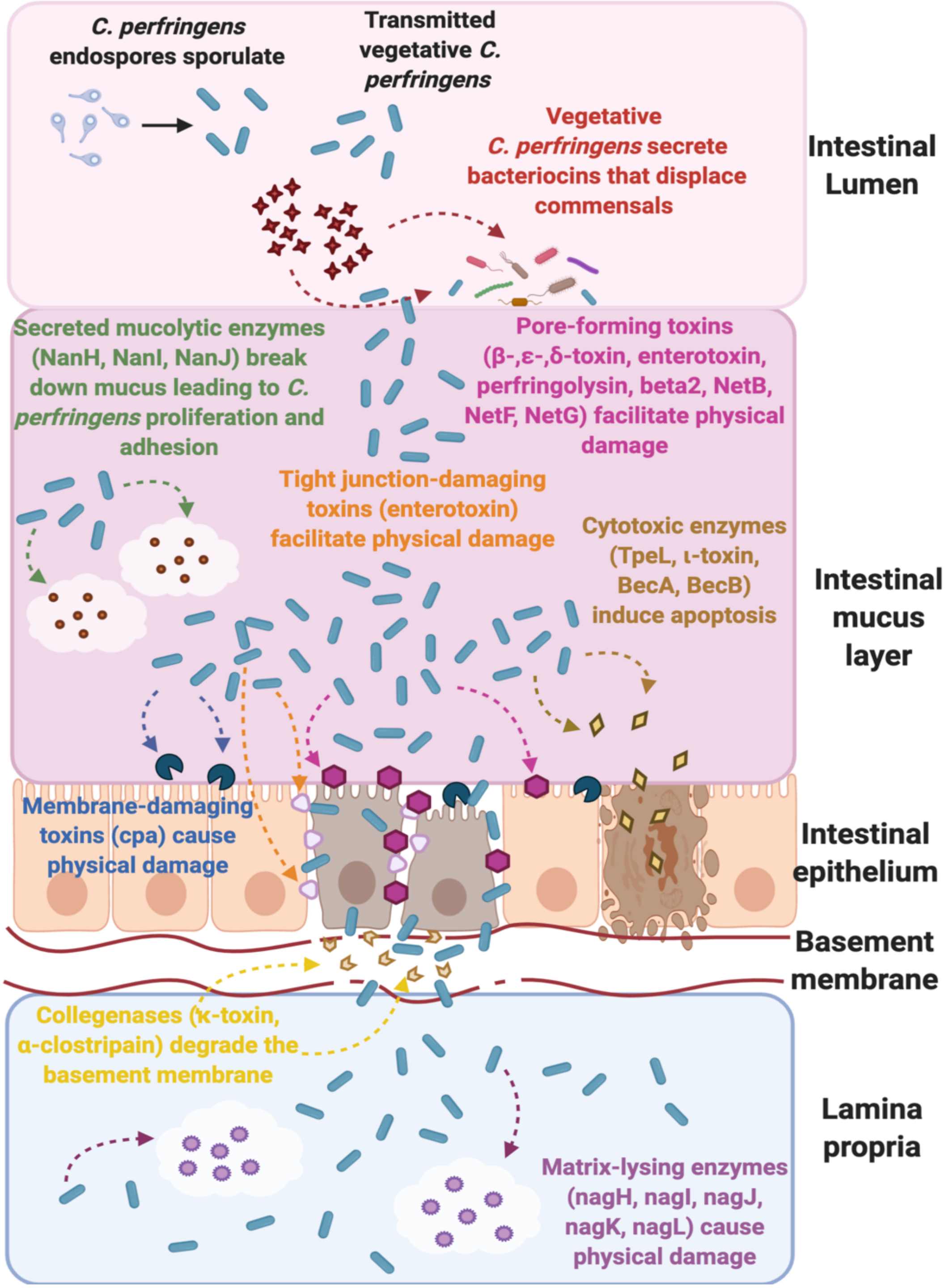


Figure 1. The pathogenesis of enteric *C. perfringens* infection is complex and involves secretion of numerous toxins and enzymes. Created with BioRender.com.

There is evidence that at the onset of infection, invading *C. perfringens* likely secrete bacteriocins that aid in the displacement of commensal *C. perfringens* (Barbara et al., 2008; Timbermont et al., 2009, 2014). Along with displacing commensals, invading bacteria must also break through the mucus barrier of the small intestine. The mucus layer is a major part of the host immune system and serves to protect the host from the external environment of the lumen and consists of O-glycosylated mucin glycoproteins as well as antimicrobial peptides (McGuckin et al., 2011). *C. perfringens* produce not only two specific glycoside hydrolases that likely play a role in O-glycan degradation but also additional enzymes capable of degrading other mucins (Lepp et al., 2010; Fujita et al., 2011; Ficko-Blean et al., 2012). These enzyme genes are encoded on a plasmid that has been labeled NELoc-1, or Necrotic Enteritis pathogenicity locus 1 (Lepp et al., 2010). This locus also contains genes responsible for a zinc metalloproteases with likely mucinase activity that is shown to be a protective antigen against NE (Kulkarni et al., 2007; Lepp et al., 2010; Nakjang et al., 2012). A second NE pathogenicity locus contains additional glycosyl hydrolases, indicating that colonization and degradation of the mucus layer is critical in NE pathogenesis.

Degradation of the mucus layer not only strips at the host's protective layers, but also provides ample nutrients for *C. perfringens* growth (Van Immerseel et al., 2009; McGuckin et al., 2011; Ficko-Blean et al., 2012). As the bacteria increases in concentration and reaches a critical density, various quorum sensing and auto-inducing peptides are produced that upregulate the production of the VirR-VirS regulon leading to the production of numerous toxin genes that contribute to disease such as *plc, netB, cpb2*, and *pfoA* (Ohtani et al., 2009; Ohtani and Shimizu, 2015). Membrane disrupting toxins, pore-forming toxins, and mucolytic enzymes likely play a role in the next steps of pathogenesis. Histopathological studies have demonstrated that after the superficial mucosal layer is degraded, *C. perfringens* then colonize the submucosa in a biofilm-like manner (Vidal et al., 2015). Interestingly, studies have suggested that pathological damage likely starts at the basement membrane and lateral domain of the enterocytes, before spreading through the lamina propria (Olkowski et al., 2006, 2008). The epithelium is not damaged until afterwards. This indicates that there are likely factors affecting the extracellular matrix and tight junctions of the intestine (Olkowski et al., 2008).

The last element of infection is adhesion. *C. perfringens* ability to adhere and proliferate has been linked to its production of specific collagenases and sialidases. For example, Wade et al., have demonstrated that specific netB+ isolates of *C. perfringens* possess a putative fimbrial adhesion VR-10B operon that is crucial for virulence (Wade et al., 2016). Similarly, sialidases such as NanI play a role in exposing adhesion-related receptors on enterocyte cell surfaces (Chiarezza et al., 2009; Li and McClane, 2014).

2.3.2. Host response to *C. perfringens*

The immune response to *C. perfringens* and its secreted toxins is still poorly understood. Infection primarily takes place in the small intestine where the pathogen interacts with the enteric immune system. The immune systems in neonatal chickens is poorly developed at hatch and matures rapidly until four to six weeks post-hatch (Mast and Goddeeris, 1999; Panda et al., 2015). Lu et al. demonstrated that Toll-like receptors (TLRs) likely play a role in early detection of *C. perfringens* and the activation of immune responses, with the upregulation of TLR1 type 1 as soon as two days after infection (Lu et al., 2009).

There is conflicting data on the adaptive immune response to NE infection. This is in part because the infection is so multi-faceted that it is difficult to isolate variables and compare studies to each other due to differing multi-factorial challenge models. For example, studies have demonstrated that infection with *C. perfringens* alone induces the expression of interferon (IFN)-α, IFN-γ, IL-1β, IL-2, IL-10, IL-12, IL-13, IL-17 and TGF-β4 but if challenged with both *Eimeria tenella* and *C. perfringens* one to two days after initial challenge, expression levels of all of these cytokines are significantly repressed (Collier et al., 2008; Park et al., 2008). This suggests that co-infection with *Eimeria* represses the ability of *C. perfringens* to induce an effective inflammatory response which may contribute to the exacerbated effect of co-infection on broiler chickens. It is important to note that co-challenge with *Eimeria* spp. is extremely common (Mcdevitt et al., 2006; Collier et al., 2008; Bortoluzzi et al., 2019). Conversely, studies have also demonstrated the downregulation of IL-1β, IL-2, IL-16, IL-18, and IFN-γ after *C. perfringens* infection (Zhou et al., 2009). Generally, adaptive immune defenses at the mucosal surface are mediated by activation of lymphocytes and local secretion of IgA (Muir et al., 2000). In multiple vaccination studies, there was a strong mucosal IgA response against various immunogenic proteins in birds partially protected against NE (Kulkarni et al., 2007, 2010; Lee et al., 2012; Emami et al., 2019).

There is also significant evidence from vaccination studies that IgY plays a role in protection from NE. Healthy birds had higher levels of IgY against NetB and alpha toxin than chickens displaying signs of clinical NE (Lee et al., 2011, 2012). As a whole, multiple studies have indicated that both cell-mediated and antibody-mediated immune responses are responsible for host defense against *C. perfringens*, with genes involved in inflammation, humoral immunity, antigen recognition, and immune-related metabolic processes consistently upregulated (Lu et al., 2009; Sarson et al., 2009; Lee et al., 2011).

2.3.3. Epidemiology

While *C. perfringens* is the etiological agent that causes NE, infection alone is not sufficient to induce disease (Figure 2). Rather, a combination of predisposing factors are necessary to produce an environment optimal for infection (Moore, 2016). Only once established does *C. perfringens* cause damage resulting in NE. There are many predisposing factors that have been experimentally shown to play a role in creating this ideal environment and increasing the incidence and/or severity of NE (Moore, 2016).

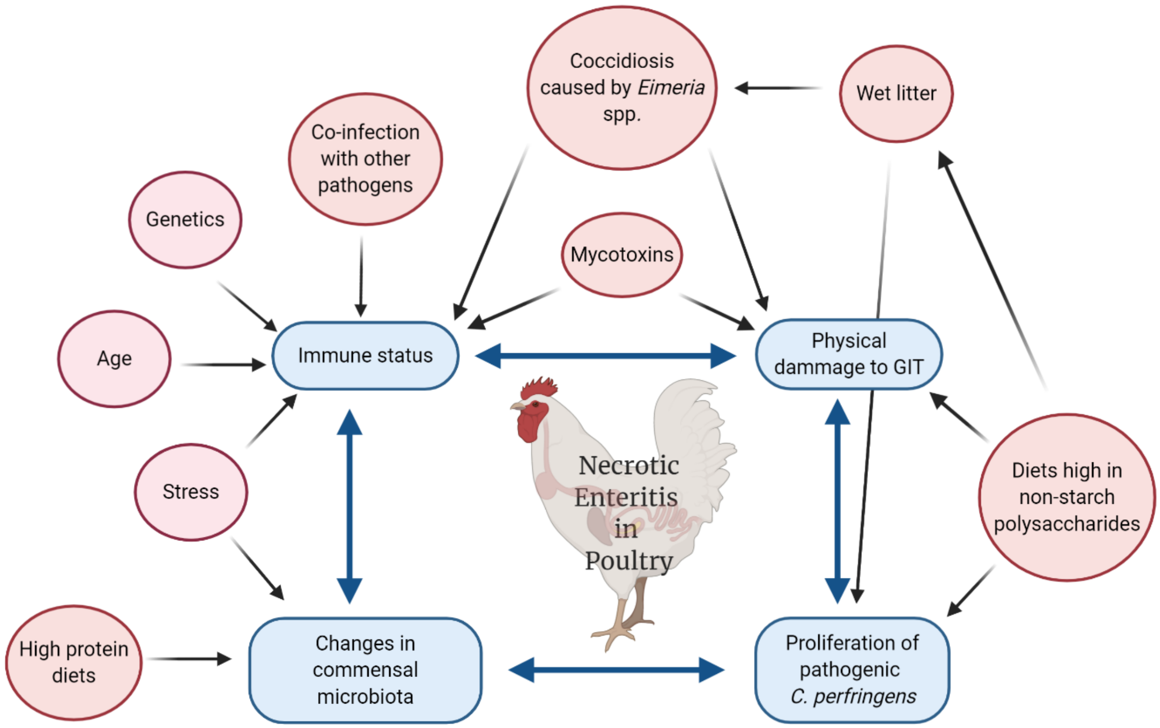


Figure 2. The pathogenesis of Necrotic Enteritis in poultry is multi-factorial, with multiple pre-disposing factors playing a role. Adapted from Moore, 2016. Created with BioRender.com.

Dietary factors can play a role in NE, the most obvious being the presence of high levels of wheat, barley, and fishmeal in the diet. Birds fed diets high in cereal grains such as wheat, rye, oats, and barley are more likely to suffer from NE than those fed corn-based diets (Branton et al., 1987; Riddell and Kong, 1992).

This is due to the presence of non-starch polysaccharides that increase digesta viscosity and transit time and provide ample nutrient for *C. perfringens* (Annett et al., 2002; Moore, 2016). Furthermore, there is evidence that the presence of non-starch polysaccharides in the diet leads to increased water intake resulting in wet litter, an environment that encourages both *C. perfringens* and *Eimeria* species, yet another predisposing factor of NE (Hermans and Morgan, 2007; Shirzadi et al., 2009; Moftakharzadeh et al., 2017). Diets high in animal protein, specifically fishmeal, have also been linked to increased susceptibility to NE, as high protein levels can induce changes in the microbiota and possibly alter the pH of the GIT (Keyburn et al., 2006; Cooper et al., 2010; Wu et al., 2010, 2014).

Immune status plays a large role in predisposing birds to NE. In general, chickens are protected from NE until maternal antibodies decline at around two to three weeks (Lovland et al., 2004; Crouch et al., 2010; Keyburn et al., 2013a). Infections that are immunosuppressive, such as Marek's disease and infectious bursal disease can potentially increase the severity of NE (Williams et al., 2003; Lee et al., 2011; Timbermont et al., 2011). Other stressors such as overcrowding, ammonia buildup, and physiological s tress can lead to immunosuppression and alter the risk of developing NE (Hoerr, 2010; Tsiouris et al., 2015; Tsiouris, 2016). While not a disreputable feature, there is evidence that specific breeds of chicken may be more susceptible to NE based on differences in immune responses (Jang et al., 2013; Hong et al., 2014; Swaggerty et al., 2016). Co-infection with *Salmonella enterica* serovar Typhimurium may also increase the susceptibility of birds to NE by modulating the host immune response and inducing intestinal damage from persistent inflammation (Hassan and Curtiss, 1994; Henderson et al., 1999; Shivaramaiah et al., 2011; Latorre et al., 2018).

Co-infection with other pathogens, specifically, *Eimeria* spp., is likely the most important factor in NE infection. *Eimeria* are apicomplexan obligate intracellular intestinal protozoan parasites that are capable of causing disease in a vast range of animals such as cattle, poultry, dogs, cats, sheep and goats (Chapman et al., 2013; Constable, 2015; López-Osorio et al., 2020). Seven species of *Eimeria* have demonstrated ability to cause coccidiosis in poultry, with *E. tenella, E. maxima, and E. acervulina* being the most economically significant to chickens (Gerhold, 2014; López-Osorio et al., 2020). The infection of each strain is cite specific, with *E. maxima* contributing the most to NE (Jang et al., 2013; Adhikari et al., 2020; López-Osorio et al., 2020). The life cycle of *Eimeria* occurs in stages that vary slightly from species to species. Generally, sporulated oocysts are ingested and sporocysts are released during mechanical digestion in the gizzard. Trypsin and bile acids released in the duodenum promote the excystation of sporozoites which can then penetrate specific mucosal cells the epithelium. Sporozoites become trophozoites and undergo schizogony. The daughter cells (merozoites) then rupture, individually penetrate more mucosal cells and reproduce multiple times causing mass physical damage to the GIT. As the daughter cells mature they differentiate into male and female forms and produce unsporulated oocysts which are then passed in the feces. The cycle of ingestion, replication, and shedding repeats, quickly infecting an entire flock (López-Osorio et al., 2020).

Coccidial infection, specifically with *E. maxima*, predisposes birds to NE due to physical damage to the epithelium, which compromises GIT integrity (Gerhold, 2014; Wu et al., 2014; Moore, 2016; Bortoluzzi et al., 2019; Adhikari et al., 2020). This leaves the intestinal basal layer susceptible to the external environ2ment, a factor that has been linked to early NE infection (Olkowski et al., 2008). Physical damage may also expose extracellular matrix which may encourage adhesion of *C. perfringens* (Awad et al., 2000; Li and McClane, 2014; Wade et al., 2015). Furthermore, damage caused by *E*. maxima not only allows the leakage of plasma into the GIT, but also increases mucin production, both protein-rich nutrient sources optimal for *C. perfringens* growth (Immerseel et al., 2004; Collier et al., 2008; Forder et al., 2012; Moore, 2016).

2.3.4. Current control strategies

The resurgence of previously controlled diseases has led to the intensive investigation and commercialization of multiple methods to control and improve animal health, including pre- and probiotics, organic acids, nutrition-based feed additives, immunomodulators, and vaccines (Van Immerseel et al., 2009; Cheng et al., 2014; Caly et al., 2015; Marquardt and Li, 2018). Ultimately, a vaccine could offer additional and more consistent protection that could supplement current control mechanisms.

2.3.5. Current vaccine strategies

Due to the economic relevance of NE, there has been significant amounts of research into protective vaccines. Thompson et al. demonstrated the fundamental principle that infection with *C. perfringens* induced protection against subsequent challenge (Thompson et al., 2006). This study demonstrated the potential for live vaccination but highlighted that a balance between attenuation and protection must be struck. Vaccination with live avirulent strains, typically produced by removing key virulence genes), has yielded mixed results (Thompson et al., 2006; Mishra and Smyth, 2017). As with any live-attenuated vaccine, there is the fear of regained virulence. In *C. perfringens* this fear is exacerbated due to demonstrated instances of plasmid transfer *in vivo and in vitro*, converting an avirulent strain into a virulent one (Brynestad et al., 2001; Hughes et al., 2007).

Protein-based vaccines, including toxoids, recombinant vaccines, and subunit vaccines have been investigated as potential vaccine candidates. Because the pathogenicity of NE is linked to the production of specific toxins, much of the NE vaccine research has been focused on the antigenicity of these toxins. Subcutaneous vaccination with recombinant α-toxin can reduce NE lesions, while intramuscular vaccination with alpha toxoid followed by a boost with active toxin is protective against NE challenge (Kulkarni et al., 2007; Cooper et al., 2009; Jang et al., 2012). While there is evidence that α-toxin can be protective, studies have demonstrated that it does not play a primary role in pathogenesis (Keyburn et al., 2006). It is notable that the only commercial vaccine for NE has been a toxoid vaccine (*Clostridium perfringens* type A alpha-toxoid, Netvax™), although it is no longer available as of 2014 (EMA, 2009, 2014). This vaccine, approved for use in the European Union (EU) was an adjuvanted vaccine composed of formaldehyde-inactivated *C. perfringens* type A alpha toxoid with light mineral oil, sorbitan oleate, polysorbate 80, benzyl alcohol and triethanolamine used as an adjuvant. When administered via two spaced out intramuscular injections to laying hens, the vaccine conferred partial protection to the offspring (EMA, 2009; Keyburn et al., 2013a).

Discovery of netB toxin in 2008 shifted focus to this toxin. Specifically, recombinant netB toxin vaccines have proven to be mildly protective against NE challenge (Fernandes da Costa et al., 2013; Keyburn et al., 2013b, 2013a). Culture supernatants have been studied as potential vaccines for the prevention of NE, with slightly better, but still limited success (Kulkarni et al., 2007; Lanckriet et al., 2010; Saleh et al., 2011; Mot et al., 2013). In these studies, cell free supernatant, either crude or inactivated, provided increased protection over isolated proteins, demonstrating that the inclusion of multiple antigens is crucial for protection (Kulkarni et al., 2007). Furthermore, Lanckriet et al., demonstrated that protective characteristics of the CFS was not dependent on the production of alpha or netB toxin (Lanckriet et al., 2010). These studies highlight that a singular, isolated, antigen is unlikely to be protective against NE. Rather, multiple antigens as is the case in culture supernatant, are likely necessary for protection.

Attenuated or avirulent vector-based vaccines have also been explored. Attenuated *Salmonella* strains can safely serve as carrier vaccines to prevent disease by expressing specific antigens. These vaccines are typically considered safe because attenuation is induced by deletions in metabolism genes, preventing the strain from overgrowing and causing disease (Spreng et al., 2006). The disadvantage of this vaccine scheme is that once again, only a singular antigen is presented, limiting its protective ability. In studies using modified *Salmonella enterica* serovar Typhimurium, birds were protected from a mild challenge, but not protected against a severe challenge (Kulkarni et al., 2008, 2010).

2.4. Ionizing radiation

Ionizing radiation is energy that possesses sufficient energy to ionize molecules that it interacts with. There are three main types of ionizing radiation currently used in commercial settings: Gamma-ray, X-ray, and electron beam (eBeam) (Pillai and Shayanfar, 2017). Gamma-rays are electromagnetic radiation composed of photons that are emitted from the nucleus of a radioactive source. In most commercial settings, this source is either Cobalt-60 or Cesium-137. X-rays are also electromagnetic radiation composed of photons. Electron beam irradiation is composed of highly energetic electrons that are produced from a nucleus (beta decay) or from a machine-source. Due to increasing acquisition, maintenance, and disposal costs associated with radioactive sources as well as security concerns, gamma-irradiation is slowly being replaced by machine generated sources of irradiation, such as eBeam (Pillai and Shayanfar, 2017; Pillai and Bhatia, 2018). From a commercial standpoint, eBeam technology is an attractive processing technology because of its low costs, low environmental footprint, on/off nature, and its diversity as a non-thermal processing technique.

2.4.1. Electron beam irradiation

High energy eBeam technology is currently used in the food and medical device industry for its ability to inactivate microorganisms. It is also commonly used for material transformation applications, such as in wire and cable curing and gemstone coloring. In the food industry, this technology is regularly used for phytosanitary treatment, shelf-life elongation, pathogen inactivation, and occasionally terminal sterilization (Pillai and Shayanfar, 2017; Pillai and Bhatia, 2018). In the medical device industry, this technology is used to sterilize single-use medical devices and laboratory consumables (IIA and GIPA, 2017).

In commercial settings, eBeam irradiation is generated using highly specialized accelerators (Figure 3). In an accelerator, typically a linear accelerator (LINAC), electrons generated from commercial electricity are accelerated to approximately 99.999% of the speed of light in a vacuum tube supplemented with microwaves, resulting in energies up to 10 MeV (Mega electron volts) (Miller, 2005). These highly energetic electrons are then focused and pulsed uniformly over a material, solid or liquid ((Miller, 2005; Pillai and Shayanfar, 2017)). When the electrons interact with a molecule leading to its ionization, the ejected electron becomes energized, going on to interact with and ionize an adjacent molecule. This chain reaction continues until the energy has fully dissipated (Miller, 2005).

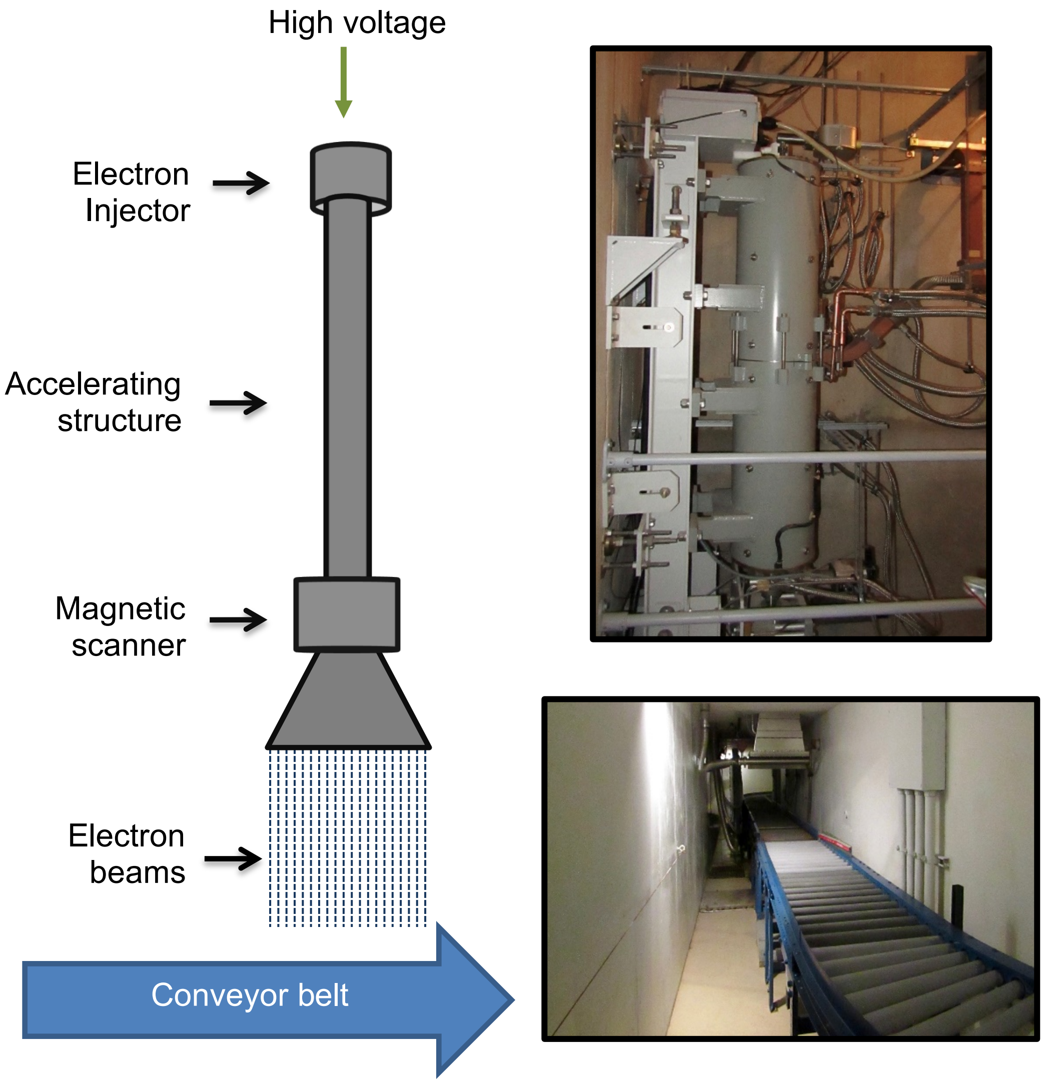


Figure 3. A schematic and photographs of a 10 MeV, 18 kw high energy electron beam linear accelerator at the National Center for Electron Beam Research at Texas A&M University.

2.4.2. Effect of IR on microorganisms

The effect of irradiation on a material depends on the composition of the molecule (Miller, 2005). High energy eBeam technology damages microorganisms through direct and indirect methods. Direct damage is caused as a result of interactions between energetic electrons and the molecules within an organism, while indirect damage is caused as a result of interactions with products of water radiolysis (Urbain, 1986; Miller, 2005; Tahergorabi et al., 2012). When an electron from the beam interacts with a material, molecules are ionized, ejecting valance electrons, a proportion of the energy of the primary electron is passed to the ejected electron. This ejected electron continues to cause a cascade of similar ionization events until all energy has been fully dissipated. It is for this reason that eBeam doses are measured in kilograys (kGy = 1000 gy), with one gy equivalent to 1 J/kg, or absorbed energy/mass.

In microorganisms, DNA is the largest molecule, resulting in it being the primary target of direct ionization events (Figure 4). The ionization of DNA results in the cleavage of the phosphodiester bonds along the DNA backbone. While single-stranded breaks are repairable, double stranded breaks are much harder for an organism to repair and overcome. Due to excessive shearing of the nucleic acid, the microorganism is inactivated (Tahergorabi et al., 2012). The other major target of eBeam in a microorganism is water, leading to the production of radiolytic water products. Water radiolysis generates a diverse array of highly reactive, but short lived free radical species such hydroxyl radicals, hydrogen peroxide, hydrogen, hydrate electrons, and hydrated protons. The summary equation for water radiolysis is presented below (Equation 1) with the quantity of each product per 100 eV of energy absorbed shown in parenthesis.

Equation 1. $e^- + H_2O \rightarrow {}^*OH\ (2.7) + e_{aq}^-\ (2.7) + {}^*H\ (0.55) + H_2\ (0.55) + H_2O_2\ (0.71) + H_3O^+\ (2.7)$

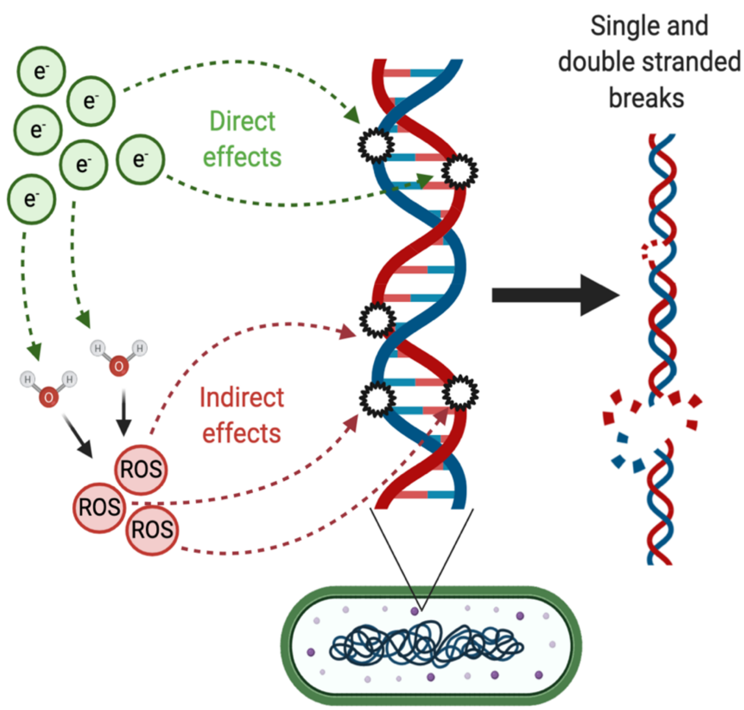


Figure 4. Electron Beam technology inactivates microorganisms by causing the shearing of DNA directly through the effects of electrons and indirectly through the generation of reactive oxygen species (ROS) such hydroxyl radicals, hydrogen peroxide, hydrogen, hydrate electrons, and hydrated protons. Created with BioRender.com.

Indirect damage results from interaction of these reactive oxygen species (ROS) with cellular components results in the most molecular damage in cell, as opposed to incident electrons. Hydroxyl radicals (*OH) are extremely short lived however during their short time, they can cause significant damage to molecules in their immediate surroundings (Mavragani et al., 2019). Superoxide radicals (O_2-*) are also generated by the radiolysis of water, and it is hypothesized that these molecules accumulate within a microbial cell causing severe damage to proteins such as enzymes with exposed iron-sulfur clusters (Keyer and Imlay, 1996; Popović-Bijelić et al., 2016). For example, methionine and cysteine have been shown to be especially susceptible to ionizing radiation (Reisz et al., 2014). Superoxide radicals also reacts with endogenous nitric oxide within a cell, forming reactive nitrogen species (RNS) such as a peroxynitrite anion ($ONOO^-$), nitrogen dioxide (NO_2*), and dinitrogen trioxide (N_2O_3), which cause further damage to the DNA and are the primary agents of damage to proteins within the bacteria (Daly, 2009). This protein damage can have significant effects on the microorganism's ability to function.

Together, direct and indirect mechanisms of damage lead to the inactivation of the cell due to the high number of single and double stranded breaks (Tahergorabi et al., 2012). Assuming a hypothetical genome size of 3.5 million base pairs, a dose of 1 kGy would cause approximate 200 single stranded breaks and 14 double stranded breaks, per copy of a bacteria's genome (Miller, 2005; Alsharifi and Müllbacher, 2010). This extent of damage is irreparable to most microorganisms, inactivating the microbe by preventing replication. The damage done to the microorganism is extremely rapid. Direct damage from chemical bonds are cleaved within 10^{-14} – 10^{-12} seconds of exposure. Within one ps (10^{-12} s), superoxide and hydrogen peroxide radicals are formed. By about 1 ms after exposure, the reactions of most ROS are complete (Singh and Singh, 1982; Reisz et al., 2014).

While cells are inactivated due to damage to their nucleic acid, previous studies in our laboratory as well as others have demonstrated that the cellular membrane remains intact (Vemulapalli et al., 2000; Magnani et al., 2009; McReynolds et al., 2012; Bordin et al., 2014; Jesudhasan et al., 2015a; Hieke and Pillai, 2018; Bhatia and Pillai, 2019). Furthermore, there is significant evidence that in cells treated with ionizing radiation, there is sustained metabolic activity after treatment (Ahn et al., 1962; Hiramoto et al., 2002; Magnani et al., 2009; Secanella-Fandos et al., 2014; Hieke and Pillai, 2018). This has been demonstrated using genomic and metabolomic studies (Trampuz et al., 2006; Magnani et al., 2009; Praveen, 2014; Secanella-Fandos et al., 2014; Hieke and Pillai, 2018; Bhatia and Pillai, 2019). Taken together, this state is considered a Metabolically Active, yet Non-Culturable (MAyNC) state as has broad applications in vaccine development. MAyNC cells are inactivated, but maintain cell membrane integrity, and could therefore potentially function as a killed vaccine that also carries properties of a live-attenuated vaccine. Because eBeam maintains metabolic activity and membrane integrity, MAyNC cells may be specifically well-suited for diseases that require immune recognition of multiple pathogen epitopes. Furthermore, due to the flexibility of eBeam technology, it could be used to create a vaccine platform that could be adapted to specific diseases or even outbreak-specific strains.

2.4.3. Vaccines inactivated using ionizing radiation

The use of ionizing radiation as a method to attenuate or inactive microorganisms for the use as vaccines is not novel, with reports of gamma and x-ray-inactivated vaccine research dating to back to the mid-20th century (Moore and Kersten, 1936; Jordan and Kempe, 1956; Tumanyan et al., 1958; Carpenter, 1959; Kaplan, 1960; Ward et al., 1960; Kalenina and Abidov, 1963). The advantage of ionizing-radiation vaccines, or radio-vaccines, is that because they are inactivated, they may be able to retain immunogenicity when stored at non-refrigerated temperatures (Praveen, 2014; Scherließ et al., 2014). There is also evidence that vaccines inactive by gamma irradiation or eBeam retain their immunogenicity after lyophilization or spray drying, further eliminating the need for a cold-chain (Praveen, 2014; Scherließ et al., 2014; Sir Karakus et al., 2020). This in turn could result in lower costs of manufacturing and distribution, and increased accessibility to remote areas (Orr et al., 2014; Lloyd and Cheyne, 2017). Furthermore, this technology is easily scalable with the capability to inactivate large quantities of preparations (Fertey et al., 2020b). Due to its ability to commercial capabilities, numerous patents related to radio-vaccines have been filed (Table 3).

Table 3. A selection of patents relating to radio-vaccines

Patent #	Country	Year	Status[a]	Title
US3657415A	USA	1969	Expired	Canine hookworm vaccines
DE3853854T2	Germany	1988	Expired	Vaccine against group b *Neisseria meningitidis*, gammaglobulin and transfer factor
AU706213B2	Australia	1996	Ceased	Method for obtaining a vaccine with wide protective range against group b *Neisseria meningitidis*, the resulting vaccine, gammaglobulin and transfer factor
AU6320001A	Australia	2001	Published	Gamma irradiation of protein-based pharmaceutical products
KR20030034517A	South Korea	2001	Granted	*Burkholderia gladioli* k4 having antifungal activity, preparation method of its mutant by gamma radiation and the mutant thereof
US20060147460A1	USA	2002	Granted	Anticancer vaccine and diagnostic methods and reagents
KR101173871B1	South Korea	2004	Granted	Modified free-living microbes vaccine compositions and methods of use thereof
US20050175630A1	USA	2004	Abandoned	Immunogenic compositions and methods of use thereof
US8173139B1	USA	2009	Granted	High energy electron beam irradiation for the production of immunomodulators in poultry
CA2733356C	Canada	2009	Granted	Influenza vaccines
US8282942B2	USA	2010	Granted	*Toxoplasma gondii* vaccines and uses thereof
US20130122045A1	USA	2010	Abandoned	Cross-protective influenza vaccine
US20150209424A1	USA	2011	Abandoned	Inactivated varicella zoster virus vaccines, methods of production, and uses thereof
JP2014520117A	Japan	2012	Granted	Vaccine composition comprising inactivated chikungunya virus strain

Table 3. Continued

Patent #	Country	Year	Status[a]	Title
AU2012211043B2	Australia	2012	Published	Combination vaccines
US10080795B2	USA	2013	Granted	Method for inactivating viruses using electron beams
WO2014155297A2	WIPOb[b]	2014	Published	Systems and methods for viral inactivation of vaccine compositions by treatment with carbohydrates and radiation
WO2014165916A1	WIPO[b]	2014	Published	Methods and compositions for inducing an immune response
DE102015224206B3	Germany	2015	Granted	Irradiation of biological media in transported foil bags
KR20180036987A	South Korea	2016	Published	Vaccine composition
WO2018167149A1	WIPO[b]	2018	Ceased	Method for irradiating mammalian cells with electron beams and/or x-rays
DE102016216573A1	Germany	2016	Published	Inactivation of pathogens in biological media
Wo2019191586a2	Canada	2019	Published	Irradiation-inactivated poliovirus, compositions including the same, and methods of preparation

[a] Status as of November, 2020 [b] World Intellectual Property Organization

Interest in radio-vaccines has increased significantly recently, with investigations into the creation of vaccines for bacterial, viral, and protozoan diseases (Table 4). While many of the researched vaccine candidates have been inactivated with gamma-irradiation, there has been significantly less research conducted on eBeam inactivated vaccines. Among all of the research conducted on irradiated vaccines, the most progress has been obtained from *Plasmodium* sporozoites attenuated with irradiation to protect against malaria. First examined in 1967 using x-ray irradiation, this idea has evolved considerably over the last 50+ years to its current iteration in phase 2 clinical trials using gamma-attenuated sporozoites (Nussenzweig et al., 1967; Clyde, 1990; Rieckmann, 1990; Hoffman et al., 2002; Luke and Hoffman, 2003; Seder et al., 2013; Arévalo-Herrera et al., 2016). Studies using gamma-irradiated *Listeria monocytogenes* have demonstrated that unlike other inactivation methods such as heat or formalin, irradiation better preserves antigenic properties and stimulated robust T cell responses (Datta et al., 2006).

In multiple studies investigating the immune response to gamma-irradiated *Brucella* spp., investigators found that gamma-irradiated cells were metabolically active and inactivated cells were able to induce a significant cellular immune response and were protective upon challenge (Ahn et al., 1962; Vemulapalli et al., 2000; Sanakkayala et al., 2005; Magnani et al., 2009; Surendran et al., 2010; Moustafa et al., 2011). Radio-vaccines have even been investigated as a response to pandemics outbreaks such as SARS, MERS, and SARS-CoV-2, as they can be manufactured extremely rapidly and tailored to specific strains (Beniac et al., 2007; Agrawal et al., 2016; Durante et al., 2020; Mullbacher et al., 2020; Sir Karakus et al., 2020; Turan et al., 2020).

Table 4. Vaccines against bacteria, viruses, and protozoa have been developed using ionizing radiation

Type of Pathogen	Pathogen	Inactivation Method	Inactivation Dose	Model	Notes	Source
Bacteria	*Brucella abortus*	Gamma	4 kGy	Mice	Irradiated strains induced less of an immune response than live strains	(Surendran et al., 2010)
Bacteria	*Brucella abortus*	Gamma	3 kGy	Mouse	Antigen specific Th1 response	(Sanakkayala et al., 2005)
Bacteria	*Brucella abortus*	Gamma	2.5 kGy	Mice	Stimulated IFN-gamma and Th1 cells	(Oliveira et al., 1994)
Bacteria	*Brucella abortus*	Gamma	3.5 kGy	Mice	Protective upon challenge	(Dabral et al., 2014)
Bacteria	*Brucella abortus, B. melitensis, and B. suis*	Gamma	3.5 kGy	Mice	Protective upon challenge	(Moustafa et al., 2011)
Bacteria	*Brucella melitensis*	Gamma	3.5 kGy	Mouse	Cytotoxic T cell response and protective against challenge	(Magnani et al., 2009)
Bacteria	*Listeria monocytogenes*	Gamma	6 kGy	Mouse	Induced protective T cell responses	(Datta et al., 2006)
Bacteria	*Mannheimia haemolytica*	Gamma	2-20 kGy	Rabbit	Protection upon challenge	(Ahmed et al., 2016)
Bacteria	*Orientia tsutsugamushi*	Gamma	2 kGy	Mice	Partially protective upon challenge	(Jerrells et al., 1983)
Bacteria	*Orientia tsutsugamushi*	Gamma	3 kGy	Mice	Protective upon challenge	(Eisenberg and Osterman, 1978)
Bacteria	*Pasteurella tularensis*	X-ray	10 kGy	Mice	Partially protective upon challenge	(Gordon et al., 1964)
Bacteria	*Rhodococcus equi*	Electron Beam (High Energy)	4-5 kGy	Horse	Produced cell-mediated and upper respiratory mucosal immune response	(Bordin et al., 2014)

Table 4. Continued

Type of Pathogen	Pathogen	Inactivation Method	Inactivation Dose	Model	Notes	Source
Bacteria	*Rhodococcus equi*	Electron Beam	5 kGy	Horse	Not protective upon challenge	(Rocha et al., 2016)
Bacteria	*Rodentibacter pneumotropicus*	Electron Beam (Low Energy)	20 kGy	Mice	Protective upon challenge and reduced colonization	(Fertey et al., 2020a)
Bacteria	*Salmonella* Enteriditis	Electron Beam (High Energy)	2.5 kGy	Chicken	Protective upon challenge and reduced colonization	(Jesudhasan et al., 2015a)
Bacteria	*Salmonella* Typhimurium	Electron Beam (High Energy)	2.5 kGy	Chicken	Heterophil-mediated innate immune response	(Kogut et al., 2012)
Bacteria	*Salmonella* Typhimurium	Electron Beam (High Energy)	7 kGy	Mouse	Stimulated innate immune markers and reduced colonization	(Praveen, 2014)
Bacteria	*Salmonella* Typhimurium	Gamma	10-80 kGy	Chicken	Protective upon challenge	(Begum et al., 2011)
Bacteria	*Shigella dysenteriae*	X-ray	Not reported	Rabbits	Bacteria that were treated for a longer time were non-toxic and protective upon challenge	(Moore and Kersten, 1936)
Bacteria	*Staphylococcus aureus*	Gamma	2.5-2.9 kGy	Mice	Induced specific antibody production, but not protective upon challenge	(van Diemen et al., 2013)
Bacteria	*Staphylococcus aureus*	Gamma	25-40 kGy	Mice	Induced B and T cell-dependent protection against challenge	(Gaidamakova et al., 2012)

Table 4. Continued

Type of Pathogen	Pathogen	Inactivation Method	Inactivation Dose	Model	Notes	Source
Bacteria	*Streptococcus pneumoniae*	Gamma	12 kGy	Mice	Protection upon challenge mediated by B-cells and innate IL-17 response	(Babb et al., 2016)
Bacteria	*Streptococcus pneumoniae*	Electron Beam	25 kGy	Rabbit and Mice	Immunogenic and protective upon challenge	(Pawlowski and Svenson, 1999)
Protozoa	*Eimeria tenella*	Electron Beam (Low Energy)	0.1-0.5 kGy	Chicken	Partially protective upon challenge	(Thabet et al., 2019)
Protozoa	*Eimeria tenella*	X-ray	0.2 kGy	Chicken	Protective upon challenge	(Jenkins et al., 1991)
Protozoa	*Plasmodium berghei*	X-ray	0.02-0.15 kGy	Mouse	Protective upon challenge	(Nussenzweig et al., 1967)
Protozoa	*Plasmodium falciparum*	Gamma	0.12-0.15 kGy	Human	Long-lasting protective immunity	(Hoffman et al., 2002)
Protozoa	*Plasmodium gallinaceum*	X-ray	0.005-0.2 kGy	Mosquito	Sporozoites from irradiated oocysts were non-infective	(Ward et al., 1960)
Virus	Human Respiratory syncytial virus (RSV)	Electron Beam (Low Energy)	20 kGy	Mice	Reduction in viral load upon challenge	(Bayer et al., 2018)
Virus	Influenza A virus	Gamma	12.6 kGy	Mice	Induced cytotoxic T cells and protective upon against challenge	(Müllbacher et al., 1988)

Table 4. Continued

Pathogen	Inactivation Method	Inactivation Dose	Model	Notes	Source	Pathogen
Virus	Influenza A virus	Gamma	10-40 kGy	Mice	Cross-reactive and cross-protective cytotoxic T cell responses	(Furuya et al., 2011)
Virus	Influenza A virus	Gamma	10 kGy	Mice	Protective upon challenge; freeze-drying did not affect cross-protective immunity	(Furuya et al., 2010)
Virus	Influenza A virus	Gamma	50 kGy	Mice	Intranasal vaccination conferred complete protection	(David et al., 2017)
Virus	Influenza A virus	Electron Beam (Low Energy)	30 kGy	Mouse	Elicited a protective immune response	(Fertey et al., 2016)
Virus	Influenza A virus	Electron Beam	25-40 kGy	Nonhuman primate	Elicited seroconversion	(Scherließ et al., 2014)
Virus	Influenza A virus	Gamma	10 kGy	Mice	Protective upon heterotypic challenge	(Alsharifi et al., 2009)
Virus	Middle Eastern Respiratory Virus (MERS)	Gamma	50 kGy	Mice	Caused lung immunopathology upon challenge	(Agrawal et al., 2016)
Virus	Polio Virus	Gamma	45 kGy	Mice	Protective upon challenge	(Tobin et al., 2020)
Virus	Rotavirus	Gamma	50 kGy	Mice	Induced a specific neutralizing-antibody response	(Shahrudin et al., 2018)

Table 4. Continued

Pathogen	Inactivation Method	Inactivation Dose	Model	Notes	Source	Pathogen
Virus	Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2)	Gamma	50 kGy	Mice	Adjuvanted vaccine elicited T and B cell responses	(Sir Karakus et al., 2020)
Virus	Vaccinia virus	Gamma	0-15 kGy	Rabbit	Inactivated virus was immunogenic	(Kaplan, 1960)
Virus	Venezuelan Equine Encephalitis Vaccine	Gamma	80-100 kGy	Guinea Pig	Protective upon challenge	(Reitman et al., 1970)
Virus	Venezuelan Equine Encephalitis Vaccine	Gamma	50 kGy	Mice	Protective against subcutaneous challenge and partially protective against aerosol challenge	(Martin et al., 2010)
Virus	White Spot Syndrome Virus	Electron Beam	13 kGy	Shrimp	Protective upon challenge	(Motamedi-Sedeh et al., 2017)
Virus	Zaire ebola virus	Gamma	100 kGy	Nonhuman primate	Not protective upon challenge	(Marzi et al., 2015)
Virus	Zaire ebola virus	Gamma	60 kGy	Nonhuman primate	Not protective upon challenge	(Geisbert et al., 2002)

Electron beam technology has been investigated as a method to generate vaccine-like immunomodulators against *Salmonella* Typhimurium in a mice model (Praveen, 2014). This concept has been expanded to demonstrating the immunomodulatory and protective effects of eBeam-inactivated *Salmonella* Enteriditis and Typhimurium in chickens and *Rhodococcus equi* in neonatal fouls (Kogut et al., 2012; McReynolds et al., 2012; Bordin et al., 2014; Praveen, 2014; Jesudhasan et al., 2015b; Rocha et al., 2016). This concept has also been used to explore the use of low energy eBeam as an inactivation for vaccine development with considerable success (Fertey et al., 2016; Bayer et al., 2018; Thabet et al., 2019).

3. CHARACTERIZATION OF METABOLICALLY ACTIVE YET NON-CULTURABLE *C. perfringens*

3.1. Overview

Clostridium perfringens causes Necrotic Enteritis (NE) in poultry, causing significant mortality and costing the global poultry industry approximately $6 billion, annually. Vaccines have been proposed as an alternative control strategy for NE, however a vaccine against NE in poultry remains elusive. With significant amounts of research demonstrating that there are multiple antigens that may play a role in inducing NE in poultry, there is a need for a robust vaccine that is broadly antigenic and immunogenic. Electron beam (eBeam) irradiation is processing tool commonly used in the food and medical device industry to inactive microorganisms. When a microorganism is inactivated by eBeam, it is in a Metabolically Active, yet Non-culturable (MAyNC). Although the cell is inactivated and unable to replicate, the bacterial membrane remains intact and the cells remain metabolically active. Thus, MAyNC bacteria possess the characteristics of a potential vaccine: Because they are inactivated, they are unable to replicate and cause disease within a host, but because they are intact and metabolically active, they may retain their ability to elicit an immune response within a host.

The focus of this study was to formulate and characterize a MAyNC state of a putative *C. perfringens* vaccine candidate. Ten *C. perfringens* strains isolated from NE outbreaks were chosen to create the vaccine candidates and were combined into two cocktails, A and B. Quantitative real-time PCR demonstrated that the strains used in these studies had genetic potential to produce a wide range of virulent clostridial toxins such as α-toxin, netB toxin, and two zinc metalloproteases. The D-10 value, or dose required for a 90% reduction in microbial titer, was determined to be approximately 0.294 kGy for *C. perfringens* AUD1, 0.272 kGy for cocktail A, and 0.328 kGy for cocktail B. Subsequently, a dose of 10 kGy was used to inactivate the vaccine formulation to ensure complete inactivation. When treated at 10 kGy, the eBeam-treated *C. perfringens* (EBCP) had sheared DNA, and were unable to replicate, even when inoculated into a nutrient-rich media and incubated at ideal growth conditions for 10 days. The metabolic activity of EBCP immediately after 10 kGy eBeam treatment and over the course of 10 days following treatment was examined using the AlamarBlue assay. Furthermore, EBCP retained their membrane integrity after 10 kGy eBeam. These experiments demonstrated that eBeam-inactivated *C. perfringens* are in a MAyNC state. Because the EBCP retain their membrane integrity, the presence of surface antigens coupled with metabolic activity may be able to elicit an immune response if administered to a bird and be used an a protective immunomodulator against Necrotic Enteritis in Poultry.

3.2. Introduction

Electron beam (eBeam) treatment is a form of ionizing radiation, meaning the electrons are sufficiently energized to ionize molecules that they interact with. When the electrons interact with a molecule leading to its ionization, the ejected electron becomes energized, going on to interact with and ionize adjacent molecules. This chain reaction continues until the energy has fully dissipated (Miller, 2005). The effect of irradiation on a material depends on the composition of the molecule. High energy eBeam technology damages microorganisms through direct and indirect methods. Direct damage is a result of interactions of molecules with energized electrons, while indirect methods involve interactions with radiolytic water products. Since the largest molecule in a microorganism is its nucleic acid, it is the primary target of direct ionization events and is severely damaged after eBeam exposure (Urbain, 1986; Mavragani et al., 2019). The single and double stranded breaks in the DNA of a microorganism are irreparable and lead to a loss of replicative ability (Nikjoo et al., 1994). Indirect damage results from interaction of molecules with free radical species generated when the energized electrons collide with water and other organic molecules. These extremely reactive, but short-lived free radical species include reactive oxygen species (ROS) such as hydroxyl radicals, hydrogen peroxide, hydrogen, hydrate electrons, and hydrated protons (Miller, 2005). The reactive species cause further damage to the microorganism’s nucleic acid and other cellular components. While cells are inactivated due to damage to their nucleic acid, previous studies have shown that the cellular membrane remains intact, leading to a metabolically active, yet non-culturable (MAyNC) state (Vemulapalli et al., 2000; Magnani et al., 2009; Bordin et al., 2014; Hieke and Pillai, 2018; Bhatia and Pillai, 2019). This MAyNC state can be harnessed to generate vaccine-like immunomodulators (Kogut et al., 2012; McReynolds et al., 2012; Bordin et al., 2014; Praveen, 2014; Jesudhasan et al., 2015b; Rocha et al., 2016).

Necrotic enteritis (NE) is a disease caused by the Gram-positive, anaerobic, endospore-forming bacterium *Clostridium perfringens*. Commonly referred to as a “flesh-eating” bacterium, the hallmark signs of a *C. perfringens* infection include decayed tissue whether the infection is dermal, for example in gas gangrene in humans or gangrenous dermatitis in poultry, or enteric, as is the case in NE in poultry. The species can create up to 23 different toxins and virulent enzymes, although no individual strain can express all of the toxins (Kiu and Hall, 2018).

The pathogenesis of NE is complex with conflicting claims of different toxins and virulent enzymes claimed as the most important factor in disease. For example, for decades, α-toxin was considered the primary agent responsible for NE, but more recently, there have been numerous studies demonstrating that α-toxin knockout mutants can still induce NE (Al-Sheikhly and Truscott, 1977; Fukata et al., 1988; Keyburn et al., 2006). The same uncertainty has followed the discovery of the netB toxin in 2008, with conflicting data supporting and refuting it as the primary virulence factor of NE (Keyburn et al., 2008, 2010a; Abildgaard et al., 2010; Bailey et al., 2014; Rood et al., 2016). Even more recently, studies have discussed the importance of other minor clostridial toxins such as tpel, Cp beta-2 toxin, perfringolysin-O, and various other proteins (Cooper et al., 2010; Allaart et al., 2012; Coursodon et al., 2012; Bailey et al., 2014; Verherstraeten et al., 2015; Yang et al., 2018; Wade et al., 2020). Ultimately, there are most likely multiple virulence factors that influence the pathogenesis of NE, further emphasizing the importance of using novel approaches that incorporate a variety of virulent antigens when developing a vaccine for this disease.

The objective of this study was to characterize the MAyNC state of eBeam-treated *C. perfringens* using DNA fragmentation analysis as well as membrane integrity and metabolic activity assays. The hypothesis of these studies was that high energy electron beam treatment would render a cocktail of *C. perfringens* strains metabolically active, yet non-culturable.

3.3. Material and Methods

3.3.1. Bacterial cultures

The *Clostridium perfringens* strains JGS 1473, JGS 4101, JGS 4064, JGS 1235, JGS 1521, were kindly provided by Dr. J. Glenn Songer from the University of Arizona. *C. perfringens* AUD1, was kindly provided by Dr. Audrey McElroy at Texas A&M University. *C. perfringens* CP 1, CP 2, CP 3, and CP 4 were isolated and stored as previously described (McReynolds et al., 2004). Aliquots of each culture were stored as freezer stocks at -80 °C. For each experiment, overnight cultures were prepared by transferring a single loop from a freezer stock to growth medium (Fluid Thioglycolate Medium (FTG) or Reinforced Clostridial Medium (RCM) and incubating overnight anaerobically at 37 °C in a COY anaerobic chamber. For specific experiments, strains were combined to create two cocktails of strains. Cocktail A consisted of *C. perfringens* CP 1, CP 2, CP 3, and CP 4 while Cocktail B was composed of *C. perfringens* strains JGS 1473, JGS 4101, JGS 4064, JGS 1235, JGS 1521, and AUD1.

3.3.2. Electron beam treatment and dosimetry

Electron beam inactivation dosing experiments was performed at the National Center for Electron Beam Research at Texas A&M University in College Station, TX. Samples were treated with a 10 MeV, 18 kW Electron Beam Linear Accelerator (LINAC). Alanine dosimeters were used to measure the absorbed eBeam dose and read using a Bruker e-scan spectrometer. All dosimeters used were calibrated to traceable standards. Preliminary dose mapping studies were conducted to ensure that the dose uniformity ratio (DUR), or the ratio of the maximum dose to the minimum absorbed dose, of the sample bags was as close to one as possible. Sample bags that were 2.5 in x 2.5 in and filled with 5.5 ml of sample had a DUR of 0.99 so this sample packaging configuration was used for all experiments. A DUR of approximately one ensures that dose deposition throughout the sample is uniform, which is extremely important for all studies conducted in these experiments.

To comply with all Texas A&M University biosafety regulations, all biological samples were double packaged in heat sealed Whirl-Pak bags (Nasco, New York, NY). Samples were then placed inside a “specimen transport” bag that was rated up to 95 kPa. Subsequently, only triple-sealed samples were allowed to be treated at the eBeam facility.

3.3.3. Toxin typing

Quantitative real-time PCR was used to determine the genetic potential of the ten strains to produce relevant toxin genes. The strains were confirmed as *C. perfringens* isolates using the 16s rRNA gene.

3.3.4. DNA extraction and quality control

DNA was extracted from triplicate overnight cultures of each strain using the Qiagen DNeasy UltraClean Microbial Kit. The manufacturer’s instructions were followed. DNA quantity and quality was analyzed using a NanoDrop ND-1000 spectrophotometer and stored at -20 °C until further use.

3.3.5. Quantitative real-time PCR analysis

Quantitative Real-time PCR was used to detect the presence of major toxin genes such as *cpa*, *cpb, etx*, and *iap*, as well as minor toxin genes such as *cpe, netB, cpb2, tpeL, zmpA, and zmpB.* Quantitative real-time PCR (qPCR) was conducted using an Applied Biosystems 7900HT PCR system. The 20 μl reaction mixture contained 10 μl SYBR Green Master Mix (Applied Biosystems), 0.5 μM forward and reverse primer, and 10 ng template DNA. Two technical replicates along with negative and non-template controls were included on each plate. Primer pairs used are listed in Table 5. The qPCR amplification cycles were as follows: 1 cycle of 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 60 °C for 30 s (Yang et al., 2018). Melt curve analysis was used to confirm the identity of the amplified product. Samples were considered positive for a gene if the cycle threshold (Ct) value was below 44.

Table 5. Primer pairs used for qPCR toxin-typing

Gene	Primer name	Sequence (5' - 3')	Reference
cpa	Forward	AGTCTACGCTTGGGATGGAA	(Yang et al., 2018)
	Reverse	TTTCCTGGGTTGTCCATTTC	
cpb	Forward	TCCTTTCTTGAGGGAGGATAAA	
	Reverse	TGAACCTCCTATTTTGTATCCCA	
etx	Forward	TGGGAACTTCGATACAAGCA	
	Reverse	TTAACTCATCTCCCATAACTGCAC	
iap	Forward	AAACGCATTAAAGCTCACACC	
	Reverse	CTGCATAACCTGGAATGGCT	
cpb2	Forward	AGATTTTAAATATGATCCTAACC	
	Reverse	CAATACCCTTCACCAAATACTC	
cpe	Forward	GGGGAACCCTCAGTAGTTTCA	
	Reverse	ACCAGCTGGATTTGAGTTTAATG	
tpeL	Forward	ATATAGAGGCAAGCAGTGGAG	
	Reverse	GGAATACCACTTGATATACCTG	
netB	Forward	TGATACCGCTTCACATAAAGGTTGG	
	Reverse	ATAAGTTTCAGGCCATTTCATTTTTCCG	
16s rRNA	Forward	CATCATTCAACCAAAGGAGCAATCC	
	Reverse	CATTATCTTCCCCAAAGACAGAGC	
zmpA	Forward	AGGTTCCGGTAGCGTAAG	(Wade et al., 2020)
	Reverse	TAGCGTTTGCCCAGTCAG	
zmpB	Forward	CTTTCTGCTACAACAATTGATG	
	Reverse	TTACTTACCCTTGCTTGATG	

3.3.6. Inactivation kinetics

In order to measure the sensitivity *C. perfringens* to eBeam treatment, the decimal reduction value, or D-10 value, was calculated (dose required for a 90% reduction in microbial titer) of *C. perfringens* strain AUD1, cocktail A, and cocktail

B. Overnight cultures of each culture were grown individually in FTG. For the cocktails, the appropriate strains were combined in equal volumes and 5.5 ml aliquots of each sample were aseptically packaged in sterile Whirl-Pak bags (Nasco, New York, NY) and exposed to varying eBeam doses. After treatment, the eBeam-inactivated *C. perfringens* (EBCP) cells were serially diluted in FTG, plated on Blood Agar (Trypticase Soy Agar supplemented with 5% Sheep's Blood), and incubated anaerobically at 37 °C for 24 hrs. The D-10 value was estimated by plotting the number of surviving organisms ($\log_{10}$ CFU/ml) as a function of measured dose (kGy). The slope of the curve was calculated using a linear regression analysis and the negative reciprocal of the slope was determined to be the D-10 value ref needed(Jaiswal, 2016). Biological triplicate cultures were used for each trial. For AUD1 analysis, two independent eBeam treatment trials were conducted. The cocktail D-10 experiments were conducted once.

3.3.7. Determining EBCP inactivation dose

Based on the calculated D-10 value and typical titer of an overnight culture, *C. perfringens* AUD 1 was grown in FTG and was eBeam-treated at target doses of 0, 2.5, 7, and 10 kGy to determine whether these doses could inactivate the entire culture. Cultures were plated immediately after treatment. Treated samples were enriched by spiking 9 ml of fresh FTG broth with 1 ml EBCP and incubated anaerobically at 37 °C for up to 10 days. Five 100 µl aliquots of the enriched samples were plated on day 0, 3, 7, and 10 to check for multiplication of EBCP. Samples were plated on TSA with 5% sheep's blood and biological triplicate replicates were used. Based on the inactivation kinetics and the results of this experiment, an eBeam dose of 10 kGy was used for generating EBCP for all future experiments.

3.3.8. Quantifying nucleic acid damage

To assess nucleic acid damage after eBeam treatment, *C. perfringens* AUD1 grown in FTG was exposed to a target dose of 10 kGy. After treatment, samples were kept on ice and DNA was extracted from untreated and treated samples within 30 minutes of treatment, using the Qiagen DNeasy UltraClean Microbial Kit. The manufacturer's instructions were followed. DNA quantity and quality were analyzed using a NanoDrop ND-1000 spectrophotometer and stored at -20 °C until fragment analysis. Nucleic acid quality (fragment analysis) was conducted at the Texas A&M Genomics and Bioinformatics Service (TxGen) using an Agilent Fragment Analyzer (Agilent, Santa Clara, CA) system.

3.3.9. Membrane integrity

Membrane integrity of the EBCP AUD1 was assessed using the LIVE/DEAD BacLight Bacterial Viability kit (ThermoFisher, Molecular Probes, Eugene, OR) for microscopy. This assay uses SYTO®9, a green fluorescent dye, and propidium iodide, a red fluorescent dye, to differentiate between intact and compromised cell membranes. The manufacturer's instructions were followed with some small modifications. In preliminary experiments, the agar present in FTG inhibited staining using this kit. Furthermore, *C. perfringens* AUD1 was extremely sensitive to centrifugation with 1-2 log decreases with each centrifugation cycle. Because this assay emphasizes removing all traces of background media, for this experiment, *C. perfringens* AUD1 was grown using RCM, a less complex media that would require fewer washes. Biological replicates were used. Briefly, 20 ml of eBeam-treated and untreated samples were concentrated via centrifugation at 4,000 x g for 10 minutes and pellets were resuspended in 2 ml 0.85% NaCl. One ml of the untreated suspension was added to 20 ml 0.85% NaCl. To generate a negative control, 1 ml of this suspension was boiled at 100 °C for 10 minutes prior to being added to 20 ml of isopropyl alcohol. All samples were incubated at room temperature for one hour, briefly vortexing to mix every 15 minutes. Samples were then washed one time via centrifugation at 4,000 x g and resuspended in 5 ml 0.85% NaCl. The optical density (OD) of each sample was measured and all samples were normalized to an optical OD600 of 0.5.

SYTO®9 and propidium iodide were mixed in a 1:1 ratio and 3 µl of dye mixture was added to 1 µl of prepared bacterial suspension. The samples were vortexed to mix and incubated at room temperature in the dark for 15 minutes. After incubation, 5 µl of culture was placed on a clean glass slide with a cover slip and observed using a fluorescence microscope (Olympus BX50, Japan) equipped with a digital camera (Olympus qcolor3, Olympus, USA). Images were taken at 1000x lens magnification with oil.

3.3.10. Metabolic activity

The metabolic activity of EBCP was measured using the alamarBlue™ Cell Viability assay (ThermoFisher Scientific, Waltham, MA) which uses the natural reducing environment of a cell tot measure viability. Resazurin, the active ingredient of alamarBlue™ and a blue non-fluorescent compound is reduced upon entering a metabolically active cell into resorufin, a red highly-fluorescent compound. The manufacturer's instructions were followed. In preliminary experiments, the presence of sodium Thioglycolate (a reducing agent) and resazurin in FTG skewed the results of the experiment. Therefore, in all metabolic activity experiments, *C. perfringens* was grown in RCM. The metabolic activity of all EBCP strains was measured immediately (within 30 minutes) after eBeam treatment. Briefly, 90 µl of each sample was added to a black, clear bottom 96-well plate along with 10 µl of 10X alamarBlue™ reagent. Untreated *C. perfringens* and sterile FTG broth were used as control. The plate was incubated in the dark at 37 °C for 1 hr. After incubation, fluorescence was read using a plate reader at an excitation wavelength between 540-570 and emission wavelength between 580-610 nm. Multiple unpaired student's t-tests were conducted to determine whether metabolic activity was statistically different between untreated and treated samples.

To measure the metabolic activity of EBCP AUD1 over a period of 10 days, 1 ml aliquots of EBCP, live *C. perfringens*, and heat/isopropyl killed samples were incubated anaerobically at 4, 25, and 37 °C for 10 days. Heat/isopropyl killed controls were generated by heating 1 ml live *C. perfringens* AUD1 at 100 °C for 10 minutes prior to being placed in 10 ml isopropyl alcohol for 1 hr. These samples were then washed twice with RCM via centrifugation at 4,000 x g to remove any traces of isopropyl alcohol and resuspended in fresh RCM. Anaerobic conditions were generated using BD GasPaks (Becton Dickinson, Franklin Lakes, NJ) and anaerobic conditions were confirmed using colorimetric test strips placed inside containers. Metabolic activity of all samples was measured on days 0, 2, 4, 6, 8, and 10 using the previously described method. After aseptically sampling, samples were placed back in their respective environment for the duration of the experiment.

3.4. Results and Discussion

3.4.1. Determining toxin type of *C. perfringens*

The objective of this experiment was to understand the genetic potential of each *C. perfringens* strain to produce multiple major and minor toxins. The results of the analysis are presented in Table 6. All strains were confirmed as *C.*

perfringens strains. Strains JGS 1473, JGS 1235, JGS 4064, and CP 1 were classified as Type A strains while JGS 1521, JGS 4104, AUD 1, CP 2, CP 3, and CP 4 were classified as Type G strains, using the updating typing system (Kiu and Hall, 2018; Rood et al., 2018). All strains were negative for *cpb2, tpeL,* and *cpe*. It is important to note that for this experiment, it was not possible to obtain *C. perfringens* strains that had previously been screened for each of the target genes to use as positive controls, and the negative results for a gene in all samples could be false negatives due to inaccurate primer design or PCR cycle conditions. For example, JGS 1473, 4104, 1235, and 1521 have previously been identified as *cpb2* positive by other investigators but under the conditions and primers used in the present study (Table 5), none of the strains were positive for the presence of this gene (Cooper, 2007; Barbara et al., 2008; Cooper et al., 2010; Lepp et al., 2010). The source and previously published characteristics of each strain is presented in Table 7.

Table 6. *C. perfringens* strains used had a variety of toxin genotypes

	16s rRNA	***cpa***	***cpb***	***etx***	***iap***	***netB***	***cpb2***	***tpeL***	***cpe***	***zmpA***	***zmpB***
JGS 1521	+	+	-	-	-	+	-	-	-	+	+
JGS 4104	+	+	-	-	-	+	-	-	-	+	+
JGS 1473	+	+	-	-	-	-	-	-	-	+	+
JGS 1235	+	+	-	-	-	-	-	-	-	+	+
AUD 1	+	+	-	-	-	+	-	-	-	+	+
JGS 4064	+	+	-	-	-	-	-	-	-	-	+
CP 1	+	+	-	-	-	-	-	-	-	-	+
CP 2	+	+	-	-	-	+	-	-	-	+	+
CP 3	+	+	-	-	-	+	-	-	-	+	+
CP 4	+	+	-	-	-	+	-	-	-	+	+

Table 7. Summary of previously published characteristics of *C. perfringens* strains used in vaccine formulations

	Source		**Type**	**Virulence Genes**				**Antibiotic resistance ?**	**Virulent (produces lesions)?**	**Sources**
				pil A1	*pil A2*	*cpb2*	*netB*			
JGS 1473	JGS	Healthy Chicken normal flora	A	+	+	+	-	Bacitracin	No	(Cooper, 2007; Cooper et al., 2010; Lepp et al., 2010)
JGS 4104	JGS	Turkey NE	A	+	-	+			Yes	(Cooper, 2007; Barbara et al., 2008)
JGS 4064	JGS	Chicken NE	A	+	-	-			Yes	(Cooper, 2007; Barbara et al., 2008)
JGS 1235	JGS	Chicken Ulcerative enteritis	A	-	+	+			Yes	(Cooper, 2007; Barbara et al., 2008)
JGS 1521	JGS	Chicken NE	A	-	-	+		Bacitracin	Yes	(Cooper, 2007; Barbara et al., 2008; Lepp et al., 2010; Rahimi et al., 2011)
AUD 1	AM	Chicken NE, Virginia	A						Yes	Unpublished
CP 1	USDA	Chicken NE	A						Yes	(McReynolds et al., 2004, 2007)
CP 2	USDA	Chicken NE	A						Yes	(McReynolds et al., 2004, 2007)
CP 3	USDA	Chicken NE	A						Yes	(McReynolds et al., 2004, 2007)
CP 4	USDA	Chicken NE	A						Yes	(McReynolds et al., 2004, 2007)

Cocktails A and B were composed such that they contained strains with the genetic potential to produce a variety of toxins or virulence factors. Because the pathogenicity of *C. perfringens*-NE is still not completely understood, the rationale for preparing these cocktails was that they contained a variety of strains with varied virulence profiles. This could be beneficial since the current hypothesis is that numerous toxins and enzymes are likely important virulence factors in NE (Allaart et al., 2012; Coursodon et al., 2012; Bailey et al., 2014; Yang et al., 2018). For example, two zinc metalloproteases encoded by the genes *zmpA* and *zmpB* have been recently implicated in the pathogenesis of NE (Wade et al., 2020). Out of the ten screened isolates used in the present study, all were positive for *zmpB*, a chromosomally encoded gene, and 80% of isolates were positive for *zmpA*, a plasmid-borne gene.

3.4.2. Determining the inactivation kinetics of EBCP

The sensitivity of *C. perfringens* to eBeam treatment was investigated by exposing *C. perfringens* AUD1, cocktail A, and Cocktail B to specific eBeam doses and enumerating the survivors. The D-10 value, or the eBeam dose required for a 90% reduction in microbial population, was calculated as the inverse reciprocal of the slope of each line. The D-10 value of *C. perfringens* AUD1 was calculated to be 0.294 kGy (Figure 5). The D-10 value of cocktail A was 0.272 kGy and the D-10 value of cocktail B was calculated to be 0.328 kGy (Figure 6 A and B). The D-10 values presented here are similar to those previously reported (Grant and Patterson, 1992; Monk et al., 1995; Van Gerwen et al., 1999).

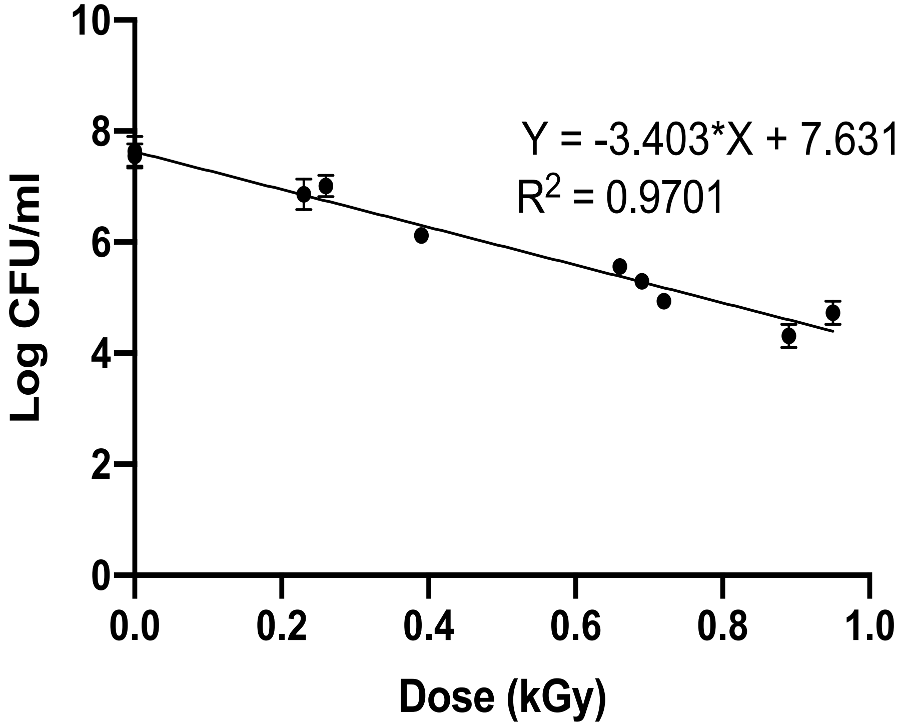
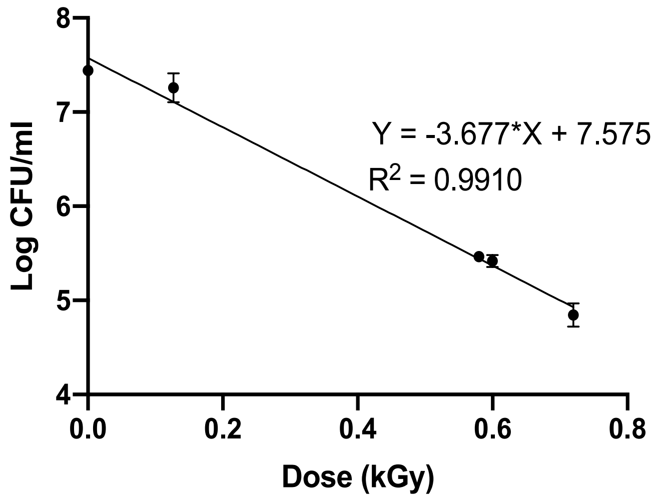


Figure 5. The D-10 value of *C. perfringens* AUD1 is 0.294 kGy.

A)



B)

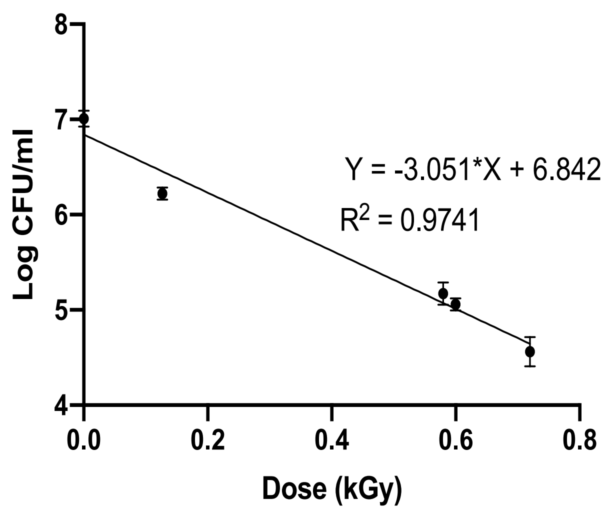


Figure 6. The D-10 value of two *C. perfringens* cocktails was determined to be 0.272 and 0.328 kGy, respectively. A) Cocktail A has D-10 value of 0.272 kGy B) Cocktail B has a D-10 value of 0.328 kGy.

3.4.3. Determining EBCP inactivation dose

Triplicate biological replicates of EBCP AUD 1 treated at various doses was enriched in fresh media to determine the lethal inactivation dose (Table 8). Based on a calculated D-10 of 0.294 and a typical overnight culture titer of approximately 10^8 CFU/ml, the minimum dose required for an eight log reduction was theoretically 2.35 kGy. Therefore, 2.5 kGy was chosen as the absolute minimum possible dose required for complete inactivation. However, this dose failed to fully inactivate the culture, even immediately after eBeam treatment (Table 8). For each plating 500 µl was plated from each sample in 100 µl aliquots, in order to improve detection sensitivity to 2 CFU/ml. On day ten, one replicate of the EBCP treated with 7 kGy had one plate out of five with two colonies growing. This suggests that at 7 kGy, there were either a very low number of surviving cells that were only detectable after ten days of enrichment. Alternatively, vegetative cells present on day 10 may have been the result of the germination of *C. perfringens* spores that were not inactivated at 7 kGy. A high titer of spores would be unexpected from cultures grown in an ideal nutrient-rich medium at the appropriate temperature and anaerobic conditions, as sporulation is typically triggered by environmental factors, nutrient starvation, or the presence of inorganic phosphate (Philippe et al., 2006; Li et al., 2016). However, there is always the possibility for very low levels of spores in overnight cultures. *C. perfringens* spores are more resistant to eBeam treatment, with the D-10 of *C. perfringens* spores reported to be between 2.3-3.0

kGy (Midura et al., 1965; Clifford and Anellis, 1975). To avoid the possibility of even small number of spores being present (resulting in incomplete inactivation) an eBeam dose of 10 kGy was chosen to generate EBCP for all future experiments. Enrichment of triplicate biological replicates *C. perfringens* treated with 10 kGy was repeated a second time to ensure reproducibility.

Table 8. *C. perfringens* was inactivated at 10 kGy

Target Dose	Absorbed Dose	Day 0	Day 3	Day 7	Day 10
0 kGy	0 kGy	+	+	+	+
2.5 kGy	2.573 kGy	+	+	+	+
7 kGy	7.126 kGy	-	-	-	+
10 kGy	9.821 kGy	-	-	-	-

3.4.4. eBeam causes significant DNA shearing

When IR interacts with a molecule, it causes the production of ROS and ionization cascades which can cause clustered damage. In the case of DNA, this leads to clustered single and double-stranded breaks that due to the rate and clustering of damage, are irreparable by the cell (Goodhead, 1989; Nikjoo et al., 1994; Mavragani et al., 2019). Previous studies have demonstrated shearing of DNA, primarily through the use of gel electrophoresis. Using a Fragment Analyzer to visualize DNA fragmentation is commonly used as a quality step in genomics studies (Ponce et al., 1999; Nachamkin et al., 2001; Rodríguez and Vaneechoutte, 2019; Sidstedt et al., 2019). In this study, this technique was used as a novel method to quantify the fragmentation of DNA caused by IR. *C. perfringens* AUD1 at a concentration of 3.7 x 10^8 CFU/ml was eBeam treated at 9.96 kGy. Nucleic acid extracted from untreated and treated samples had vastly different profiles after treatment (Figure 7A and Figure 7B). The majority of DNA extracted from untreated samples was in large fragments with approximately 14% in fragments larger than 60,000 base pairs (bp) in length and approximately 15% around 23,569 bp in length. DNA extracted from EBCP was significantly smaller in length, with approximately 71% in fragments around 5,632 bp in length and approximately 17% in fragments around 162 bp in length. The DNA of EBCP appears to be in very small fragments, as demonstrated by the shoulder in the figure, rather than the distinct peaks seen in the untreated samples.

Due to the clustering of damage, DNA is sheared into short fragments (Holley and Chatterjee, 1996). DNA from gamma-irradiated *Bacillus atrophaeus* variant *globigii* and *Yersinia pestis* appeared as large smears when subjected to agarose gel electrophoresis, indicating a large number of small fragments (Broomall et al., 2016). When using optimal mapping, a technique that arrays long (>50 kb) fragments onto slides for direct observation, no fragments of this length were observed (Broomall et al., 2016). The advantage of using a fragment analyzer for this analysis is the quantitative aspect of the technique. Rather than just visualizing a smear on a gel, the proportion of a sample at various fragment sizes can be quantified.

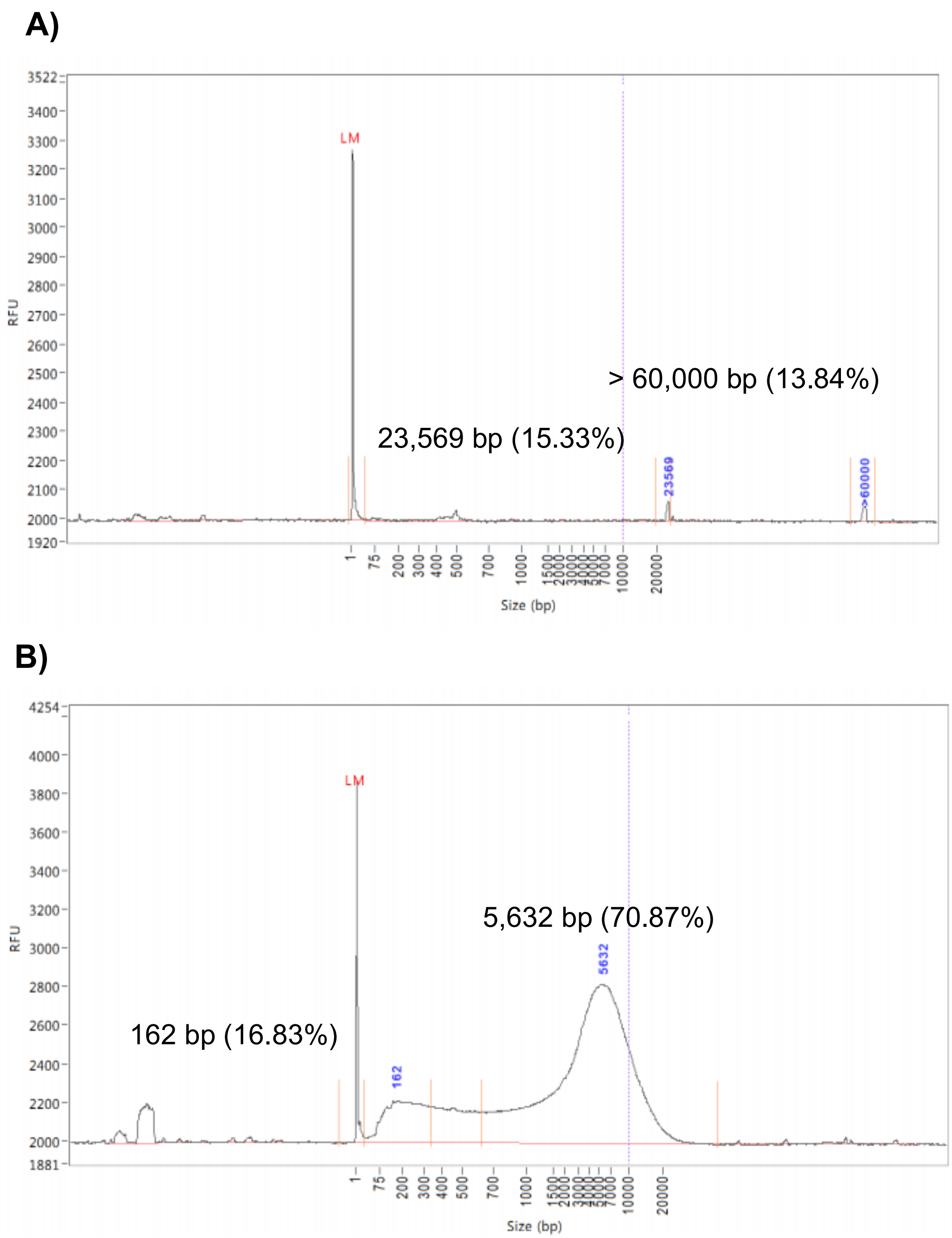


Figure 7. Treatment of *C. perfringens* AUD1 at 10 kGy resulted in shredded DNA. A) Untreated control B) EBCP treated at 9.96 kGy

3.4.5. Membrane integrity of *C. perfringens* was maintained after eBeam treatment

The Live/Dead *Bac*Light™ assay was used to investigate whether eBeam treatment causes damage to the cell membrane at 10 kGy. The membrane integrity of 3.08 x 10^8 CFU/ml EBCP (10.08 kGy) was assessed with live and heat and isopropyl-killed *C. perfringens* used as live and dead controls, respectively (Figure 8). The *Bac*Light™ assay utilizes two dyes to differentiate between intact and compromised membranes. SYTO®9 is used to stain all membranes green, while propidium iodide can only enter and stain cells with compromised membranes. When used together in equal proportions and visualized using a fluorescent microscope, cells with intact membranes fluoresce green and cells with damaged membranes fluoresce red. EBCP maintained intact cell membranes as indicated by the majority of cells staining green (Figure 8A). A few red cells were visible in both the treated and untreated samples, likely indicative of natural cell death (Figure 8A and Figure 8B). Various studies have been conducted demonstrating that eBeam treatment does not compromise the cellular membrane of bacteria, primarily by using microscopy (Bordin et al., 2014; Praveen, 2014; Jesudhasan et al., 2015b; Hieke and Pillai, 2018). Similarly, gamma irradiation has also been shown to not damage the membrane of bacteria (Sanakkayala et al., 2005; Magnani et al., 2009; Moustafa et al., 2011). Other methods at examining membrane damage after exposure to ionizing radiation have been conducted and paint a more nuanced picture of membrane integrity after treatment. Broomall et al. demonstrated that after exposure to lethal doses of gamma irradiation, intact *B. atrophaeus* spores struggled to retain various stains such as malachite green or crystal violet, both of which bind to anionic components in the cell wall or spore coat by charge-based interactions (Broomall et al., 2016). This suggests that there may be structural polymeric components of the cell wall which are damaged during treatment. Furthermore, metabolomic and transcriptomic studies of eBeam-treated *E. coli* and *Salmonella* show increased production of metabolites and up-regulation of genes responsible for membrane repair, suggesting that there may be damage to the membrane that is undetectable by microscopy (Hieke, 2015; Bhatia and Pillai, 2019).

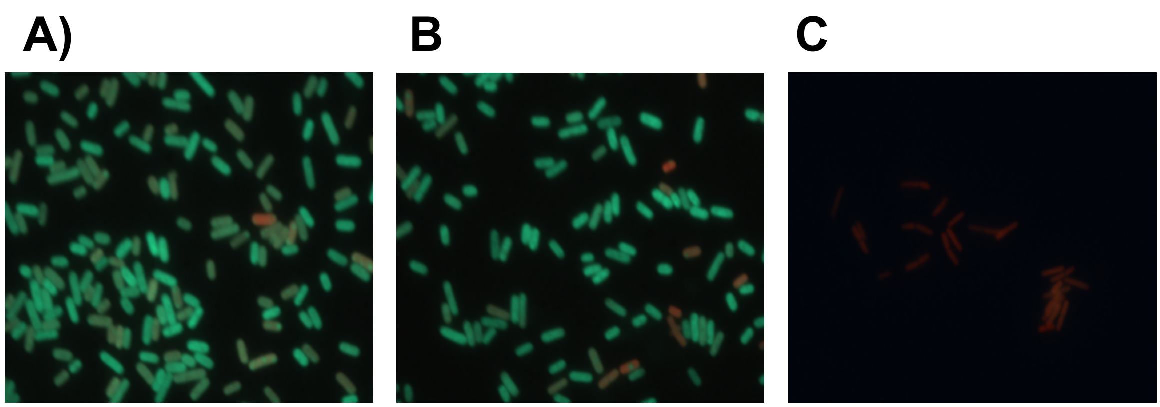


Figure 8. EBCP maintained their membrane integrity after eBeam treatment. A) EBCP (10.08 kGy) B) Live *C. perfringens* AUD1 C) Heat and isopropyl killed *C. perfringens* AUD1.

3.4.6. Metabolic activity is sustained in EBCP

The metabolic activity of EBCP immediately after irradiation and over ten days was examined using the AlamarBlue™ reagent (Figure 9 and Figure 10). This reagent uses the inherent reducing capacity of metabolically active cells to convert resazurin (blue, non- or weakly fluorescent) to resorufin (red, highly fluorescent). The presence of this redox reaction indicates that the cell is metabolically active. The starting titer of each strain is shown in Table 9. Immediately after eBeam treatment (11.52 kGy), the metabolic activity of all strains did not differ from their respective untreated control ($P > 0.05$).

Table 9. Starting titer of *C. perfringens* for metabolic activity assay

***C. perfringens* strain**	**Average CFU/ml**
AUD 1	1.10×10^8
JGS 1473	3.50×10^7
JGS 1235	1.22×10^8
JS 4104	2.91×10^8
JGS 4064	1.96×10^8
JGS 1521	1.34×10^8
CP 1	3.82×10^8
CP 2	2.43×10^8
CP 3	2.38×10^8
CP 4	4.55×10^8

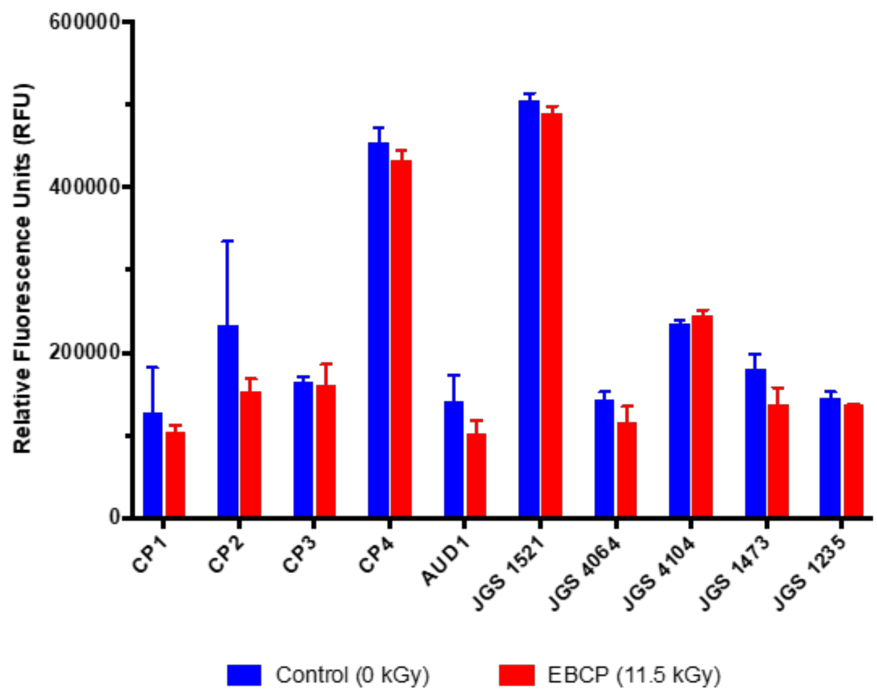


Figure 9. EBCP remained metabolically active immediately after eBeam treatment.

In order to examine the shelf-life of EBCP exposed to 10.08 kGy eBeam dose, 3.31 x 10^8 CFU/ml was incubated anaerobically various temperatures. When stored at various temperatures after treatment over ten days, the metabolic activity of EBCP AUD 1 had similar trends as the untreated control (Figure 10 A-C). Significant differences ($P < 0.05$) in metabolic activity between untreated controls and EBCP on each day are indicated by an asterisk. On day 0, immediately after irradiation, the metabolic activity of EBCP was higher than the untreated controls ($P < 0.05$). The metabolic activity was most stable in samples stored at 4 °C, with the metabolic activity at day 0 similar to that of day 10 in both the untreated and treated samples. In comparison, in samples stored at room temperature (25 °C) or 37 °C, both untreated controls and EBCP AUD 1 were at or below the level of the heat/isopropyl alcohol control by day 10. These results suggest that while EBCP remain metabolic activity after treatment, unless stored at 4 °C, the metabolic activity will quickly decrease to non-detectable levels within ten days. Alternatively, because the metabolic activity of the untreated samples also decreased to below detectable limits when stored at room temperature and at 37 °C, the loss of metabolic activity in EBCP may not be due to eBeam treatment. For example, nutrients in the media may have been fully depleted causing cell death.

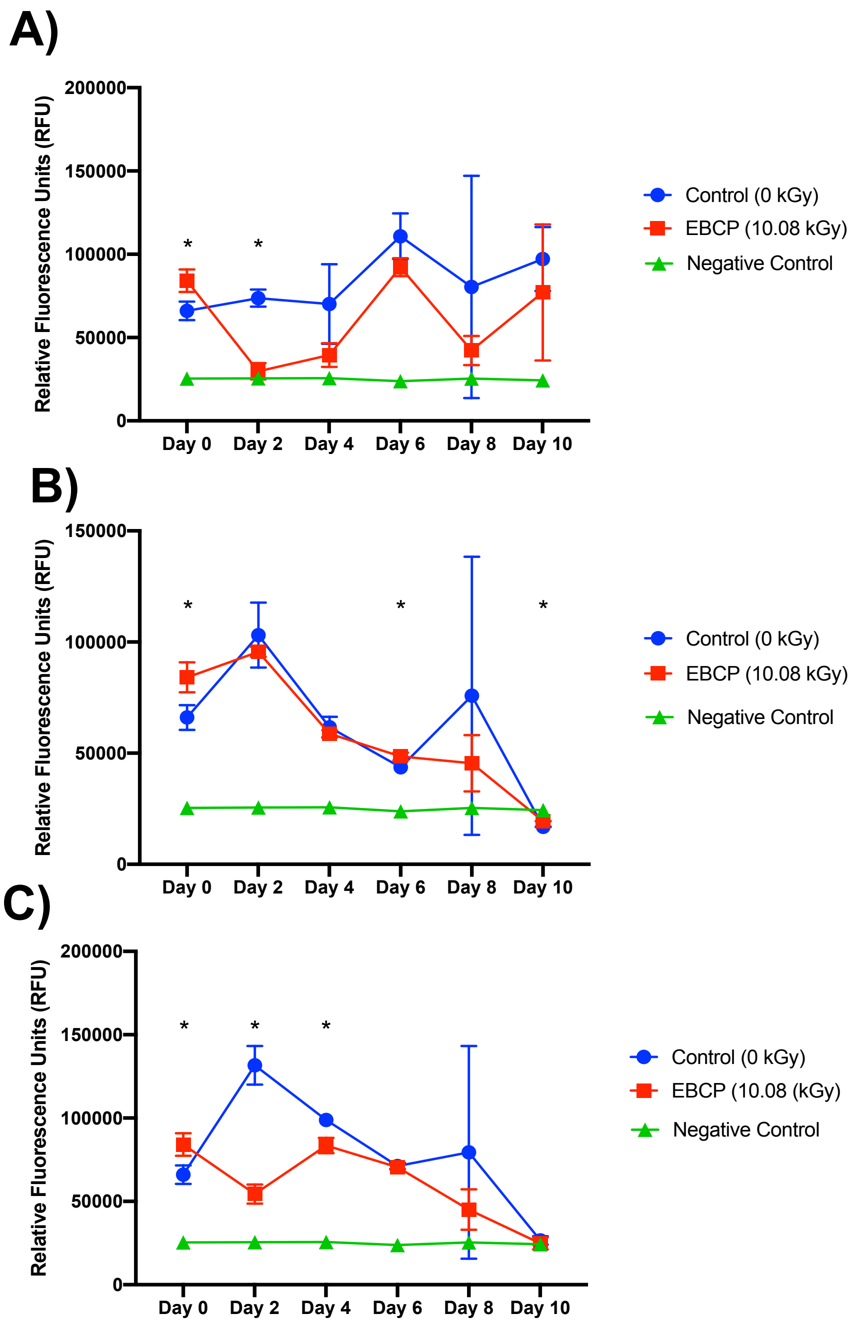


Figure 10. Metabolic activity of EBCP AUD1 was sustained for multiple days after eBeam treatment. A) EBCP stored at 4 °C B) EBCP stored at 25 °C C) EBCP stored at 37 °C. * indicate statistically significant differences ($P < 0.05$).

Electron beam treatment at 10 kGy inactivated *C. perfringens*, completely hindering its ability to replicate. Despite not being able to replicate, EBCP remained intact and metabolically active after 10 kGy eBeam treatment. Furthermore, metabolically activity was sustained for multiple days post treatment indicating that any metabolic activity seen immediately after treatment was not residual carryover. These results are similar to the results of metabolic activity studies conducted with *Escherichia coli* K-12 where the metabolic activity of *E. coli* was sustained for up to nine days following treatment, as demonstrated using alamarBlue™ as well as ATP assays (Hieke and Pillai, 2018). Studies have demonstrated that ionizing radiation does not significantly hinder cellular functions. Gamma irradiated cells maintained oxidative function and the ability for nucleic acid and protein syntheses (Hiramoto et al., 2002; Magnani et al., 2009). Furthermore, despite shredded nucleic acid, sustained metabolic activity is indicative that there are portions of genomes intact enough to sustain cellular functions (Trampuz, 2006; Magnani et al., 2009; Secanella-Fandos et al., 2014). Bacteria exposed to electron beam treatment have similar features. Studies examining the metabolomic state of inactivated *E. coli* and *Salmonella* Typhimurium have shown that immediately after treatment, cells are extremely metabolically active with metabolomic fluxes continuing even 24 hours after eBeam (Bhatia and Pillai, 2019).

Electron beam treatment at 10 kGy inactivated *C. perfringens. H*owever, despite being completely inactivated, cells were still intact and metabolically active after treatment. Furthermore, EBCP exhibited metabolic activity for at least ten days post-eBeam dosing when stored at 4 °C. Together, these experiments demonstrate that EBCP are in a MAyNC state. This state has potential to be used as an immunomodulator in poultry. Because the EBCP retain their membrane integrity, the presence of surface antigens coupled with metabolic activity was hypothesized to be able to elicit an immune response if administered to a bird. Furthermore, because they are inactivated and cannot replicate, it was hypothesized that the cells would not be able to cause disease in the bird.

4. METABOLOMIC ANALYSIS OF METABOLICALLY ACTIVE YET NON CULTURABLE *C. perfringens*

4.1. Overview

Clostridium perfringens is the causative agent of Necrotic Enteritis (NE) in poultry. This disease is of significant relevance to the poultry industry, and there is a need for preventive vaccines against this disease. Ionizing radiation is a processing tool that is used to inactivate microorganisms in multiple industries. High Energy Electron Beam (eBeam) irradiation, a form of ionizing radiation, causes significant damage to an organism's nucleic acid through primary ionization events as well as through damage from highly reactive products of water radiolysis. Although the microorganism is inactivated and non-culturable, it remains intact and metabolically active (MAyNC). *C. perfringens* inactivated by eBeam that are in this state have potential to be used as a vaccine candidate to protect poultry from NE. Thus, the overall goal of this study was to determine the metabolomic state of untreated *C. perfringens* strains grown in different anaerobic environments, and then understand the metabolomic state of eBeam-inactivated *C. perfringens* (EBCP) immediately after eBeam inactivation. Untargeted metabolomic analysis of six biological replicates of three *C. perfringens* strains demonstrated that there are very few differences between bacteria grown in an anaerobic chamber and bacteria grown in an anaerobic jar. Of the 928 primary metabolites that were detected overall, all metabolites detected at significantly different ($P < 0.05$) levels in bacteria grown in each environment were unidentifiable using publicly available databases. Untargeted metabolomics was also used to examine the metabolomic state of EBCP immediately after eBeam (10.08 kGy). Immediately after eBeam, *C. perfringens* AUD1 had a vastly different metabolomic profile when compared to untreated controls. Of 745 metabolites detected in these samples, ten metabolites (L-Methionine sulfoxide, D-Cysteine, Phenethylamine, Bis2-ethylhexyl phthalate, Acetylcholine, Adenosine, L-Histidine, Pilocarpine, 3-2-Hydroxyethylindole, and L-Valine) were detected at significantly different levels when compared to untreated controls ($P < 0.05$). These results suggest that the term Metabolically Active, yet Non-culturable is an appropriate descriptor of *C. perfringens* inactivated at 10 kGy eBeam.

4.2. Introduction

The enteric pathogen *Clostridium perfringens* is the causative agent of Necrotic Enteritis in poultry, a disease that is estimated to cause up to $6 billion in global economic losses annually (Wade and Keyburn, 2015). With an increase in outbreaks of this disease, the need for control measures, such as vaccines, is evident (Mot et al., 2014). Ionizing radiation, such as high energy Electron beam (eBeam) causes numerous single and double stranded breaks in the DNA of the organism, preventing DNA replication and thus inactivation of the organism.

Despite being inactivated with shredded nucleic, the cells remain intact and are metabolically active. This state is defined as metabolically active, yet non-culturable (MAyNC). Furthermore, eBeam-inactivated MAyNC cells not only exhibit defined gene expression patterns even 24 hours after inactivation, but maintain other functions such as the ability to propagate bacteriophages (Hieke, 2015; Hieke and Pillai, 2018; Bhatia and Pillai, 2019). Because this state induced by eBeam has properties of both a viable cell and an inactivated one, it is an attractive method for inactivating a culture for vaccine production (Gaidamakova et al., 2012). Because it is inactivated, a MAyNC bacteria possesses the safety aspects of an inactivated vaccine, but also because it is intact and metabolically active, it retains the immunogenicity of a live-attenuated vaccine (Kogut et al., 2012; Jesudhasan et al., 2015a).

There were two specific objectives in these experiments, namely, objective 1: Determine the effect of anaerobic growth environment on *C. perfringens* prior to eBeam treatment. The hypothesis of this study was that the metabolomic profile of *C. perfringens* grown in different anaerobic conditions would be distinct. It was important to explore whether the growth conditions would have an impact on *C. perfringens* that could be used in vaccine formulations, as the growth environment could impact the virulence of the organism. Furthermore, a metabolomics approach would provide valuable information on the state of the organism under control anaerobic conditions.

Objective 2: Examine the metabolomic state of eBeam-inactivated *C. perfringens.* The hypothesis of this experiment was that MAyNC *C. perfringens* would possess distinct metabolomic markers that distinguish them from untreated cells. Since an underlying hypothesis of this research was to explore the possibility of using MAyNC cells as immunomodulators, it was imperative that these cells were analyzed at the molecular level. While previous studies have observed consistent gene expression patters in MAyNC bacteria, the mechanism behind the metabolic state of MAyNC bacteria is still poorly understood. Thus, it was important to examine the metabolomic state of eBeam-inactivated *C. perfringens* that would be used in vaccine formulations. To investigate both of these objectives, untargeted metabolomics was used to examine the metabolomic state after growth in varying anaerobic environments or after eBeam exposure. Untargeted metabolomics was chosen as this would provide an insight to the metabolic pathways that are functional within these cells (Patti et al., 2012; Ribbenstedt et al., 2018).

4.3. Materials and Methods

4.3.1. Effect of growth environment on the metabolomic state of *C. perfringens*

4.3.1.1. Bacterial cultures

To investigate the effect of anaerobic environment on *C. perfringens,* strains JGS 1235, JGS 1473, and JGS 4104 were grown in two different anaerobic conditions. Strains were kindly provided by Dr. J. Glenn Songer from the University of Arizona. Aliquots of each culture were stored as glycerol stocks at -80 °C. One hundred microliters from a thawed -80 °C glycerol stock was transferred to 25 ml Fluid Thioglycolate Medium (FTG). Samples were then incubated in an anaerobic chamber or an anaerobic jar. The anaerobic chamber (COY, Grass Lake, MI) was purged and flushed with two gasses prior to use according to manufacturer instructions: compressed nitrogen gas was used as a background gas, and a gas mixture containing 5% H_2, 5% CO_2, and 90% N_2 was used as the circulating gas. BD EZ anaerobe GasPak^{TM} (Cat. # 260678, Becton Dickinson, Franklin Lakes, NJ) along with an anaerobic indicator strip (BD BBL GasPak^{TM} Becton Dickinson, Franklin Lakes, NJ) were used in anaerobic jars. Samples were incubated for 18 hours at 37 °C, centrifuged at 3,500 x g for 10 min and then resuspended in Phosphate Buffered Saline (PBS). One ml from each sample was used to determine the culture titer using serial dilutions in FTG and plating on blood agar (TSA with 5% sheep’s blood). Remaining samples in PBS were stored at -80 °C until being shipped to UC Davis on ice. Six biological replicates per sample were conducted for each strain.

4.3.1.2. Metabolite extraction, detection, and identification

Primary metabolites from 2 ml of all samples were extracted, detected, and analyzed using an untargeted metabolomics approach using gas chromatography-time-of-flight-mass spectrometry (GC-TOF MS) at the University of California Davis NIH West Coast Metabolomics Center. Six biological replicates were used for each sample and each biological replicate was run three times on the GC-MS as technical replicates. Amino acids, hydroxy acids, carbohydrates, sugar acids, sterols, aromatic compounds, nucleosides, amines, and other co-purifying compounds were extracted, detected, and analyzed using previously published methods (Fiehn et al., 2008, 2010). Briefly, extraction of samples was conducted using degassed acetonitrile:isopropanol (3:3:2, v/v/v). Samples were centrifuged and the solvent was allowed to evaporate until completely dry. The removal of membrane lipids and trigylcerides was facilitated by acetonitrile:water (1:1). Internal standards C08-C30 FAMEs were added and the sample was derivatized using methoxyamine hydrochloride in pyridine, followed by N-methyl-N-trimethylsilyltrifluoroacetamide to allow for the trimethylsilylation of acidic protons.

An Agilent 6890 gas chromatograph (Agilent, Santa Clara, CA) fitted with a Restek Rtx-5Sil MS column (30 m x 0. 25 mm length by internal diameter with 0.25 µm film composed of 95% dimethyl/5%diphenylpolysiloxane) and a 10 m integrated guard column (Restek, Bellefonte, PA) was used to separate compounds. A volume of 0.5 µl was injected into a mobile phase composed of pure helium (>99.9% purity) at a flow rate of 1 ml per minite using a Gerstel MPS2 automatic liner exchange system (Mülheim an der Ruhr, Germany). The temperature was gradually increased to 250 °C at a rate of 12 °C/s. The column was held constant at 50 °C for one minute, then increased to 330 °C at a rate of 20 °C per min, and held at 330 °C for five minutes. A Leco Pegasus IV mass spectrometer (St. Joseph, MI) at -70 eV ionization energy, 1800 V detector voltage with a 230 °C transfer line and 250 °C ion source was used at a unit mass resolution of 17 spectra/sec from 80-500 Da.

Raw data files were pre-processed immediately after data acquisition using ChromaTOF vs 2.32 software. Apex masses were matched using the BinBase metabolomics database. Identified metabolites were reported if present in at least 10% of samples. Data was reported as peak heights for the quantification ion (m/z value) at the specific RI (retention index). Peak heights were used as they are more accurate than peak areas at quantifying metabolites present at low concentrations. Raw data was transformed by dividing each raw metabolite value by the sum intensities of all known metabolites in that sample and multiplying the resulting value by a constant to obtain an integer. This transformation was done to disregard unknown samples that could contain artifacts or chemical contaminants (Fiehn et al., 2008).

4.3.1.3. Statistical analysis

Analysis of primary identified and unknown metabolites was performed using MetaboAnalyst 4.0, a web-based metabolomics processing tool (Xia and Wishart, 2016; Chong et al., 2018). Using the data filtering feature to filter out variables that are likely baseline noise, data was filtered by mean intensity to filter out very metabolites with very small values. Data was then normalized by log transformation (generalized log transformation) and pareto scaling (mean centered and divided by the square root of the standard deviation of each variable). Students t-tests were conducted for each metabolite and adjusted P-values (false discovery rate - FDR) was used to determine significant features. Each strain was analyzed individually and collectively. Partial Least Squares – Discriminant Analysis (PLS-DA) was used to analyze differences between treatment groups.

4.3.2. **Effect of eBeam treatment (10 kGy) on the metabolomic state of *C. perfringens***

4.3.2.1. Bacterial cultures

To investigate the effect of eBeam on the metabolomic profile of *C. perfringens,* overnight cultures of strain AUD1 were grown in Fluid Thioglycolate Medium, incubating overnight anaerobically at 37 °C in an anaerobic chamber. Five and a half ml of sample aliquots were packaged in sterile whirl-pak bags (Nasco, New York, NY) and heat sealed. Sample bags were packaged once again in a second sterile whirl-pak bag and then placed in 95 kPa specimen transport bags, as according to Texas A&M biosafety regulations on transporting organisms. After eBeam treatment, 1 ml aliquots of untreated (0 kGy) and treated (10 kGy) samples were serially diluted in FTG and plated on blood agar to determine starting titers and to confirm inactivation, respectively. Remaining samples were stored at -80 °C until metabolite extraction. Six biological replicates were used in each treatment group

4.3.2.2. Electron beam treatment

Electron beam treatment was conducted at the National Center for Electron Beam Research at Texas A&M University in College Station, TX, using a 10 MeV, 18 kW Electron Beam Linear Accelerator (LINAC). Alanine (L-α-alanine) dosimetry was used to measure the absorbed eBeam dose and read using a Bruker e-scan spectrometer. Samples were treated at a target dose of 10 kGy and immediately stored at -80 °C until metabolite extraction.

4.3.2.3. Metabolite extraction, detection, and identification

Metabolite extraction, detection, and identification was conducted at the Integrated Metabolomics Analysis Core at Texas A&M University. Metabolites were extracted from the cultures using multiple chloroform and methanol extractions. Briefly, 4 ml of each culture was pelleted by centrifugation at 10,000 x g for 10 minutes, and resuspended in 800 µl ice cold chloroform:methanol (1:1, v:v). The entire volume was then transferred to a bead-based lysis tube (Precyllys, Bertin, Rockville, MD) and homogenized for 30 seconds (Precyllys, Bertin, Rockville, MD). After homogenization, samples were immediately placed on ice for 5 min and then centrifuged at 15,000 x g for 5 min at 4 °C to pellet the homogenizer beads. The supernatant was transferred to a fresh tube, while the pellet was subjected to second round of extraction with 1:1 chloroform:methanol following the steps described above. Supernatants from the two extractions were pooled for each sample and 600 µl of sterile ice-cold water was added. Samples were vortexed to thoroughly mix and centrifuged at 4,000 x g for 10 minutes to pellet any remaining beads and cell debris and to separate the phases. The upper aqueous phase was filtered using a 0.2 µm nylon filter (Merck Millipore, Burlington, MA). Five hundred µl of the filtered aqueous phase was then passed through a

3kDa cutoff column (ThermoFisher Scientific, Waltham, MA). Extracted metabolites were stored at -80 °C until analysis.

Untargeted liquid chromatography high resolution accurate mass spectrometry (LC-HRAM) analysis was performed using a Q Exactive Plus orbitrap mass spectrometer (MS) (Thermo Scientific, Waltham, MA) coupled to a binary pump high performance liquid chromatography (HPLC) instrument (UltiMate 3000, Thermo Scientific). Samples were maintained at 4 °C before injection and 10 μL was used as an injection volume. The sheath, auxiliary, and sweep gasses were set at 50, 15 and 1, respectively, for acquisition. The S-lens radio frequency (RF) was set to 50 and the spray voltage was set to 3.5 kV (Pos) or 2.8 kV (Neg). The source and capillary temperatures were maintained at 350 °C and 350 °C respectively. Full MS spectra were obtained with a scan range of 50-750 m/z and at a resolution of 70,000 resolution at 200 m/z. Full MS scans followed by ddMS2 scans were obtained at a resolution of 35,000 (MS1) and 17,500 (MS2) with a 1.5 m/z isolation window and stepped NCE (20, 40, 60). A Synergi Fusion 4μm, 150 mm x 2 mm reverse phase column (Phenomenex, Torrance, CA) maintained at 30 °C using a solvent gradient method was used to achieve chromatographic separation. Solvent A was water (0.1% formic acid). Solvent B was methanol (0.1% formic acid). The gradient was as follows: From 0-5 min the gradient was 10% B to 40% B, from 5-7 min the gradient went from 40% B to 95% B, from 7-9 min the gradient was 95% B, from 9.0 to 9.1 min the gradient decreased from 95%

B to 10% B), and from 9.1-13 min (10% B). The flow rate was 0.4 mL per mn. Sample acquisition was performed Xcalibur (Thermo Scientific) and data analysis was performed with Compound Discoverer version 2.1 (Thermo Scientific). Detected metabolites were matched to publicly available web-based databases such as mzCloud (mzcloud.org) and ChemSpider (chemspider.com) (Pence and Williams, 2010; Little et al., 2012).

4.3.2.4. Statistical analysis

Statistical analysis was conducted of metabolites was performed using MetaboAnalyst 4.0, a web-based metabolomics platform (Xia and Wishart, 2016; Chong et al., 2018). The data filtering feature was used to filter out variables by mean intensity to filter out metabolites with very small values that are likely background noise. Data was then normalized by logarithmic transformation and pareto scaling. T-tests were conducted for each metabolite and the FDR was used to determine significant metabolites. PLS-DA was used to analyze differences between treatment groups.

4.4. Results and Discussion

4.4.1. Metabolomic analysis of *C. perfringens* grown in different anaerobic environments

The effect of growth environment on three strains of *C. perfringens* was examined using untargeted primary metabolomics. Primary metabolites were extracted from cultures that were grown overnight in two different anaerobic growth environments and analyzed using GC-TOF-MS (Appendix A, Appendix B, and Appendix C). The starting titer for each strain in each environment is shown in Table 10. During GC-TOF-MS analysis, there was a sample injection failure in one replicate of JGS 1473 grown in the anaerobic chamber and therefore this sample replicate was excluded from analysis. From the 17 samples analyzed, 928 primary metabolites were detected, although 760 metabolites (81.9%) were unidentifiable using publicly available databases such as PubChem and KEGG. Each strain was analyzed individually to determine the effect of the anaerobic environments on metabolomic profile.

Table 10. Titer of *C. perfringens* strains for growth environment metabolomics

	Starting titer (Log_{10}CFU/ml)	
	Anaerobic Chamber	**Anaerobic Jar**
JGS 1235	7.68 ± 0.10	7.47 ± 0.24
JGS 1473	7.94 ± 0.05	7.85 ± 0.08
JGS 4104	7.95 ± 0.07	7.73 ± 0.02

In *C. perfringens* JGS 1235 there were no significant differences in any metabolite concentration between the treatment groups when 0.05 was used as the cutoff for FDR values. When significance was decreased to 0.1, five unknown metabolites had significant differences in concentration: Unknowns 5290, 111255, 223203, 88847, and 223208 (Figure 11). PLS-DA analysis showed that while there the biological replicates were clustered together, there was still overlap between the two treatment groups (Figure 12). For *C. perfringens* JGS 1473, all differences in metabolite concentration were statistically insignificant ($P > 0.1$). When all metabolites were considered in PLS-DA analysis, the two treatment groups were distinct from each other (Figure 13). For *C. perfringens* JGS 4104, the concentration of three metabolites were statistically different between treatment groups ($P < 0.05$) (Figure 14). When all metabolites were analyzed using PLS-DA, the two anaerobic treatment groups were distinct from each other (Figure 15). These data suggest that while the metabolomic profile of *C. perfringens* strains grown in different anaerobic environments may be distinct, there are very few if any metabolites produced at significantly different concentrations. When all strains are grouped together, the difference between samples grown in an anaerobic chamber and samples grown in an anaerobic jar decreases further with significant overlap in samples (Figure 16). Furthermore, because all metabolites that were statistically significant were unknown compounds, it is difficult to determine if these are biologically significant metabolites.

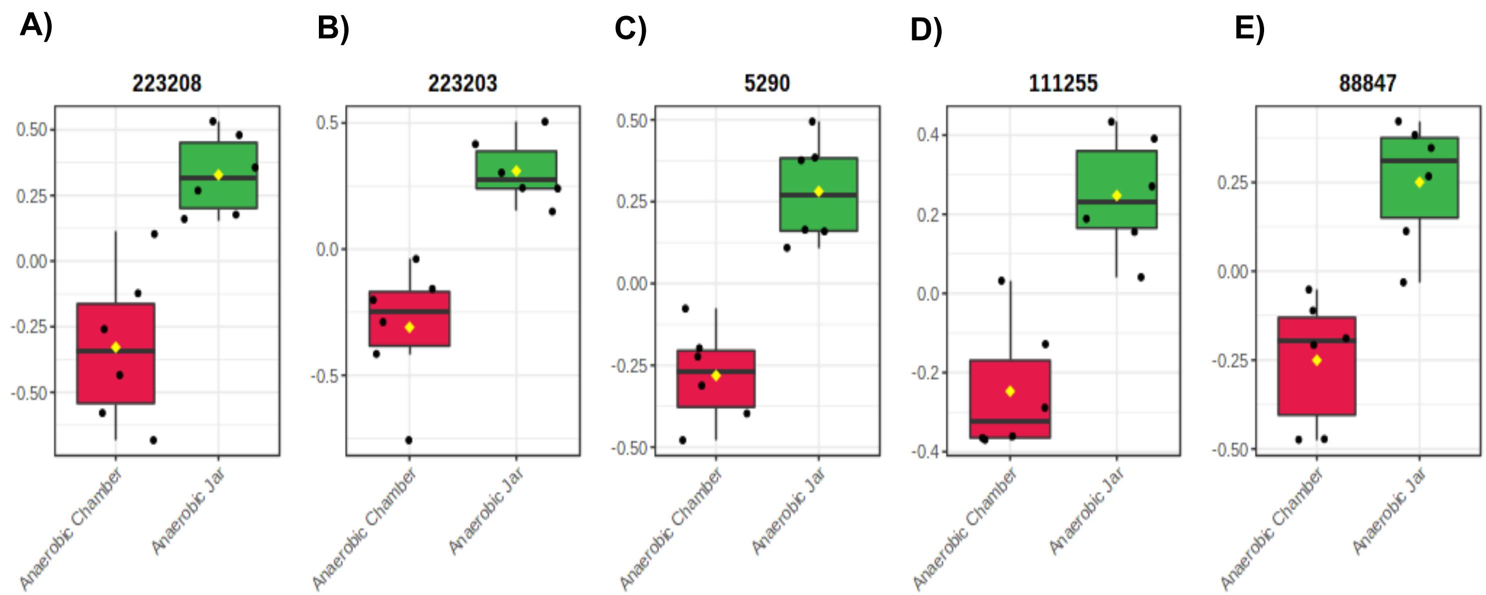


Figure 11. Five unidentifiable metabolites produced by *C. perfringens* JGS 1235 were produced in significantly different concentrations ($P < 0.1$) when the bacterium was grown in an anaerobic chamber or an anaerobic jar. A) Unidentified 223208 B) Unidentified 22303 C) Unidentified 5290 D) Unidentified 111255 E) Unidentified 88847.

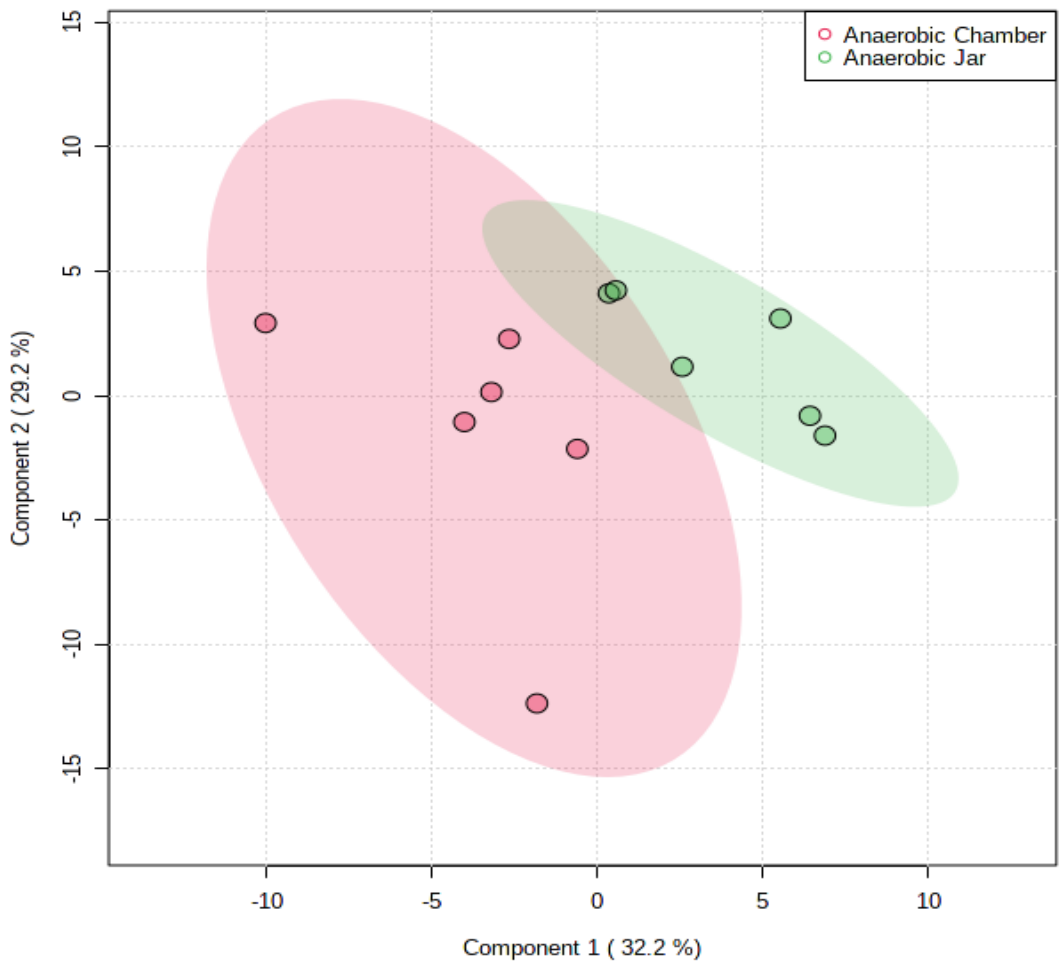


Figure 12. PLS-DA of the metabolome of *C. perfringens* JGS 1235 grown in an anaerobic chamber or anaerobic jar suggests slight differences. The variance explained by each component is shown on each axis in parenthesis.

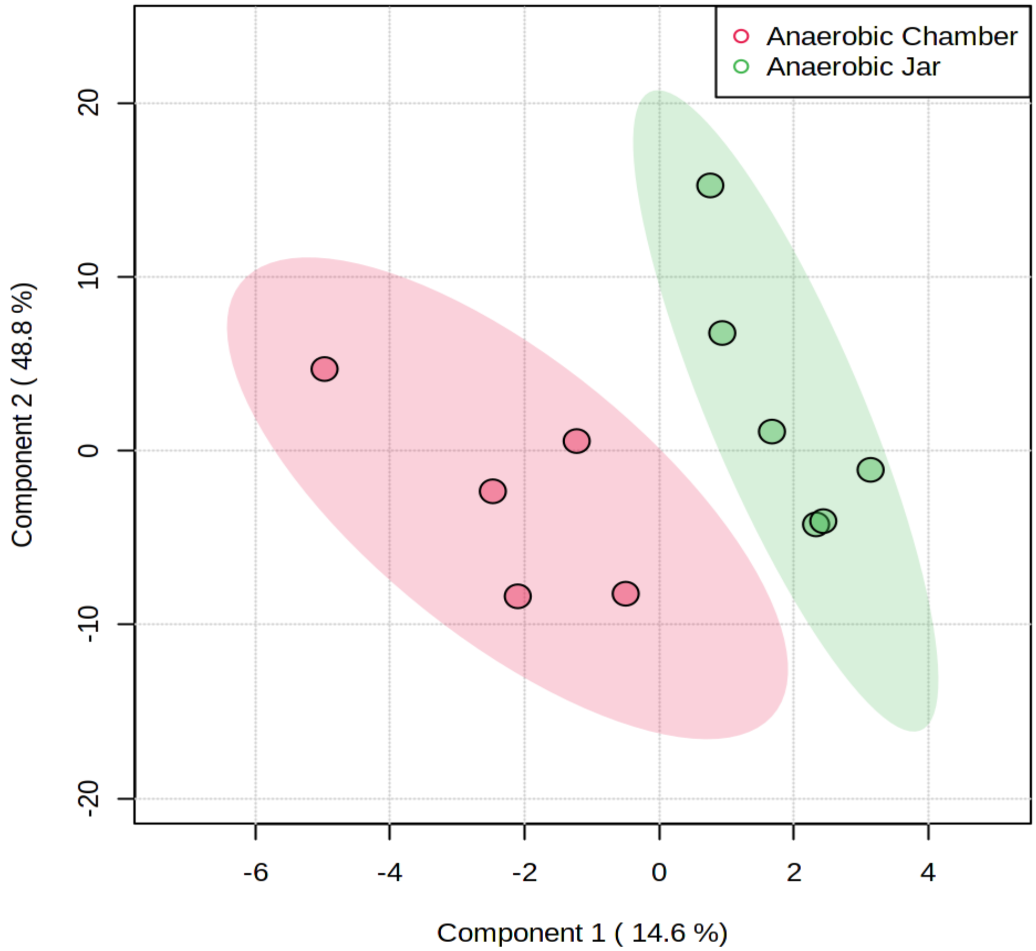


Figure 13. PLS-DA of the metabolome of *C. perfringens* JGS 1473 grown in an anaerobic chamber or anaerobic jar suggests slight differences. The variance explained by each component is shown on each axis in parenthesis.

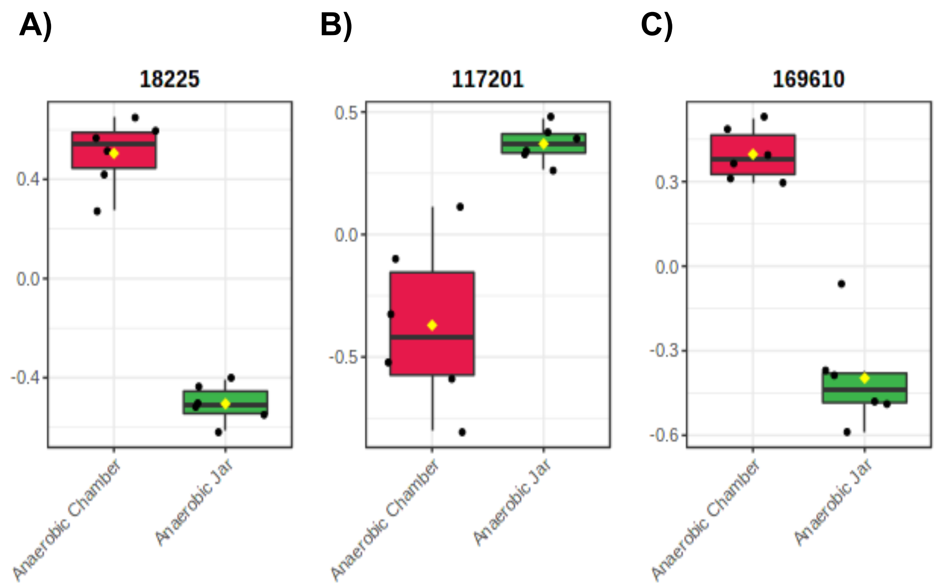


Figure 14. Three unidentifiable metabolites were produced at significantly different concentrations ($P < 0.05$) when *C. perfringens* JGS 4104 was grown in an anaerobic chamber or anaerobic jar. A) Unidentified 18225 B) Unidentified 117201 C) Unidentified 169610.

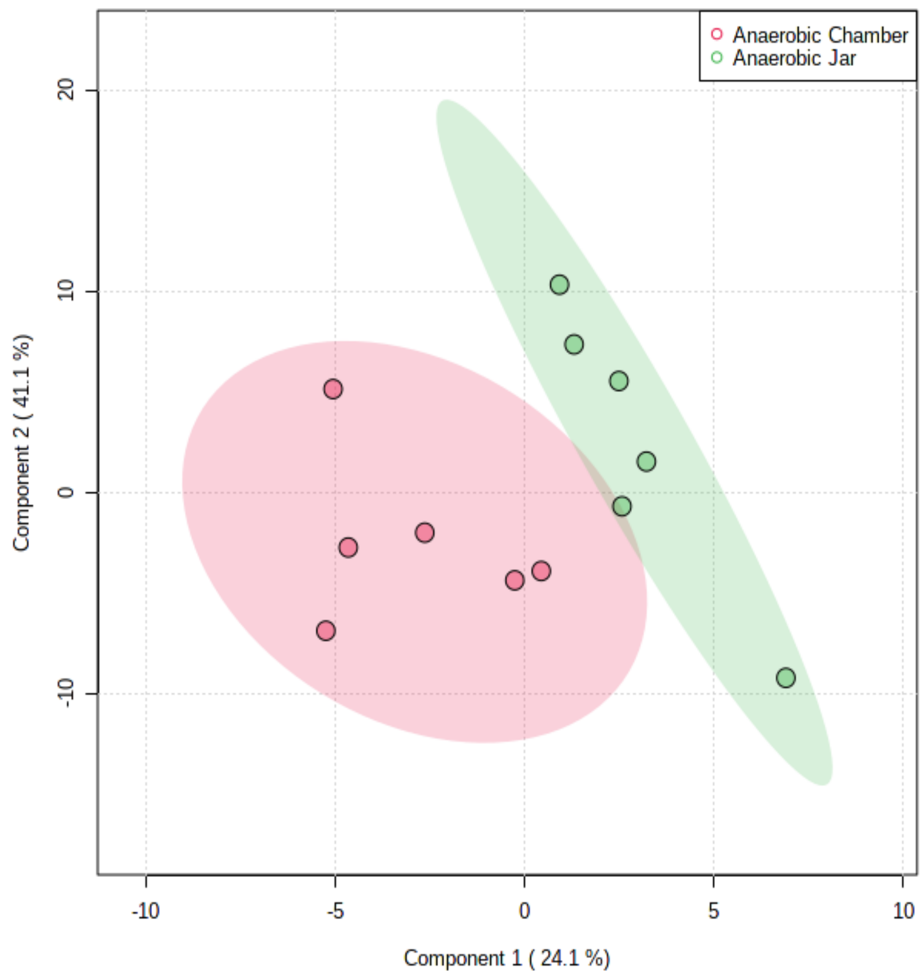


Figure 15. PLS-DA of the metabolome of *C. perfringens* JGS 4104 when grown in an anaerobic chamber or anaerobic jar suggests slight differencese. The variance explained by each component is shown on each axis in parenthesis.

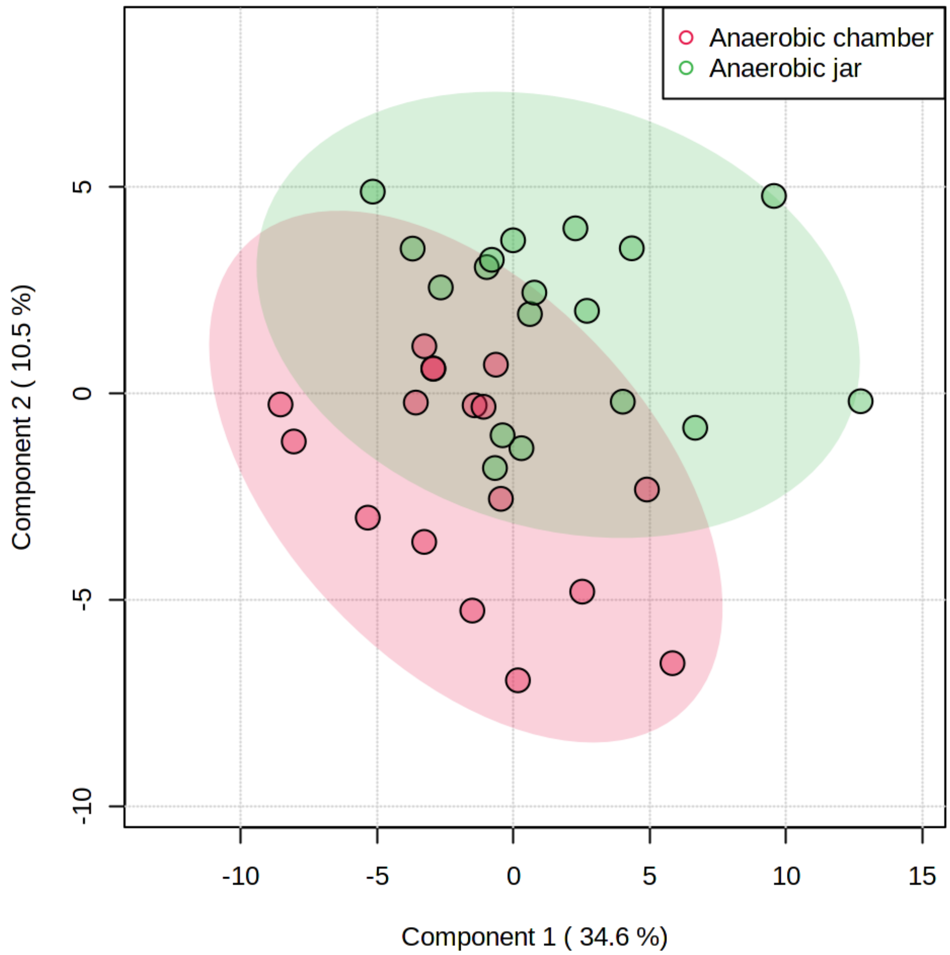


Figure 16. PLS-DA of the metabolome of all *C. perfringens* strains (JGS 1235, JGS 1473, and JGS 4104) grown in an anaerobic chamber or anaerobic jar. The variance explained by each component is shown on each axis in parenthesis.

The effect of two anaerobic environments were studied in this study. GasPaks[TM] are ready-to-use gas-generating sachets that contain inorganic carbonate, activated carbon, ascorbic acid, and water. When exposed to air, the sachets are activated and rapidly reduces the oxygen concentration and increases the concentration of carbon dioxide within the sealed container. The packets generate an environment that is <1% oxygen within 2.5 hours and ≥13% carbon dioxide within 24 hr. The advantage of the anaerobic sachets are that they are a low-cost per unit, mobile, easy-to-use anaerobic system that does not require specialized equipment. In an anaerobic chamber, a hydrogen gas mix reacts with a palladium catalyst to remove excess oxygen. The gas mix containing H_2 gas circulates through the palladium catalyst and removes O_2 by forming a water molecule. Oxygen levels are maintained below 5 ppm.

In an anaerobic chamber, cells are in an anaerobic environment from the very start of incubation, while in the anaerobic jar oxygen levels are only decreased to less than 1% after 2.5 hours of incubation. Thus, the largest difference in the growth conditions corresponded with the lag and early exponential growth phases of the bacteria (Sottile and Zabransky, 1977; Meyer and Tholozan, 1999). In lag phase, cells transform their transcriptome and proteome to upregulate the production of cellular components required for biomass accumulation and cell division. Furthermore, cells must repair and replace damaged subcellular components (Rolfe et al., 2012; Bertrand, 2019). Cells start adapting to their new growth environment extremely rapidly and even transient differences in environment could have downstream impacts. For example, in studies conducted with *Salmonella* Typhimurium, 1,119 genes were differentially expressed within four minutes of liquid inoculation (Rolfe et al., 2012).

Lag phase is initiated when cells encounter new nutrients, but interestingly, studies have demonstrated that cells in lag phase primarily utilized stored glycogen rather than intracellular glucose or extracellular carbon sources (Yamamotoya et al., 2012). Yamamotoya et al., hypothesized that this allows the cell time for the translation of proteins needed for nutrient transport. While it is clear that lag phase is a formative time for a bacteria, it is less clear whether minute differences in the environment during lag phase can influence the metabolomic state of the culture in stationary phase. In this study, because *C. perfringens* was inoculated into FTG, a broth with reducing agents present, it is difficult to ascertain whether differences in the atmospheric anaerobic environment would even effect the bacteria in the medium. The results of this experiment suggest that the different anaerobic environments do not cause any significant changes in the metabolomic profile of *C. perfringens.* It is important to note that because of the large number of metabolites discovered during untargeted metabolomics, the statistical power of the individual t-tests done for each metabolite was greatly reduced due to correcting for the false discovery rate. This could be mitigated by conducting targeted analysis on only compounds of interest.

4.4.2. The metabolomic response of *C. perfringens* exposed to eBeam

Untargeted metabolomics was used to examine the effect of eBeam treatment (10.08 kGy) on *C. perfringens* AUD 1 (3.18×10^8 CFU/ml). A total of 745 metabolites were identified among the 12 samples as full matches to the publically available databases ChemSpider and mzCloud. Due to mzCloud full matches being of higher fidelity, only the 81 metabolites that were full matches to compounds in the mzCloud database were used for analysis (Appendix D). Ten (12.3%) of these metabolites were found in significantly different ($P < 0.05$) concentrations after eBeam treatment (Table 11). The concentration of five metabolites were significantly increased ($P < 0.05$) after eBeam treatment and the concentration of five metabolites were significantly decreased ($P < 0.05$) after eBeam inactivation and (Figure 17 and Figure 18). The overall metabolomic profile of untreated control *C. perfringens* and eBeam-treated *C. perfringens* (EBCP) were very different, as demonstrated during PLS-DA analysis (Figure 19). Furthermore, there was not a significant amount of variation between the biological replicates within a treatment group. Figure 20 visualizes the clustering of samples even further.

Table 11. Ten metabolites produced by *C. perfringens* AUD 1 were expressed at significantly different concentrations after eBeam inactivation (10.08 kGy)

Metabolite	**FDR (P-value)**	**Effect of eBeam**
L-Methionine sulfoxide	3.86E-05	Increase
D-Cysteine	0.0001	Decrease
Phenethylamine	0.002	Increase
Bis2-ethylhexyl phthalate	0.003	Increase
Acetylcholine	0.008	Decrease
Adenosine	0.008	Increase
L-Histidine	0.009	Decrease
Pilocarpine	0.034	Decrease
3-2-Hydroxyethylindole	0.033	Decrease
L-Valine	0.042	Increase

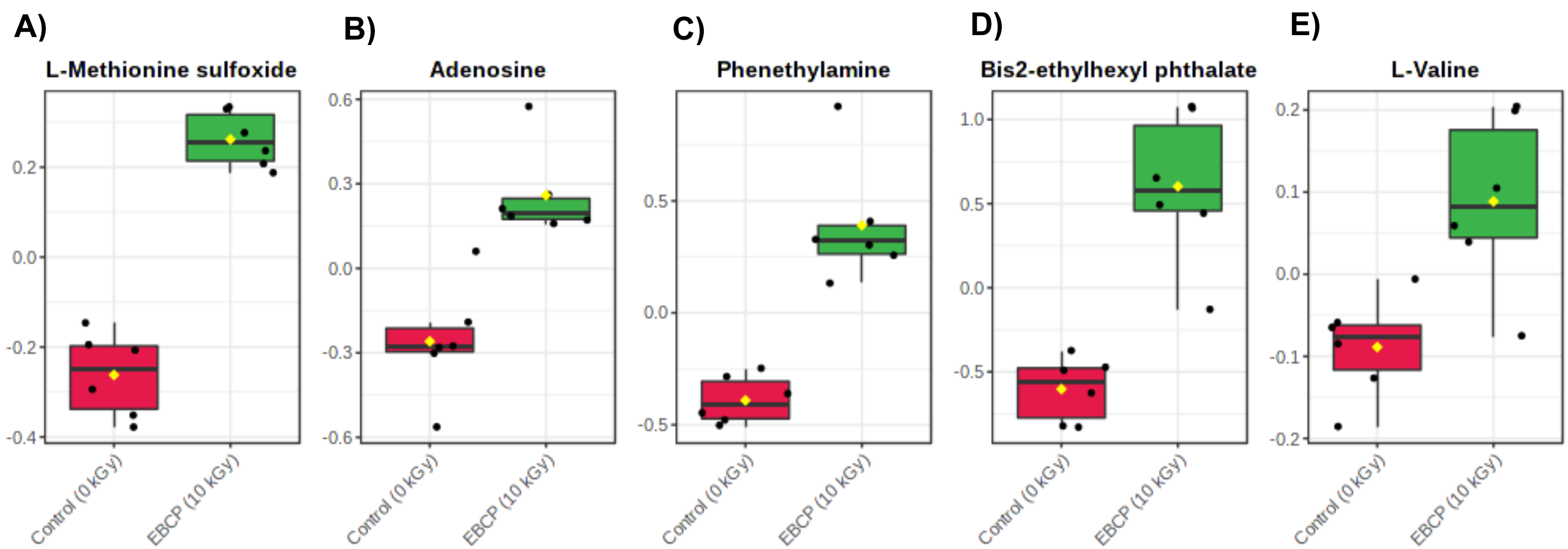


Figure 17. Five metabolites produced by *C. perfringens* AUD1 were expressed at significantly increased levels (P < 0.05) after eBeam inactivation (10.08 kGy). A) L-methionine B) Adenosine C) Phenethylamine D) Bis2-ethylhexyl phthalate E) L-Valine.

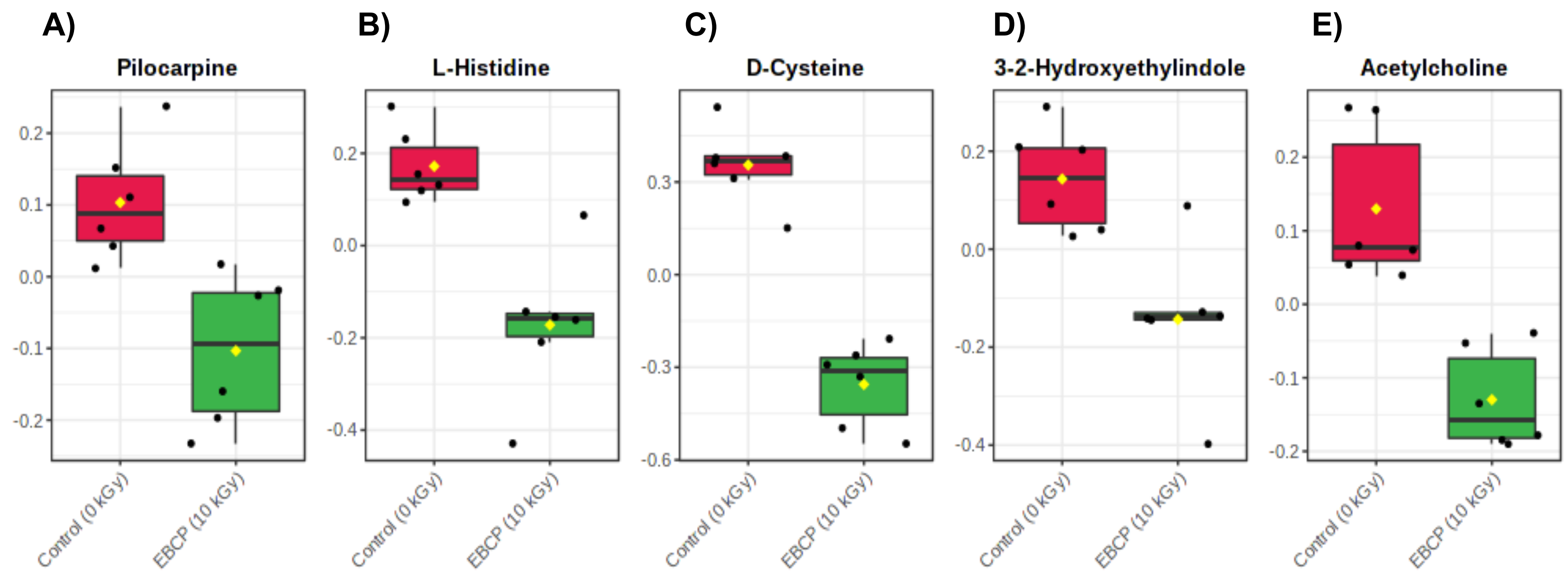


Figure 18. Five metabolites produced by *C. perfringens* AUD1 were expressed at significantly decreased levels (P < 0.05) after eBeam inactivation (10.08 kGy). A) Pilocarpine B) L-Histidine C) D-Cysteine D) 3-2-Hydroxyethylindole E) Acetylcholine.

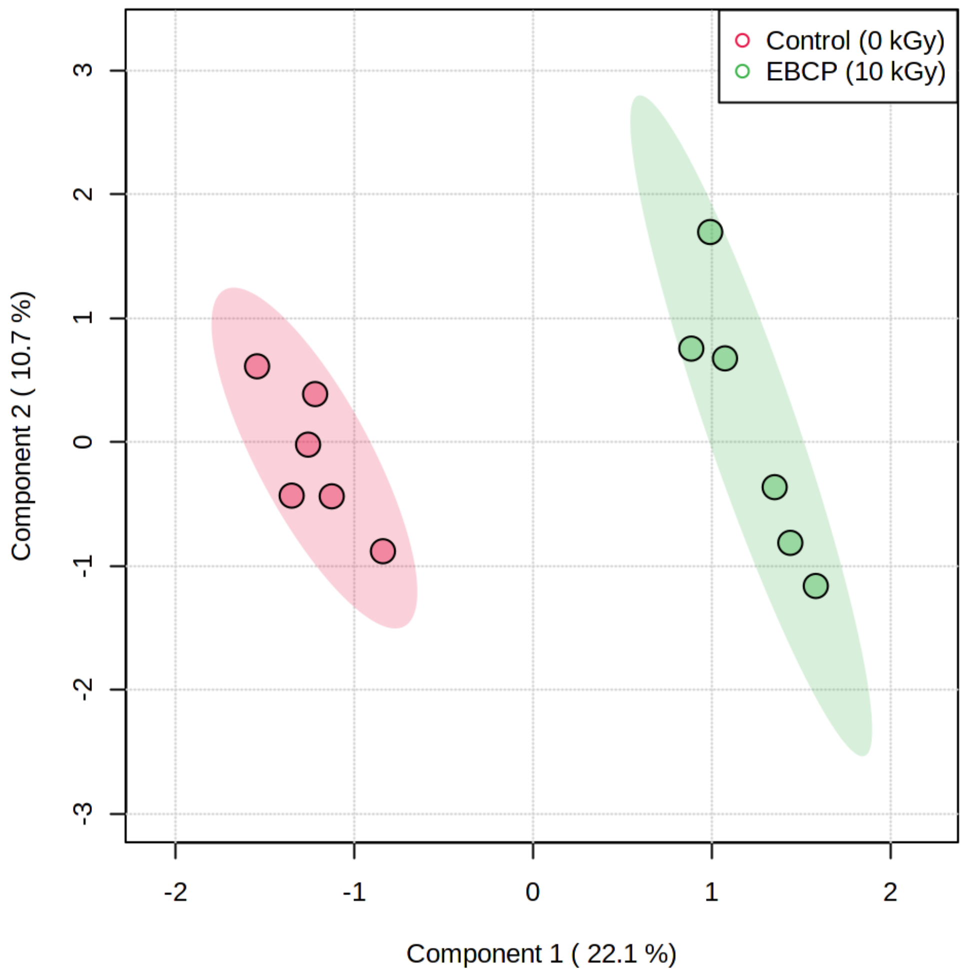


Figure 19. PLS-DA of the metabolome of *C. perfringens* AUD 1 demonstrates that metabolomic profile is distinct after eBeam inactivation (10.08 kGy). The variance explained by each component is shown on each axis in parenthesis.

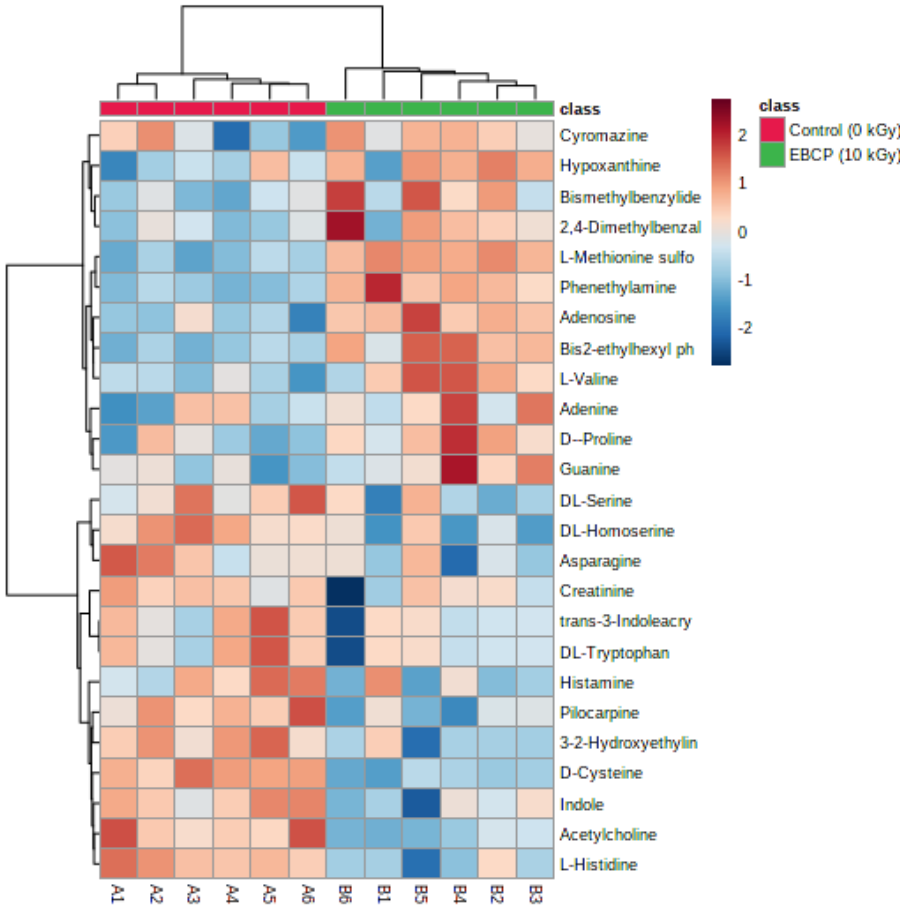


Figure 20. Metabolic shifts were evident in *C. perfringens* AUD 1 after exposure to 10.08 kGy eBeam dose. Relative differences between the 25 most significant metabolites (by P value) detected at statistically different concentrations are shown.

Untargeted metabolomics analysis is a useful tool as it provides a snapshot of the metabolism of an organism (Patti et al., 2012). This experiment clearly shows that even immediately after eBeam treatment, the metabolomic profile of EBCP is drastically different than untreated *C. perfringens*. Despite inactivation and the inability to replicate, EBCP retain their metabolic activity and have a different metabolomic profile, even immediately after eBeam. It is difficult to differentiate between metabolites that are detected at higher concentrations because they are breakdown products of molecules damaged by eBeam, and those that are produced as a metabolic response to eBeam damage. Regardless, an increase in specific metabolites after eBeam has biological significance. For example, methionine and cysteine are the most susceptible amino acids to damage caused by reactive oxygen species (ROS) due to their sulfur residues (Lavine, 1947; Lee and Gladyshev, 2011). It is therefore not surprising to see a significant decrease in cysteine after eBeam treatment (Figure 18C). Furthermore, a significant increase in L-methionine sulfoxide after eBeam treatment (both an oxidative and reductive process) is also expected, as it is the primary product of the oxidation of methionine (Figure 17A) (Lavine, 1947; Stadtman, 1993). It is hypothesized that methionine sulfoxide acts as an environmental sensing molecule, as the oxidation of exposed methionine at the surface of a bacteria stimulates the up-regulation of genes (*msrA and msrB)* involved in reversing damage caused by ROS (Bigelow and Squier, 2011; Lourenço dos Santos et al., 2018). In bacteria such as *Streptococcus pneumonia, Neisseria gonorrhoeae,* and *Eschericheia coli*, the expression of *msrA* (methionine sulfoxide reductase A) is also responsible for the production of adhesins that contribute to bacterial adhesion in a host (Wizemann et al., 1996). While little is known about the function of this gene in anaerobic bacteria, in C*lostridium oremlandii*, the *msrA* gene is likely associated with protein repair and antioxidant defense against oxygen exposure (Kim et al., 2009; Lee et al., 2015).

Adenosine, which was also found in higher concentrations after eBeam (Figure 17B), is often used by bacteria as a danger signaling molecule in microorganisms, and has demonstrated ability to stimulate germination of *Bacillus* (Dodatko et al., 2010; Drygiannakis et al., 2011; Skaldin et al., 2018). It may also be used to evade the host immune system by inhibiting secretion of IL-12, TNF-α, and neutrophil degranulation *in vivo* (Firestein et al., 1995; Bouma et al., 1997). The up-regulation of adenosine after eBeam could potentially inhibit its recognition by the host immune system, which could potentially limit its use as an immunomodulator. Increased production of Bis 2-ethylhexyl phthalate (Figure 17D), a compound with significant antimicrobial activity is interesting, although little is known about the production of this compound by bacteria (Ortiz and Sansinenea, 2018; Lotfy et al., 2019). Accumulation of phenethylamine (Figure 17C), has been identified as a stress response against acid stress in lactic acid bacteria and has also been identified as a metabolite of *C. perfringens* (Pinho et al., 2004; Barbieri et al., 2019). An increase in L-valine after eBeam treatment (Figure 17D) is interesting as L-valine is able to induce germination of spores of *C. perfringens*, but only those that carry a chromosomal enterotoxin gene, such as is the case in most NE-causing strains (Paredes-Sabja et al., 2008; Freedman et al., 2016).

There are few studies examining the metabolomic response to ionizing radiation, although the differentially expressed metabolites produced by *C. perfringens* AUD 1 are similar to those seen in previous studies (Bhatia and Pillai, 2019). Bhatia and Pillai also demonstrated that cysteine was significantly decreased after eBeam treatment (3 kGy) in *E. coli*, but after 24 hours, levels had increased slightly, indicating that the bacteria remains metabolically active and is able to replenish its stores. Unlike *C. perfringens* where methionine sulfoxide was significantly increased after eBeam, in both *E. coli* and *Salmonella,* methionine sulfoxide was decreased immediately after eBeam, with levels increasing slightly after 24 hours at room temperature, although the changes in concentration were not significant. Furthermore, unlike *C. perfringens* where L-valine was significantly increased after eBeam, in *Salmonella,* L-valine was significantly decreased immediately after eBeam-treatment (2 kGy), but 24 hours after treatment, L-valine was significantly higher than even that found in untreated (0 kGy) controls. Differences in metabolomic responses to eBeam are likely due to difference in inactivation dose, as well as the vast differences in metabolism between the anaerobic Gram-positive *C. perfringens* and the aerobic Gram-negative *E. coli* and *S.* Typhimurium. A limitation of the present study is that it only examines the state of *C. perfringens* immediately after eBeam. Expanding the scope of this study to

24-48 hours after eBeam would be beneficial to fully understand how the bacteria continues to respond to eBeam.

In conclusion, untargeted metabolomic analysis was used to examine the effect of anaerobic environment and eBeam treatment on *C. perfringens*. There were very few differences found between *C. perfringens* strains grown in an anaerobic jar and strains grown in an anaerobic chamber. *C. perfringens* that were treated with 10 kGy eBeam were inactivated and unable to replicate, but maintained their metabolic activity, with distinct metabolomic profiles seen in untreated and treated cells.

5. DEVELOPMENT OF A ROBUST NECROTIC ENTERITIS CHALLENGE MODEL

5.1. Overview

Necrotic enteritis (NE) is a disease of poultry that is of extreme relevance to the poultry industry. The causative agent of this disease is the Gram positive, anaerobic bacteria, *Clostridium perfringens*. Vaccination has been proposed as a preventative control mechanism for this disease. Specifically, High Energy Electron Beam (eBeam) inactivation has been proposed as a method to inactivate *C.* perfringens for vaccine use. Using this inactivation method, microorganisms are unable to replicate, but remain intact and in a Metabolically Active, yet Non-culturable (MAyNC) state. In order to conduct research on preventative measures against NE, such as a MAyNC vaccine candidate, inducing the disease under experimental conditions is crucial. There were two primary objectives of this study. The first objective was to establish a NE challenge model that could induce clinical NE in experimental conditions, while the second objective was to verify eBeam-inactivated (EBCP) was inactivated and would not cause disease in vivo, even in an environment that would encourage NE. A NE challenge model was established that combines numerous pre-disposing factors to induce disease. Birds were reared on used litter from a local poultry farm, fed a 55% wheat diet, administered two commercial live vaccines as immunosuppressants, and challenged with a high titer of *C. perfringens* for three consecutive days. Furthermore, feed was withdrawn for 12 hours prior to the first challenge. Lesion scores in birds challenged with all of the predisposing factors were significantly higher ($P < 0.0001$) than the lesion scores of unchallenged birds. Furthermore, when birds were challenged with EBCP in the NE challenge model, lesion scores were not significantly different than unchallenged controls, demonstrating that EBCP did not cause disease *in vivo*. The outcome of this study is that a NE challenge model was established that could be used in further studies to determine the protective nature of EBCP.

5.2. Introduction

In the poultry industry, Necrotic enteritis (NE) is a disease of concern caused by the Gram-positive, anaerobic, endospore-forming bacterium *Clostridium perfringens*. The pathogenesis of NE is complex and multi-factorial. While many toxins and virulent enzymes produced by *C. perfringens* play a role in disease, there are typically many predisposing factors that make a bird more susceptible to disease (Moore, 2016; Prescott et al., 2016a). While previously controlled by antimicrobials administered in the feed and water, concerns of antibiotic resistance has led to regulatory pressure to phase-out their use in many major poultry-producing regions of the world (Maron et al., 2013; McEwen et al., 2017). This has in turn led to an increase in the prevalence of NE and an increase in research into preventative control mechanisms (Wierup, 2001; Immerseel et al., 2004; Mot et al., 2014). In order to investigate the efficacy of interventions such as vaccines, it is necessary to induce NE under controlled experimental conditions. Despite significant research into understanding the many causes of NE in chickens, reproducibly inducing disease remains extremely difficult (Kaldhusdal et al., 1999; Shojadoost et al., 2012; Bortoluzzi et al., 2019). This may be because it is not possible to mimic all of the conditions of commercial production under experimental conditions.

Ionizing radiation, such as Electron Beam (eBeam) technology, can be used to inactivate microorganisms by causing extensive damage to nucleic acid (Holley and Chatterjee, 1996; Miller, 2005; Daly, 2012; Mavragani et al., 2019). Despite being unable to replicate, these cells remain intact and metabolically active, and can thus potentially be used as immunomodulators (Seo, 2015). Electron beam has been previously used to develop vaccine candidates against *Salmonella* spp. and *Eimeria* spp. for the poultry industry (Kogut et al., 2012; McReynolds et al., 2012; Jesudhasan et al., 2015b; Thabet et al., 2019). In a similar fashion, metabolically active yet non-culturable (MAyNC) *C. perfringens* could be used to induce a protective immune response in poultry. Prior to determining protective abilities, it was crucial to confirm that EBCP was inactivated and would not cause disease *in vivo.* The first objective of this study was to establish a robust NE challenge model that could induce NE in experimental conditions. The second objective was to verify EBCP was inactivated and would not cause disease *in vivo*, even in an environment that would encourage NE. The hypotheses of this study was that a combination of predisposing factors would contribute to a NE challenge model, and even when administered in this model, EBCP would not induce disease.

5.3. Materials and Methods

5.3.1. Bacterial cultures

The *Clostridium perfringens* strains JGS 1473, JGS 4101, JGS 4064, JGS 1235, JGS 1521, AUD1, CP 1, CP 2, CP 3, and CP 4 were used in this study as previously described. Aliquots of each culture were stored as glycerol stocks at -80 °C. Overnight cultures were prepared by transferring a single loop from a freezer stock to growth medium (Fluid Thioglycolate Medium (FTG) and incubating overnight anaerobically at 37 °C in a COY anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Strains were combined to create two cocktails of strains. Cocktail A consisted of *C. perfringens* CP 1, CP 2, CP 3, and CP 4 while Cocktail B was composed of *C. perfringens* strains JGS 1473, JGS 4101, JGS 4064, JGS 1235, JGS 1521, and AUD1. To comply with all Texas A&M University biosafety regulations, only triple-sealed samples were allowed to be treated at the eBeam facility. All biological samples were double packaged in heat sealed Whirl-

Pak bags (Nasco, New York, NY) and double-packagd samples were then placed in third “specimen transport” bag that was rated up to 95 kPa.

5.3.2. Electron beam treatment and dosimetry

Electron beam treatment was conducted at the National Center for Electron Beam Research at Texas A&M University in College Station, TX. Samples were treated with a 10 MeV, 18 kW Electron Beam Linear Accelerator (LINAC) and alanine dosimeters were used to measure the absorbed eBeam dose. Dosimeters were read using a Bruker e-scan spectrometer (Bruker, Billerica, MA). All dosimeters used were calibrated to traceable standards. Preliminary dose mapping studies were conducted to ensure uniform deposition throughout the sample bag.

5.3.3. Experimental birds

Ross 708 male broiler chicks were obtained from a commercial hatchery on day-of-hatch, weighed, banded with wing bands, and placed on used pine litter. Temperature was maintained according to breeder recommendations and additional heat was provided using heat lamps when necessary. Pens were approximately 6 ft x 8 ft. Birds were allowed water and feed ad libitum. The diet met or exceeded National Research Council guidelines for broiler chicks.

5.3.4. Experimental challenge model

A combination of methods were used to induce NE in broiler chicks. Birds were fed a 55% wheat-based broiler started diet, as outlined in Table 12. Birds were raised on used litter from a local poultry farm. Litter was plated to determine the concentration of total aerobic bacteria, total anaerobic bacteria, *Salmonella*, and *C. perfringens*. Briefly, 10 g litter was stomached with 10 ml FTG, diluted in FTG, and plated. Culture conditions are outlined in Table 13. A commercial Infections Bursal Disease vaccine (BursaVac, Merck Animal Health) and a commercial Coccidiosis vaccine (CocciVac B-52, Merck Animal Health) were used as predisposing factors in this disease model. Birds were also challenged for three consecutive days with live *C. perfringens*.

Table 12. Wheat diet formulation used in NE challenge model

Ingredient	**Percent**
Trace Minerals Premix - Poultry	0.05
TAMU Corn	8.41
TAMU Soybean	29.03
TAMU Wheat (hard)	55.00
TAMU Soybeaen oil	2.76
TAMU Vitamin Premix	0.25
Limestone	1.60
Mono-dicalcium phosphate	1.65
Salt, Plain (NaCl)	0.28
L-Lysine HCl	0.43
DL-Methionine	0.37
L-Threonine	0.18

Table 13. Culture conditions for determining bioburden of used litter

	Agar Media	Incubation Conditions		
Total Aerobic bacteria	Trypticase Soy Agar (TSA)	37 °C	Aerobic	24 hrs
Salmonella	Xylose Lysine Deoxycholate agar (XLD)	37 °C	Aerobic	48 hrs
Total Anaerobic bacteria	Blood Agar	37 °C	Anaerobic	24 hrs
C. perfringens	Shahidi Ferguson Perfringens Agar (SFP) with overlay (Harmon et al., 1971; Shahidi and Ferguson, 1971)	37 °C	Anaerobic	24 hrs

5.3.5. Experimental design

Day-of-hatch broiler chicks were assigned to one of eight treatment groups as outlined in Table 14, with each experiment group consisting of 25 birds. On day 9, groups 2 and 4-8 were vaccinated with a commercial IBD vaccine (BursaVac, Merck Animal Health) at the manufacturer's recommended dose via the ocular administration route. On day 11, a 10X dose of a commercial coccidiosis vaccine (CocciVac, Merck Animal Health) was administered via oral gavage to groups 3-8. On Day 16-18, birds were challenged with sterile FTG (groups 1-4) or ~10^7 CFU/ml Live *C. perfringens* (groups 5 and 6) via oral gavage. Groups 7 and 8 were challenged with ~10^7 CFU EBCP via oral gavage on day 16 only. Feed was withdrawn from birds for 12 hours prior to the initial challenge on day 16. Due to high mortality in group 5, the study was terminated early on Day 18. Birds were weighed on Day 0, 16, and immediately prior to termination.

Table 14. Verification of inactivation *in vivo* experimental design

	Designation	**Commercial Vaccination**	**Challenge**	**# of Birds**
Timeline		**Day 9 and 11**	**Days 16**	
Group 1	Negative Control	-	None (FTG)	25
Group 2	IBD Control	IBD vaccine only	None (FTG)	25
Group 3	CV Control	Coccidia vaccine only	None (FTG)	25
Group 4	IBD + CV Control	IBD + Coccidia vaccine	None (FTG)	25
Group 5	Live A	IBD + Coccidia vaccine	Cocktail A (Live)	25
Group 6	Live B	IBD + Coccidia vaccine	Cocktail B (Live)	25
Group 7	EBCP A	IBD + Coccidia vaccine	Cocktail A (EBCP-A)	25
Group 8	EBCP B	IBD + Coccidia vaccine	Cocktail B (EBCP - B)	25
Total				**200**

5.3.6. EBCP preparation

C. perfringens cultures were cultured individually in FTG and eBeam treated at a target dose of 10 kGy. After treatment, untreated controls were plated on blood agar to determine the starting titer of each culture. Treated samples were also plated to ensure lethal inactivation. Cultures were aseptically combined such that the final cocktails contained approximately equal concentrations of each strain. EBCP cocktails were stored at 4 °C until use.

5.3.7. Live *C. perfringens* challenge

Fresh, live *C. perfringens* cultures were prepared for each day of challenge. To avoid errors associated with unequal cell titers during challenge, equal volumes of culture were passaged one day prior to challenge and previous cultures were inoculated into fresh FTG to prepare the fresh culture for each challenge. First, overnight cultures of each *C. perfringens* strains were grown individually in FTG, as previously described. Four hundred µl of this culture was then inoculated in 40 ml of fresh FTG and incubated anaerobically for 24 hours at 37 °C. After incubation, 400 µl of each overnight culture was passaged into another 40 ml of fresh FTG and incubated anaerobically for 24 hours at 37 °C, while the rest of the culture was combined in equal volumes to create the challenge cocktails. Cultures were passaged again on the second day of challenge from the previous day’s cultures. This was done in order to have approximately equal concentrations of each culture in the challenge cocktails. The concentration of the combined cocktails was serially diluted in FTG and plated on blood agar to determine the titer of each challenge.

5.3.8. Necrotic enteritis lesion scores

Upon termination, birds were necropsied, and the small intestine was examined with a focus on the jejunum and ileum. The presence of gross lesions in the jejunum at Meckel's diverticulum were scored on a scale of 0-4 (Table 15) (Prescott, 1979; McReynolds et al., 2004; Swaggerty et al., 2016). To eliminate bias, one person scored all birds for lesions.

Table 15. Gross lesion score criteria

Lesion Score	Criteria
0	No gross lesions, healthy intestinal appearance
1	Thin-walled or friable, grey appearance
2	Thin-walled, gray appearance, small amounts of gas production, focal necrosis
3	Thin-walled, sizable patches of necrosis, gas-filled intestine, small flecks of blood
4	Severe extensive necrosis, marked hemorrhage, large amounts of gas in intestine

5.4. Results and Discussion

The objectives of this study were to establish a robust Necrotic Enteritis (NE) challenge model and to verify that two EBCP cocktails were inactivated and did not cause disease *in vivo*, even when used in a Necrotic Enteritis challenge model. Cocktail A consisted of *C. perfringens* CP 1, CP 2, CP 3, and CP 4 while Cocktail B was composed of *C. perfringens* strains JGS 1473, JGS 4101, JGS

4064, JGS 1235, JGS 1521, and AUD1. Two immunosuppressants were administered to encourage disease: a live commercial IBD vaccine and a 10X dose of a live commercial Coccidia vaccine. Birds were also housed on spent litter and fed a diet high in wheat. The starting bioburden of the litter is presented in Table 16.

Table 16. Bioburden of used litter

Organism	**Concentration (Log_{10}CFU/g) ± Standard Deviation**
Total anaerobic bacteria	7.04 ± 0.09
Total aerobic bacteria	6.60 ± 0.05
Salmonella spp.	2.01 ± 0.05
C. perfringens	2.90 ± 0.01

On day 16, birds were challenged with 3 ml of either sterile FTG, EBCP A or B (9.6 kGy), or live *C. perfringens* (Table 17). As part of the challenge model, groups 5 and 6 were challenge with live *C. perfringens* for three consecutive days. Due to high mortality in the group challenged with live cocktail A, the study was terminated early on Day 18 and birds were not challenged a third time.

Table 17. EBCP and live *C. perfringens* challenge titers

	Challenge Titer (Log_{10}CFU/ml) ± Standard Deviation	
	Day 16	**Day 17**
EBCP Cocktail A	7.47 ± 0.12	-
EBCP Cocktail B	7.33 ± 0.08	-
Live Cocktail A	8.29 ± 0.03	8.04 ± 0.06
Live Cocktail B	7.93 ± 0.03	7.94 ± 0.08

C. perfringens cocktail A produced a strong challenge with birds in this group having significantly higher lesion scores than the negative control groups (Table 18). The average lesion score of birds challenged with EBCP A were not significantly different than the negative control groups ($P > 0.05$) and were significantly lower ($P<0.0001$) than groups challenged with live cocktail A, indicating that EBCP A did not cause disease *in vivo*, even when challenged in a NE challenge model that pre-disposes birds to disease (Figure 21A). Furthermore, live cocktail A induced NE, suggesting that it could be used in a NE challenge model. Cocktail B did not cause any significant increase in lesion score when compared to controls and subsequently would not be a strong candidate for inclusion in a NE challenge model. While the live cocktail B did not produce a strong challenge, EBCP B also did not cause any increase in lesion scores, indicating that EBCP B did not cause disease *in vivo* (Figure 21B).

Table 18. EBCP did not cause gross lesions or mortality *in vivo*

Lesion Scores	Negative Control	IBD Control	CV Control	IBD + CV Control	Live A	Live B	EBCP A	EBCP B
0	21	20	24	22	8	20	23	24
1	4	5	1	2	10	5	2	1
2	-	-	-	-	4	-	-	-
3	-	-	-	-	2	-	-	-
4	-	-	-	-	-	-	-	-
Average Lesion Score	**0.16**	**0.2**	**0.04**	**0.08**	**1**	**0.2**	**0.08**	**0.04**
Mortality post-challenge	**0**	**0**	**0**	**1**	**7**	**0**	**0**	**0**

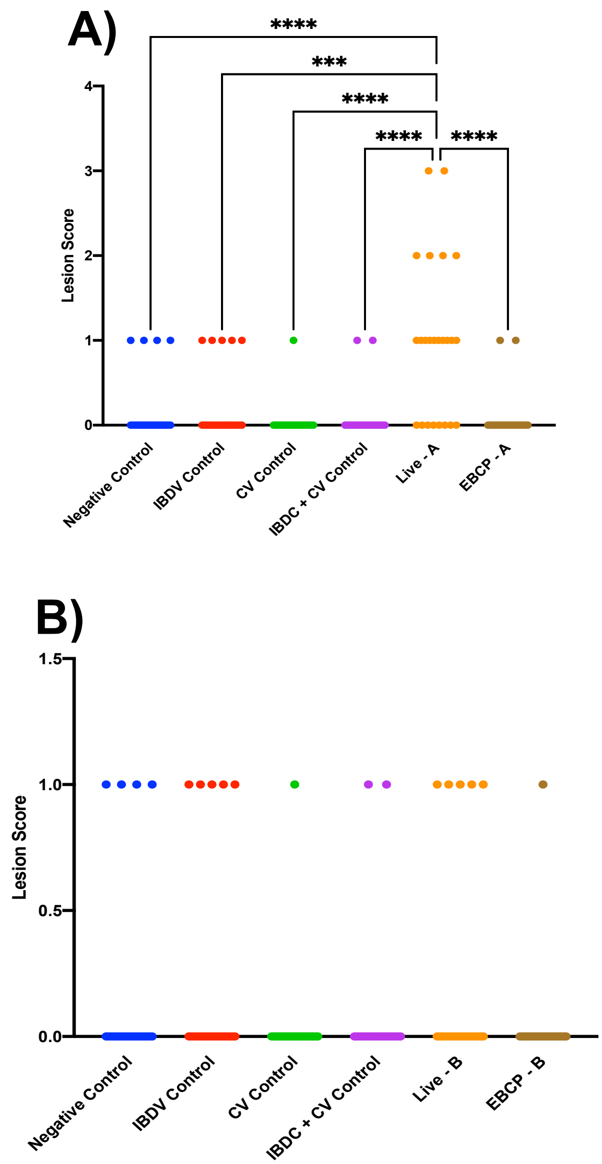


Figure 21. Challenge with EBCP cocktail A or B did not cause increased lesion scores. A) Birds were challenged with live or EBCP cocktail A B) Birds were challenged with live or EBCP cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Pen weights were recorded on the day-of-hatch, day 16 pre-challenge, and on day 18 prior to termination (Table 19). Because bird weights were taken as pen weights rather than individual bird weights, statistical analysis could not be conducted on this data set. The feed conversion ratio (FCR) on day 18 was calculated as the total feed intake/bird (kg) divided by the average bird weight on day 18. Bird weights and FCR varied between the groups throughout the study. At the time of termination (day 18), the negative control group had the lowest FCR and the group challenged with live cocktail A had the highest FCR.

Table 19. Average bird weights and FCR after EBCP challenge

	Avg. weight/bird (kg)			**Total Feed Consumption (kg)**	**Avg. Feed intake/bird (kg)**	**Day 18 FCR**
	Day 0	**Day 16**	**Day 18**			
Negative Control	0.043	0.475	0.595	20.574	0.823	1.384
IBD Control	0.041	0.443	0.503	20.484	0.819	1.630
CV Control	0.042	0.480	0.547	22.242	0.890	1.627
IBD + CV Control	0.041	0.495	0.567	19.940	0.831	1.466
Live A	0.042	0.439	0.554	23.988	0.960	1.732
Live B	0.044	0.451	0.519	19.357	0.774	1.492
EBCP A	0.043	0.499	0.571	20.077	0.803	1.407
EBCP B	0.042	0.455	0.527	20.780	0.831	1.578

5.4.1. Multiple predisposing factors were used to induce NE

The primary objective of this study was to establish a robust NE challenge model. Despite being one of the most economically important and devastating diseases for the poultry industry, reproduction of NE in experimental conditions continues to challenge researchers (Shojadoost et al., 2012). In preliminary studies, different challenge models were used to induce NE (data not shown). In one study, *C. perfringens* alone was used to induce NE. Birds were raised on fresh pine shavings, fed a normal corn/soy starter diet, and challenged only once on day 7 with live cocktail B. This did not result in any lesions or clinical signs of NE, with no detectable difference between negative and positive control groups. In a second study, birds were again raised on fresh pine shavings and fed a normal corn/soy starter diet, but also administered a live infectious bursal disease vaccine on day 11, and then challenged with live *Clostridium perfringens* on days 16-19. Once again, no difference in lesion scores or mortality was seen between the negative and positive control groups. Due to complexity of the pathogenesis of NE, multiple factors must be considered when establishing a challenge model. Ultimately, disruptions to the gut lead to increased susceptibility to NE. In the present study, used litter, a high wheat diet, feed withdrawal, two immunosuppressants, and a high *C. perfringens* titer were used to cause gut dysbiosis and induce NE.

5.4.2. High bioburden pine shavings as a predisposing factor for NE

Used pine shaving litter from a local poultry farm were used rather than fresh pine shavings in order better simulate the rearing conditions used in commercial broiler production. While the microbial makeup of litter plays a significant factor in the early colonization of the intestinal microbiota, it may also reduce the impact of enteric disease (Kers et al., 2018). Bortoluzzi and colleagues demonstrated that birds challenged with *C. perfringens* and raised on reused litter had the largest variations in intestinal microbiota diversity and composition upon challenge with *C. perfringens* (Bortoluzzi et al., 2019). Decreased alpha-diversity in the gut microbiome has been linked with poorer overall health broilers, which may explain an increased susceptibility to NE when raised on used litter (Chen et al., 2015; Kers et al., 2018). Interestingly, the role of reused litter in NE does not appear to be dependent on the concentration of *C. perfringens* in the litter with previous studies having demonstrated that *C. perfringens* actually decreases in abundance in reused litter (Wei et al., 2013; Wang et al., 2016). Rather, birds raised on fresh litter have an increased colonization of beneficial *Lactobacillus* spp., which may contribute to increased overall gut health (Cressman et al., 2010).

5.4.3. Dietary factors as predisposing factor for NE

A broiler starter diet was composed for this study such that it was comprised of 55% wheat (Table 12). High levels of wheat in the diet can cause changes in the viscosity in the gut, which can increase digesta transit time. Furthermore, cereal grains such as wheat, rye, and barley are high in non-starch polysaccharides (NSPs) which serve as an excellent carbohydrate source for *C. perfringens* leading to its proliferation. Specifically, beta-glucans and arabinoxylans increase the viscosity of the digesta and predispose chickens to NE (Kaldhusdal and Hofshagen, 1992; Dahiya et al., 2006). NSPs also interact with glycoproteins on the intestinal epithelial surface, leading to increased mucin production. This is noteworthy because *C. perfringens* produces a wide range of glycoside hydrolases that are directed at muco-oligosaccharides prominent in the intestinal mucosal layers (Ficko-Blean et al., 2012). Annett et al. demonstrated in *in vitro* experiments that *C. perfringens* incubated in digested wheat and barley diets proliferated significantly more than when incubated in corn-based diets (Annett et al., 2002). Several studies have presented similar findings in bird challenge studies (Branton et al., 1987; Riddell and Kong, 1992; Craven, 2000; Wu et al., 2010). A 55% wheat diet has previously been used in a challenge models with success (McReynolds et al., 2004, 2007; Swaggerty et al., 2016; Sharma et al., 2017).

Feed was withdrawn from chicks 12 hours prior to the first challenge. This is a less common method in NE challenge models, although it has been linked to high incidences of NE in both experimental and commercial settings (Prescott, 1979; Brennan et al., 2001; Redondo et al., 2016; Van Limbergen et al., 2020). Studies have demonstrated that even temporary feed withdrawals can cause changes in the gut microbiota, decreasing bacterial diversity in the ileum (Thompson et al., 2008). Interestingly, the opposite was demonstrated in a latter study, with feed restrictions at the time *C. perfringens* challenge leading to lowered lesion scores in challenged birds (Tsiouris et al., 2014).

5.4.4. Co-infection with *Eimeria* spp. was used as a predisposing factor

One of the most important pre-disposing factors for NE is the presence of coccidial infection caused by *Eimeria* species. In the present study, two commercial vaccines were used to introduce a mild coccidia challenge. First, a live infectious bursal disease virus (IBDV) vaccine was administered on day 9 in order to immunosuppress the birds prior to challenge on day 11 with a high dose (10x) dose of a live commercial Coccidia vaccine. Specifically, the vaccine used is composed of 5 strains of *Eimeria* oocysts: *E. acervulina, E. maxima, E. maxima MFP, E. mivati, and E. tenella.* This model has been employed previously using the commercial vaccines individually or together (McReynolds et al., 2004, 2007, 2009; Stringfellow et al., 2009; Swaggerty et al., 2016). Physical damage caused during coccidial infection disrupts the gut in multiple ways. During their endogenous stage, *Eimeria* sporozoites invade epithelial cells to reproduce, causing physical damage to the intestinal epithelium, compromising gut integrity and allowing the basal layer to be exposed (Prescott et al., 2016a, 2016b; Redondo et al., 2016). Areas of physical damage have been linked to the early stages of NE (Olkowski et al., 2006, 2008). Furthermore, physical damage may also encourage the adhesion of *C. perfringens* in the intestine, cause the leakage of plasma into the gut, and encourage mucus production (Immerseel et al., 2004; Collier et al., 2008; Forder et al., 2012; Wade et al., 2015). Altogether, coccidiosis primes the intestine for *C. perfringens*, providing an environment rich in nutrients and adhesion sites.

5.4.5. High titer *C. perfringens* was used to induce NE

The only difference in the two NE challenge models examined in this study were the *C. perfringens* strains used on days 16 and 17. Despite both cocktails including strains positive for alpha toxin and netB toxin, cocktail B failed to induce any signs of NE as measured by lesion scores and body weight. This difference highlights the critical role of *C. perfringens* in NE. Despite all of the predisposing factors working via varying mechanisms, the disease is ultimately caused by *C. perfringens* and the toxins it produces. This is further confounded by the natural presence of *C. perfringens* in healthy birds (Borda-Molina et al., 2018; Shang et al., 2018). The pathogenesis of *C. perfringens* in poultry is unclear, with seemingly conflicting data both supporting and refuting the critical role of most toxins produced by the bacteria (Timbermont et al., 2011; Prescott et al., 2016a; Rood et al., 2016). Despite being composed of strains with extremely similar toxin-profiles (Chapter III, Figure 9), the two cocktails presented in this study acted extremely differently *in vivo*, with one markedly inducing NE and one not. This suggests that there may be other factor(s) that are impacting pathogenesis whether it is yet unidentified or simply not explored in this study.

5.4.6. EBCP does not cause disease *in vivo*, even when administered in a NE challenge model

The second objective of this study was to verify that even when used in a NE challenge model that pre-disposes birds to NE, EBCP would not cause disease within the bird. Indicated by the lack of lesions and mortality, inactivated EBCP (9.6 kGy) did not cause disease *in vivo*, proving that MAyNC *C. perfringens* is indeed inactivated and does not resuscitate or regain virulence once inside the bird. This safety aspect demonstrates the potential for EBCP to be used as a vaccine-like immunomodulator in poultry. Despite being intact and metabolically active, birds did not exhibit any symptoms of disease such as increased lesion scores, decreased bird weight, or increased feed conversion ratios. These results are supported by previously published data that demonstrate that bacteria inactivated by IR do not cause disease *in vivo* (Magnani et al., 2009; Kogut et al., 2012; McReynolds et al., 2012; Praveen, 2014; Jesudhasan et al., 2015a)

In conclusion, these studies suggest that a combination of a high-wheat diet, used litter, immunosuppressants, feed withdrawal, and specific *C. perfringens* strains can induce a clinical outbreak of NE that causes mortality. This model can be used in future studies to determine the efficacy of interventions, such as vaccines. Furthermore, *C. perfringens* treated with 10 kGy of high energy eBeam are inactivated and do not cause disease in broiler chicks even in a setting that would encourage NE. The significance of these results are that eBeam is a method that can reproducibly inactivate *C. perfringens* allowing them to be evaluated as an immunomodulatory control method to prevent NE in broiler chickens.

6. INVESTIGATION INTO THE ABILITY OF METABOLICALLY ACTIVE YET NON-CULTURABLE *C. perfringens* TO PROTECT AGAINST NECROTIC ENTERITIS CHALLENGE

6.1. Overview

In order for a vaccine to be considered effective, it must be able to illicit a protective immune response in the host. In the poultry industry, Necrotic Enteritis (NE) caused by *Clostridium perfringens* is disease of extreme concern. Vaccination has been proposed as a preventative control mechanism for this disease. Electron Beam (eBeam) technology is one method for inactivating *C. perfringens* to induce a state that may be used for to generate a vaccine-like immunomodulator that is protective against NE in poultry. Two studies were conducted examining the protective nature of two cocktails of eBeam-inactivated *C. perfringens* (EBCP) in a NE challenge model. Furthermore, the addition of synthetic unmethylated phosphothiorated oligodeoxynucleotides with CpG motifs (CpG ODN) was used as an adjuvant. In both studies, upon challenge, EBCP alone was not protective against challenge, as measured by lesion scores, *C. perfringens* colonization, body weights gain, and feed conversion ratio (FCR). The adjuvanted EBCP vaccine was not only not-protective upon challenge, but caused increased *C. perfringens* colonization and increased FCRs ($P < 0.05$) when compared to unvaccinated birds. The results of these suggest that an alternative adjuvant may be required in order for an EBCP-based vaccine to be protective against NE.

6.2. Introduction

In the poultry industry, Necrotic Enteritis (NE), is a disease of extreme economic concern, causing an estimated $6 billion in economic losses annually (Wade and Keyburn, 2015). Because of its economic significance, there has been a significant amount of research into vaccines that protect chickens from NE (Lee et al., 2011; Mot et al., 2014). In order for a vaccine to be considered successful, it must be able to confer protection to a host upon challenge. Many of the published vaccines against NE for broiler chickens have included regimens that include multiple doses (prime/boost) vaccinations, *in ovo* vaccination, or the vaccination of laying hens (Lovland et al., 2004; Crouch et al., 2010; Keyburn et al., 2013*a*; Mot et al., 2014; Lillehoj et al., 2017; Mishra and Smyth, 2017). From an industrial standpoint, *in ovo* vaccination, laying hen vaccination, or day-of-hatch vaccination is preferable due to economic reasons and feasibility. From an immunological perspective, these vaccinations all have drawbacks because the embryonic immune system is not fully developed and does not fully develop for many weeks after hatch, and maternal antibodies diminish between 2-3 weeks of age (Triplett et al.; Lee et al., 2011; Allaart et al., 2012; Mot et al., 2013). Vaccination with live avirulent strains, has conferred only partial protection (Thompson et al., 2006; Kulkarni et al., 2008). Furthermore, there is the fear of attenuated *C. perfringens* regaining virulence, as virulence is primarily controlled by the presence or absence of conjugative plasmids that can be transferred *in vivo* (Brynestad et al., 2001; Hughes et al., 2007; Li et al., 2013; Rood et al., 2018). Recombinant or subunit vaccines have also failed to confer full protection, as they often contain isolated antigens, where NE is likely caused by multiple antigens (Keyburn et al., 2006, 2013a; Cooper et al., 2009, 2009; Kulkarni et al., 2010; Prescott et al., 2016a).

Electron beam (eBeam) technology has been identified as a vaccine technology that could be used to generate immunomodulators for the poultry industry (Kogut et al., 2012; McReynolds et al., 2012; Jesudhasan et al., 2015a; Thabet et al., 2019). When microorganisms are exposed to appropriate eBeam doses, they are inactivated and unable to replicate, but retain their membrane integrity and metabolic activity (Bordin et al., 2014; Praveen, 2014; Jesudhasan et al., 2015a). Thus, in this metabolically active, yet non-culturable (MAyNC) state, they possess the safety aspects of an inactivated vaccine, as well as the metabolic activity and immunogenicity of a live vaccine. Because MAyNC bacteria are inactivated and cannot replicate, they would likely require the addition of an adjuvant to stimulate the innate immune system (Mot et al., 2014).

In poultry, NE manifests in two forms: an acute clinical form that causes significant mortality, and a subclinical form that can cause chronic inflammation, decreased absorption of nutrients, leading to decreased weight gain, and a decreased feed conversion ratio. Because NE can have many effects on a bird, it is important to evaluate the efficacy of a NE vaccine on more than just decreased lesions and colonization levels. Thus, the objective of this study was to determine the protective ability of a cocktail of eBeam-inactivated *C. perfringens* strains against homologous challenge with the live *C. perfringens,* as measured by lesion scores, *C. perfringens* colonization, weight gain, and feed conversion. Furthermore, the addition of an unmethylated CpG-oligodeoxynucleotides (CpG

ODN) as an adjuvant to the vaccine formulation was explored. They hypothesis of this study was that a putative MAyNC vaccine candidate would be effective at preventing NE upon challenge with homologous live *C. perfringens*.

6.3. Materials and Methods

6.3.1. Bacterial cultures

Ten individual *C. perfringens* strains were used in this study: four field isolates designated as CP 1-4, were obtained from confirmed outbreaks of NE in Georgia (two isolates), Texas (one isolate), and Virginia (one isolate) as previously described (McReynolds et al., 2004, 2007). One isolate (AUD 1) was isolated from an NE outbreak in Virginia (unpublished data). NE isolates JGS 4104, JGS 4064, JGS 1235, JGS 1521 and a healthy chicken normal flora isolate (JGS 1473) were kindly provided by J. Glenn Songer. Glycerol stocks of each culture were stored at -80 °C until use. Overnight cultures were prepared in a COY anaerobic chamber (Coy laboratory products, Grass Lake, MI) by transferring a single loop from a freezer stock to an appropriate volume of growth medium (Fluid Thioglycolate Medium (FTG)) and incubating overnight anaerobically at 37 °C.

6.3.2. Vaccine preparation

C. perfringens cultures were cultured individually in FTG. To comply with all Texas A&M University biosafety regulations, all biological samples were triple packaged in two layers of heat-sealed Whirl-Pak bags (Nasco, New York, NY) and placed in third “specimen transport” bag that was rated up to 95 kPa. Samples were eBeam-treated at a target dose of 10 kGy. After treatment, untreated controls were plated on blood agar to determine the starting titer of each culture. Inactivated samples were also plated to ensure inactivation. Cultures were aseptically combined such that the final cocktails contained approximately equal concentrations of each strain. Cocktail A consisted of *C. perfringens* CP 1, CP 2, CP 3, and CP 4 while Cocktail B was composed of *C. perfringens* strains JGS 1473, JGS 4101, JGS 4064, JGS 1235, JGS 1521, and AUD1. The eBeam-inactivated *C. perfringens* (EBCP) cocktails were stored at 4 °C until use. EBCP cocktails were stored for less than 10 days prior to being administered.

Synthetic unmethylated oligodeoxynucleotides (ODNs) with phosphothiorated backbones were used as an adjuvant in these studies. The CpG ODN sequence used in this study was: GTC GTT GTC GTT GTC GTT (Integrated DNA Technologies, Coralville, IA) (He and Kogut, 2003). CpG ODN was used at a concentration of 20 µg/dose suspended in FTG (CpG ODN vaccine) or EBCP (EBCP + CpG vaccines).

6.3.3. Experimental design

Two individual studies were conducted to examine the protective ability of the EBCP vaccine candidates, with the second study a repeat of the first but with minor modifications. The experimental design of Study #1 and Study #2 are detailed in Table 20 and Table 21. Briefly, day-of-hatch broiler chicks were randomly assigned to one of 10 groups, with 25 birds per treatment group. Two negative control groups were kept with negative control (NC) birds receiving no experimental vaccination, immunosuppressants, or challenge, while birds in IBD/Coccidiosis vaccine control (BC) group received no vaccination or challenge but did receive immunosuppressants. Two positive control groups were also maintained that received no vaccination but were administered immunosuppressants and were challenged with live *C. perfringens* cocktail A (PCA) or cocktail B (PCB). The remaining groups were vaccinated with the appropriate experimental vaccine (EBCP, CpG ODN, or both EBCP and CpG ODN), administered immunosuppressants, and challenged with the homologous live *C. perfringens* cocktail as the vaccination. Experimental vaccines were administered as 0.25 ml oral doses. Birds were individually weighed on day 0, 9, 16, and immediately prior to termination. Negative control groups (NC and BC) were both housed in individual rooms to avoid any possible contamination and were always cared for first prior to entering any other room. Positive control birds (PCA and PCB) were also reared in their own room.

Table 20. Experimental design of EBCP challenge Study #1

Group	Day 0	Day 1	Day 9	Day 11	Day 16	Day 17	Day 18	Day 21
		Vaccinate	**Immunosuppressants**		**Challenge**			
NC	Place birds	FTG	-	-	-			Terminate
BC		FTG	IBD vaccine	10X Coccidiosis vaccine	-			
PC - A		FTG	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail A			
PC - B		FTG	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail B			
CpG - A		CpG ODN only	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail A			
CpG - B		CpG ODN only	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail B			
eBeamA - A		EBCP Cocktail A	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail A			
eBeamB - B		EBCP Cocktail B	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail B			
CpG + eBeamA - A		CpG ODN + EBCP Cocktail A	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail A			
CpG + eBeamB - B		CpG ODN + EBCP Cocktail B	IBD vaccine	10X Coccidiosis vaccine	Live Cocktail B			

Table 21. Experimental design of EBCP challenge Study #2

Group	Day 0	Day 1	Day 9	Day 11	Day 16	Day 17	Day 18	Day 22
	Place birds	**Vaccinate**	**Immunosuppressants**		**Challenge**			Terminate
NC		FTG	-	-	-			
BC		FTG	IBD vaccine	5X Coccidiosis vaccine	-			
PC - A		FTG	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail A			
PC - B		FTG	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail B			
CpG - A		CpG ODN only	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail A			
CpG - B		CpG ODN only	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail B			
eBeamA - A		EBCP Cocktail A	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail A			
eBeamB - B		EBCP Cocktail B	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail B			
CpG + eBeamA - A		CpG ODN + EBCP Cocktail A	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail A			
CpG + eBeamB - B		CpG ODN + EBCP Cocktail B	IBD vaccine	5X Coccidiosis vaccine	Live Cocktail B			

6.3.4. Experimental Birds

Ross 708 male broiler chicks obtained from a commercial hatchery on day-of-hatch were weighed, banded with wing bands, and placed on used pine litter. Temperature throughout the studies was maintained according to breeder recommendations and additional heat was provided using heat lamps when necessary. All pens were approximately 6 ft x 8 ft. Birds were allowed water and feed ad libitum. The diet met or exceeded National Research Council guidelines for broiler chicks.

6.3.5. Experimental challenge model

In both studies, birds were challenged using the NE challenge previously established (Chapter V). Briefly, a combination of a 55% wheat-based broiler started diet (Table 12), used litter, two immunosuppressant commercial vaccines (Infections Bursal Disease vaccine (BursaVac, Merck Animal Health) and Coccidiosis vaccine (CocciVac B-52) , Merck Animal Health)), and a 12 hour feed withdrawal prior to three days of live *C. perfringens* challenge were used to induce NE in birds. The concentration of total aerobic bacteria, total anaerobic bacteria, *Salmonella*, and *C. perfringens* in the used litter was determined by serial dilutions and plating. Briefly, litter (10 g) was stomached with 10 ml FTG, diluted in FTG, and plated (Table 13). The immunosuppressants were administered as follows: On days 9 and 11 respectively, all groups except the NC group received a commercial IBD vaccine (BursaVac, Merck Animal Health) at the manufacturer's recommended dose via the ocular administration route and a 5-10X dose of a commercial coccidiosis vaccine (CocciVac, Merck Animal Health) administered via oral gavage. In Study #1, birds were administered a 10X dose of the commercial coccidiosis vaccine and in the second study, birds were administered a 5X dose. Appropriate groups were challenged with 3 ml of live *C. perfringens* cocktails for three consecutive days starting on day 16.

6.3.6. Live *C. perfringens* challenge

All groups except the NC and BC groups were challenged with either live *C. perfringens* cocktail A or B for three days beginning on day 16. Fresh *C. perfringens* cultures were prepared for each challenge. Equal volumes of culture were passaged from an established overnight culture 24 hours prior to challenge. Briefly, 400 µl of a high titer overnight culture was inoculated in 40 ml of fresh FTG and incubated anaerobically for 24 hours at 37 °C. Four hundred µl aliquots of each overnight culture was passaged in 40 ml of fresh FTG and incubated anaerobically for 24 hours at 37 °C in preparation for the following day’s challenge, while the remaining culture was combined in equal volumes to create the cocktail used for challenge. This was repeated for the second and third day of *C. perfringens* challenge. This protocol was followed so that combined cocktails would have approximately equal concentrations of each culture. The concentration *C. perfringens* used in each challenge was determined by serial dilutions in FTG and plating on blood agar.

6.3.7. Necrotic enteritis lesion scores and *C. perfringens* colonization

Upon termination, birds were necropsied, and the small intestine was examined for the presence of gross lesions. The extent of necrosis was scored on a scale of 1-4 as previously described (Table 15) (Prescott, 1979; McReynolds et al., 2004; Swaggerty et al., 2016). Lesions were scored by a single individual as to eliminate bias. *C. perfringens* colonization was measured by removing 5 g of intestine cranial to Meckel's Diverticulum. Intestine samples were placed in 10 ml of FTG and samples were stomached for 30 sec, serially diluted in FTG, and plated on Shahidi-Ferguson Perfringens Agar (SFP). Immediately after plating, 10 ml of egg-yolk-free SFP agar was overlaid on the plates (Harmon et al., 1971; Shahidi and Ferguson, 1971). Plates were anaerobically incubated for 24 h at 37 °C.

6.3.8. Statistical analysis

All statistical analysis was conducted using GraphPad Prism versions 8 and 9 (GraphPad Software, San Diego, CA). The log-rank (Mantel-Cox) test was used to determine significance of survival data and the results were graphed in a Kaplan-Meir survival plot (Rich et al., 2010; Ranstam and Cook, 2017). Lesion scores and plate count data was analyzed using a non-parametric one-way ANOVA (Kruskal-Wallis test), with Dunn's multiple comparisons test used to compare means and correct for multiple comparisons. A two-way ANOVA (mixed-effects model) was used to analyze body weights, with means further compared and adjustment for multiple comparisons using Dunnett's multiple comparisons. In

Study #1, FCR was compared using a Kruskal-Wallis test as described above, and in Study #2, a mixed-effects model, as described above, was used due to the additional FCR values from day 16.

6.4. Results and Discussion

Bird challenge studies were used to examine the protective ability of eBeam-inactivated *C. perfringens* cocktails against NE challenge. Furthermore, the addition of an adjuvant to the vaccine cocktail was investigated. On day 1, birds were vaccinated with an adjuvant only (CpG ODN), EBCP (A or B), or adjuvanted (CpG ODN) EBCP (A or B). Each EBCP dose contained 1×10^7 CFU of *C. perfringens* inactivated with 10.52 kGy (Study #1) or 10.64 kGy (Study #2), and the adjuvant was administered at 20 µg/dose. As a part of the NE challenge model, birds were reared on used litter. The starting bioburden of the litter for both studies is presented in Table 22. Starting on day 16, birds were challenged with 3 ml of live *C. perfringens* cocktail A or B for three consecutive days (Table 23). In Study #2, due to high mortality, birds were not challenged for a third time on day 19.

Table 22. Bioburden of litter used in challenge studies

Organism	Concentration (Log_{10}CFU/g)	
	Study #1	Study #2
Total anaerobic bacteria	5.78 ± 0.32	5.74 ± 0.30
Total aerobic bacteria	7.30 ± 0.04	6.47 ± 0.27
Salmonella spp.	1.84 ± 0.32	2.03 ± 0.05
C. perfringens	3.04 ± 0.06	2.35 ± 0.13

Table 23. *C. perfringens* challenge titers for challenge studies

	Concentration (Log_{10}CFU/ml)			
	Study #1		Study #2	
	Cocktail A	Cocktail B	Cocktail A	Cocktail B
Day 16 challenge	6.56 ± 0.12	7.22 ± 0.19	6.48 ± 0.1	7.37 ± 0.19
Day 17 challenge	6.98 ± 0.20	5.83 ± 0.64	6.41 ± 0.22	6.44 ± 0.05
Day 18 challenge	6.77 ± 0.17	6.53 ± 0.41	-	-

6.4.1. Mortality in birds was observed pre- and post-challenge

In both studies, there was mortality before and after challenge (Table 24 and Table 25). In the first study, the differences in survival were statistically significant different in groups challenged with *C. perfringens* cocktail A ($P < 0.05$), but not statistically different in groups challenged with *C. perfringens* cocktail B ($P > 0.05$) (Figure 22 A and B). Mortality was primarily observed at the beginning of the study (days 1-7) and is likely due to non-experimental factors. Most notable was the mortality seen in the group vaccinated with adjuvanted EBCP A (CEA) on day 21. In the 24 hours prior to termination, 22.7% (5 birds/22 birds) birds in this group died. This is significant because this level of mortality was not observed even in the unvaccinated group (PCA). Due to the high mortality seen in the first study, in the second study, the severity of the NE challenge was reduced with birds receiving only a 5X dose of the commercial coccidiosis vaccine rather than the 10X dose administered in Study #1.

In the second study, there was no statistical difference in survival in groups challenged with *C. perfringens* cocktail A or B ($P > 0.5$) (Figure 23). Despite the adjustment in challenge severity, in the second study there was significant mortality starting on day 15 (before any *C. perfringens* challenge) that persisted until day 19. Dead birds were necropsied and coccidiosis lesions as well as signs of NE were evident. The mortality started approximately four days after administration of the live coccidiosis vaccine, which Is the minimum amount of time it takes for *Eimeria* spp. to become infectious (Gerhold, 2014). Furthermore, the most mortality observed during this time was in groups vaccinated with only the adjuvant (CA and CB), which stimulates the innate immune system, increasing nitric oxide production and increasing the expression of pro-inflammatory cytokines (He and Kogut, 2003; He et al., 2003, 2011). Because mortality continued on days 16-18, birds were not challenged for a third day in an attempt to avoid excessive mortality.

Table 24. Pre-challenge and post-challenge mortality in birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #1

	Mortality	
	Pre-challenge (Day 0-16)	**Post-challenge (Day 17-21)**
NC	0/25	0/25
BC	3/25	0/22
PC - A	0/25	1/25
PC - B	1/25	0/24
CpG - A	1/25	0/24
CpG - B	1/25	0/25
eBeamA - A	0/25	0/25
eBeamB - B	0/25	1/25
CpG + eBeamA - A	3/25	5/22
CpG + eBeamB - B	3/25	1/22

Table 25. Pre-challenge and post-challenge mortality in birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #2

	Mortality	
	Pre-challenge (Day 0-16)	**Post-challenge (Day 17-22)**
NC	1/25	0/24
BC	1/25	0/24
PC - A	1/25	0/24
PC - B	1/25	1/24
CpG - A	3/25	2/22
CpG - B	2/25	0/23
eBeamA - A	1/25	0/24
eBeamB - B	1/25	1/24
CpG + eBeamA - A	2/25	1/23
CpG + eBeamB - B	0/25	1/25

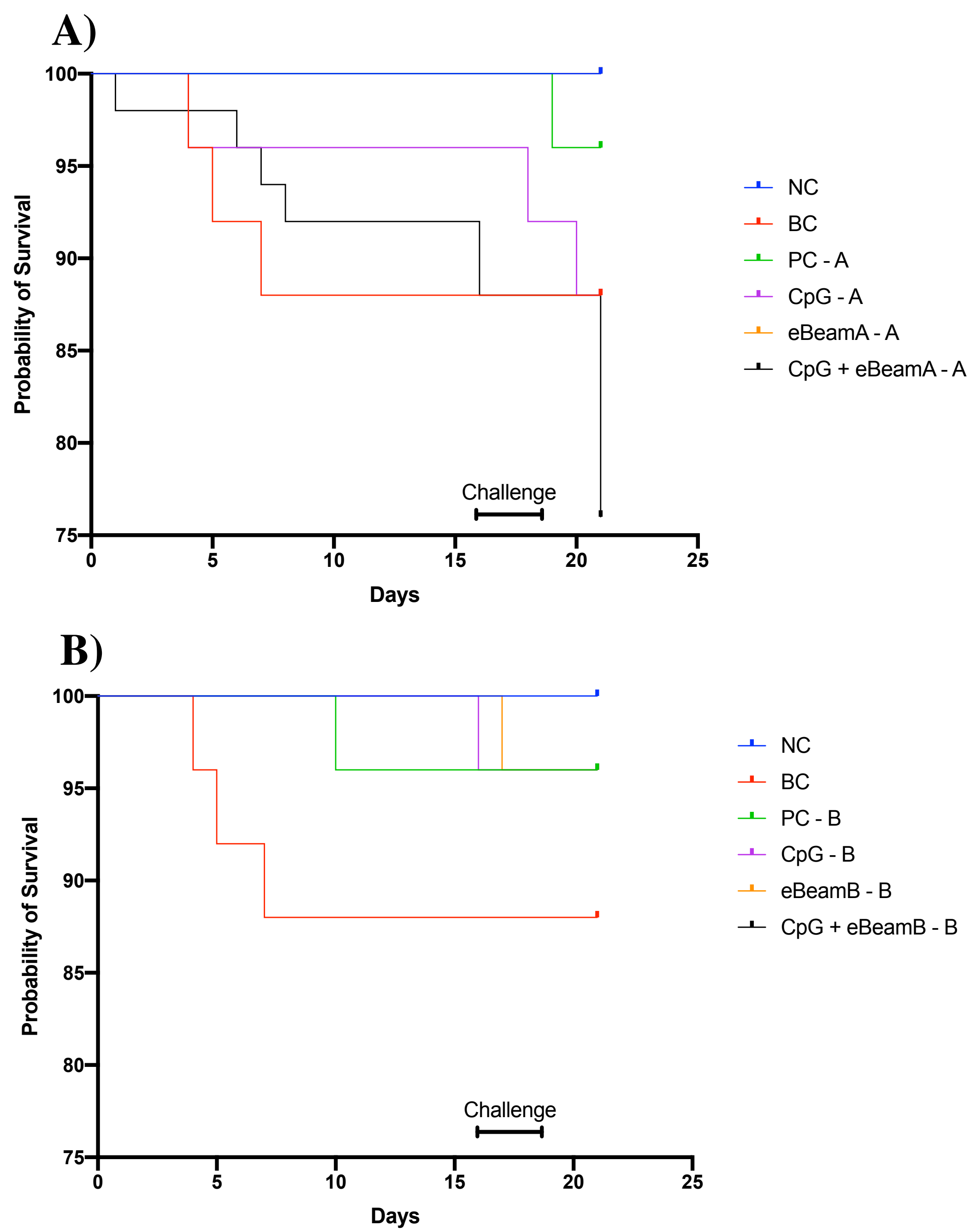


Figure 22. Probability of survival in birds vaccinated with adjuvanted or non-adjuvanted EBCP and challenged with homologous live *C. perfringens* in Study #1. A) Birds vaccinated and challenged with *C. perfringens* cocktail A B) Birds vaccinated and challenged with *C. perfringens* cocktail B.

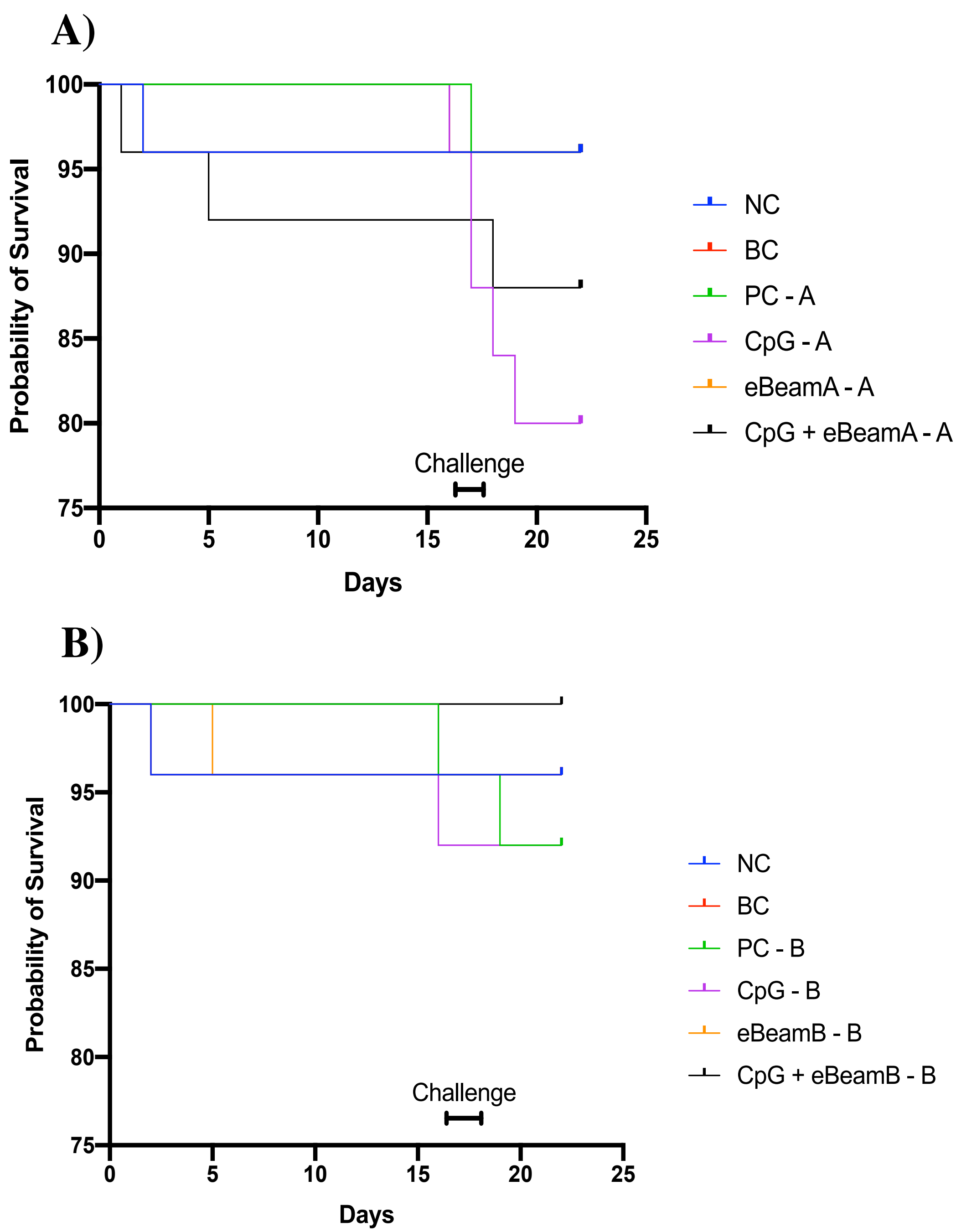


Figure 23. Probability of survival in birds vaccinated with adjuvanted or non-adjuvanted EBCP and challenged with homologous live *C. perfringens* in Study #2. A) Birds vaccinated and challenged with *C. perfringens* cocktail A B) Birds vaccinated and challenged with *C. perfringens* cocktail B.

6.4.2. Effect of vaccination on lesion scores

Upon termination of the studies, birds were necropsied, and the presence of necrotic lesions was scored on a scale of 0-4 (Table 26 and Table 27). In Study #1, vaccination with the adjuvant CpG ODN alone was the only vaccination to significantly lower lesion scores ($P < 0.05$) upon challenge with live cocktail A (Figure 24A). In birds challenged with cocktail B, there were no significant differences in lesion scores between any of the groups, indicating that cocktail B was not able to induce NE. Furthermore, live cocktail B failed to cause lesions even in the unvaccinated group, as indicated by the lack of statistically different lesion scores ($P > 0.05$) when compared to the unchallenged control (BC) (Figure 24).

In Study # 2, the baseline level of intestinal inflammation was higher than Study #1 as indicated by the high lesions scores even in the negative control (NC) group. This group was not administered any immunosuppressants and was not challenged, yet still had an average lesion score of 1.13. There was also no significant difference in lesion score between unchallenged birds (BC) and birds challenged with live cocktail A (PCA), a cocktail that has previously demonstrated the ability to induce NE (Figure 21 and Figure 24). This is likely explained by the high levels of baseline inflammation. Because the NC group also had high lesion scores, it is likely that the litter may have had high levels of pathogens that contributed to NE. When plated, the bioburden of *C. perfringens* in the litter used in this study was actually lower (2.35 ± 0.13 Log CFU/g) than the *C. perfringens* litter bioburden in previous studies (3.04 ± 0.06 and 2.90 ± 0.01 Log CFU/g (Table

16 and Table 22)), although more pathogenic strains may have been present in the litter used in this study. Alternatively, there may have been high levels of *Eimeria* spp. in the litter, although this was not measured.

Interestingly, in Study #2, vaccination with the adjuvant alone had reduced lesion scores compared to unvaccinated birds ($P < 0.001$) when challenged with *C. perfringens* cocktail B (Figure 25 B), similarly to how vaccination with the adjuvant alone yielded lower lesion scores than unvaccinated birds that were challenged with *C. perfringens* cocktail A in Study #1. While the objective of this study was to examine the protective ability of an EBCP-based vaccine, the data in these studies suggest that the adjuvant alone acts as an immunomodulator that is protective against *C. perfringens* challenge. However, birds vaccinated with the adjuvant alone also suffered the most mortality in Study #2, even prior to challenge, so further studies must be conducted to investigate this relationship further.

Table 26. Lesion scores of birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #1

Lesion Score	NC	BC	PC - A	PC - B	CpG - A	CpG - B	eBeamA - A	eBeamB - B	CpG + eBeamA - A	CpG + eBeamB - B
0	13	9	3	4	3	10	6	7	1	7
1	12	13	9	17	19	13	15	17	9	12
2	0	0	9	3	0	1	2	0	4	1
3	0	0	2	0	0	0	2	0	2	1
4	0	0	1	0	0	0	0	0	1	0
Average Lesion Score	**0.5**	**0.59**	**1.54**	**0.96**	**0.86**	**0.63**	**1**	**0.71**	**1.59**	**0.81**

Table 27. Lesion scores of birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #2

Lesion Score	NC	BC	PC - A	PC - B	CpG - A	CpG - B	eBeamA - A	eBeamB - B	CpG + eBeamA - A	CpG + eBeamB - B
0	6	3	4	1	6	12	3	6	9	9
1	10	11	4	8	8	7	7	5	4	7
2	7	7	12	10	4	4	10	12	6	7
3	1	3	4	2	2	0	3	1	3	2
4	0	0	0	2	0	0	0	0	0	0
Average Lesion Score	**1.13**	**1.42**	**1.67**	**1.83**	**1.10**	**0.65**	**1.57**	**1.33**	**1.14**	**1.08**

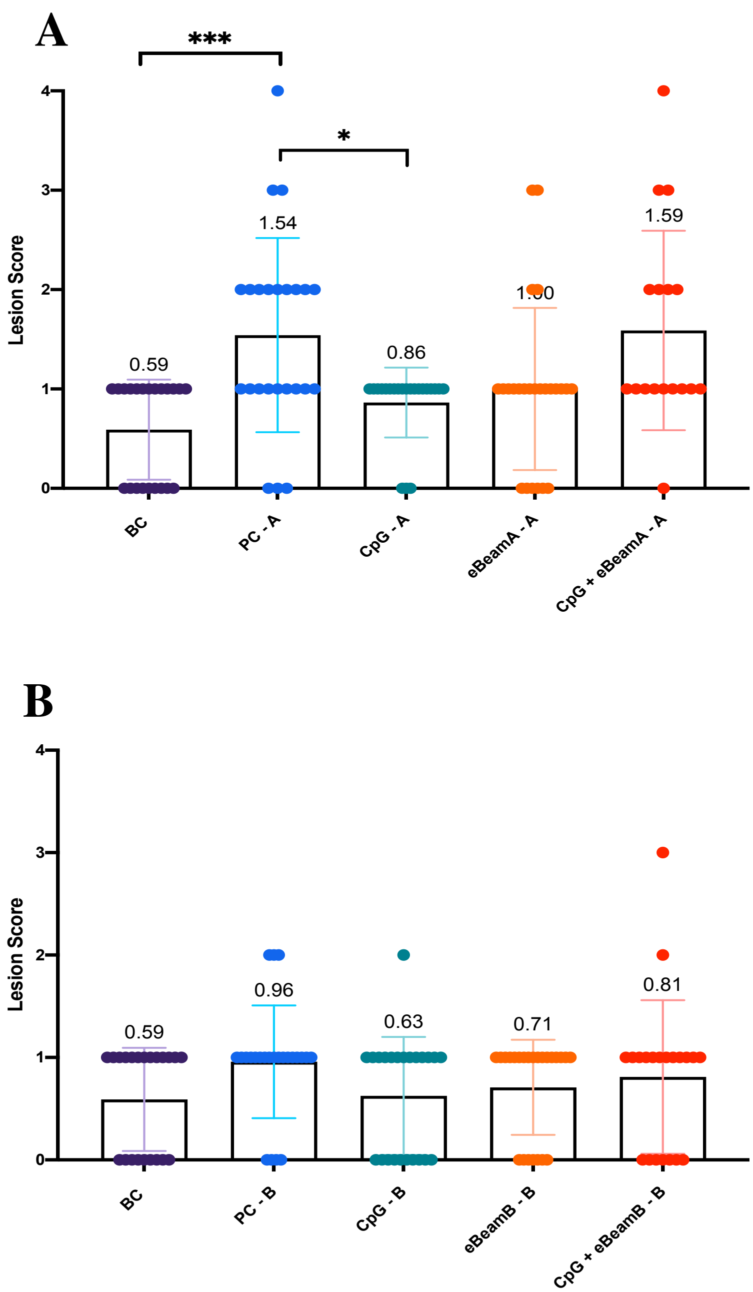


Figure 24. Lesion scores of birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study # 1. A) Birds vaccinated and challenged with *C. perfringens* cocktail A B) Birds vaccinated and challenged with *C. perfringens* cocktail B *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

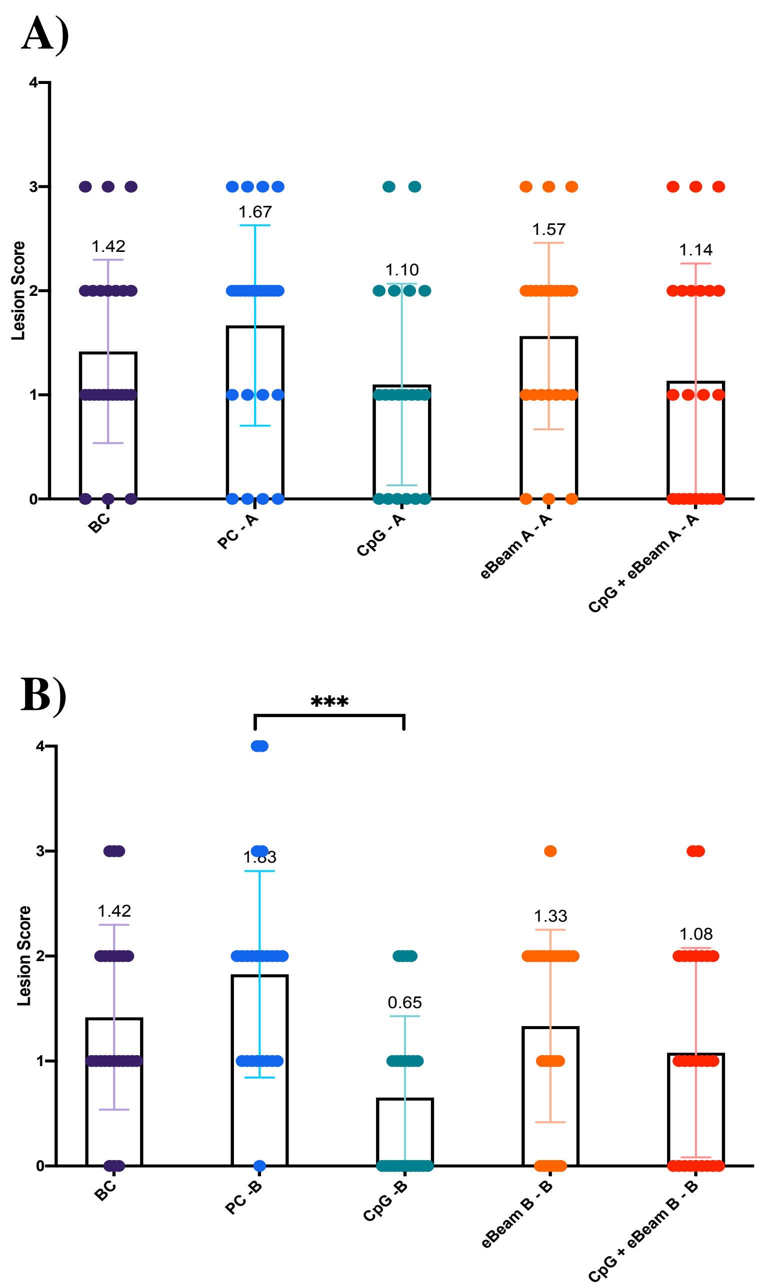


Figure 25. Lesions scores of birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #2. A) Birds vaccinated and challenged with *C. perfringens* cocktail A B) Birds vaccinated and challenged with *C. perfringen*s cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

6.4.3. Effect of vaccination on weight gain

Individual bird weights were measured throughout the studies in order to examine the effect of vaccination on weight gain and feed conversion. In Study #1, birds were weighed on days 0, 9, 16, and 21 and in Study #2, birds were weighed on days 0, 9, 16, and 22. In Study #1, there was no significant differences in body weight observed until day 21 in birds that were challenged with *C. perfringens* cocktail A. On day 21 immediately prior to termination, the weights of birds vaccinated with the adjuvant alone or EBCP A combined with the adjuvant were significantly lower than unvaccinated birds ($P < 0.05$ and $P < 0.01$, respectively) (Figure 26 A). On day 9 in birds to be challenged with *C. perfringens* cocktail B, there was a significant difference ($P < 0.05$) in weight between the PC birds (unvaccinated) and those that were vaccinated with CpG ODN, although this difference was no longer seen after day 16 (Figure 26 B).

In Study #2, while birds were randomly assigned to a group on day 0, there were statistically significant ($P < 0.05$) differences in weight even on day 0 (Figure 27 A and B). By day 9 prior to challenge, the weight of birds in the BC control group was significantly different ($P < 0.05$) than the weight of birds in the unvaccinated group that was to be challenged with *C. perfringens* cocktail A (PC – A) (Figure 27 A). This significant difference ($P < 0.05$) in weight persisted through the rest of the study and was also evident on day 16 and 22 after challenge.

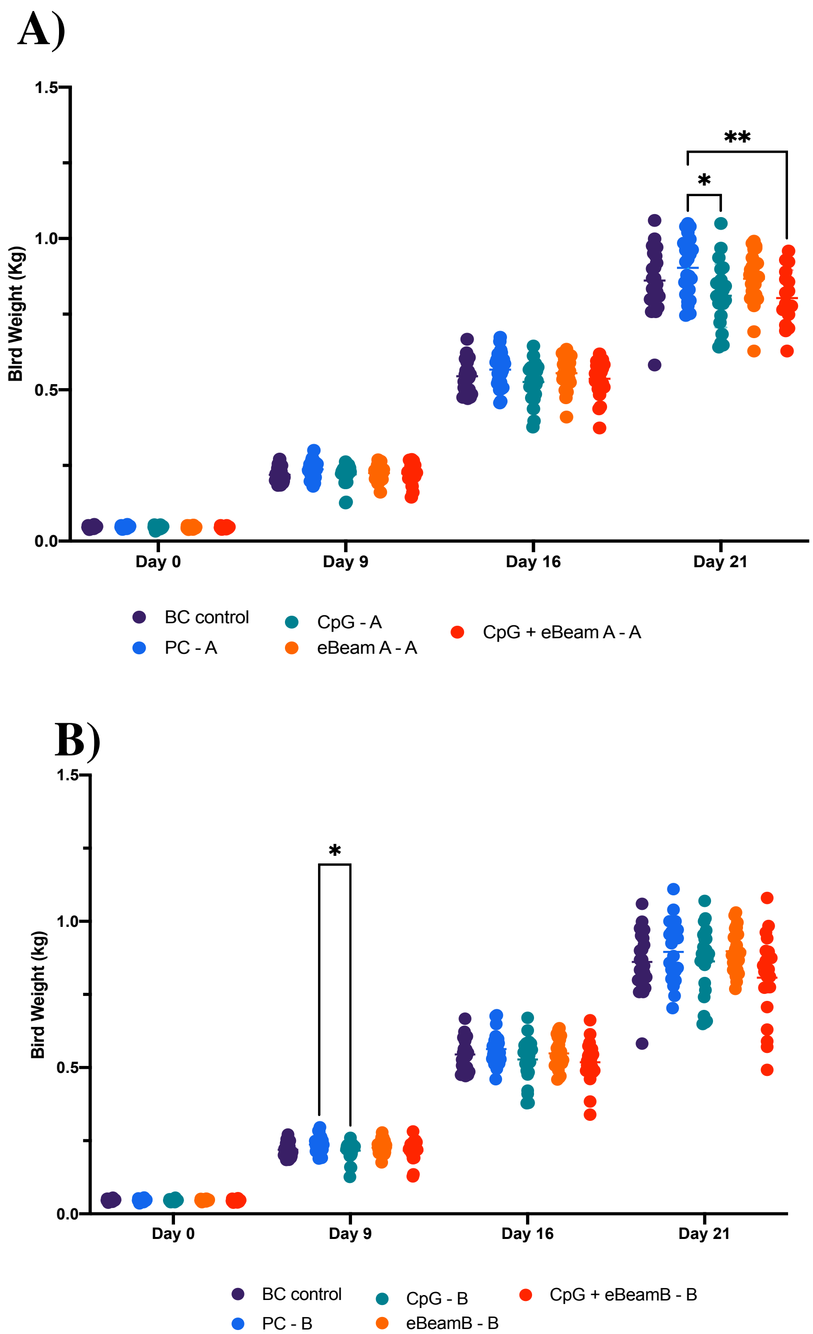


Figure 26. Body weights of birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #1 A) Birds challenged with live *C. perfringens* cocktail A B) Birds challenged with live *C. perfringens* cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

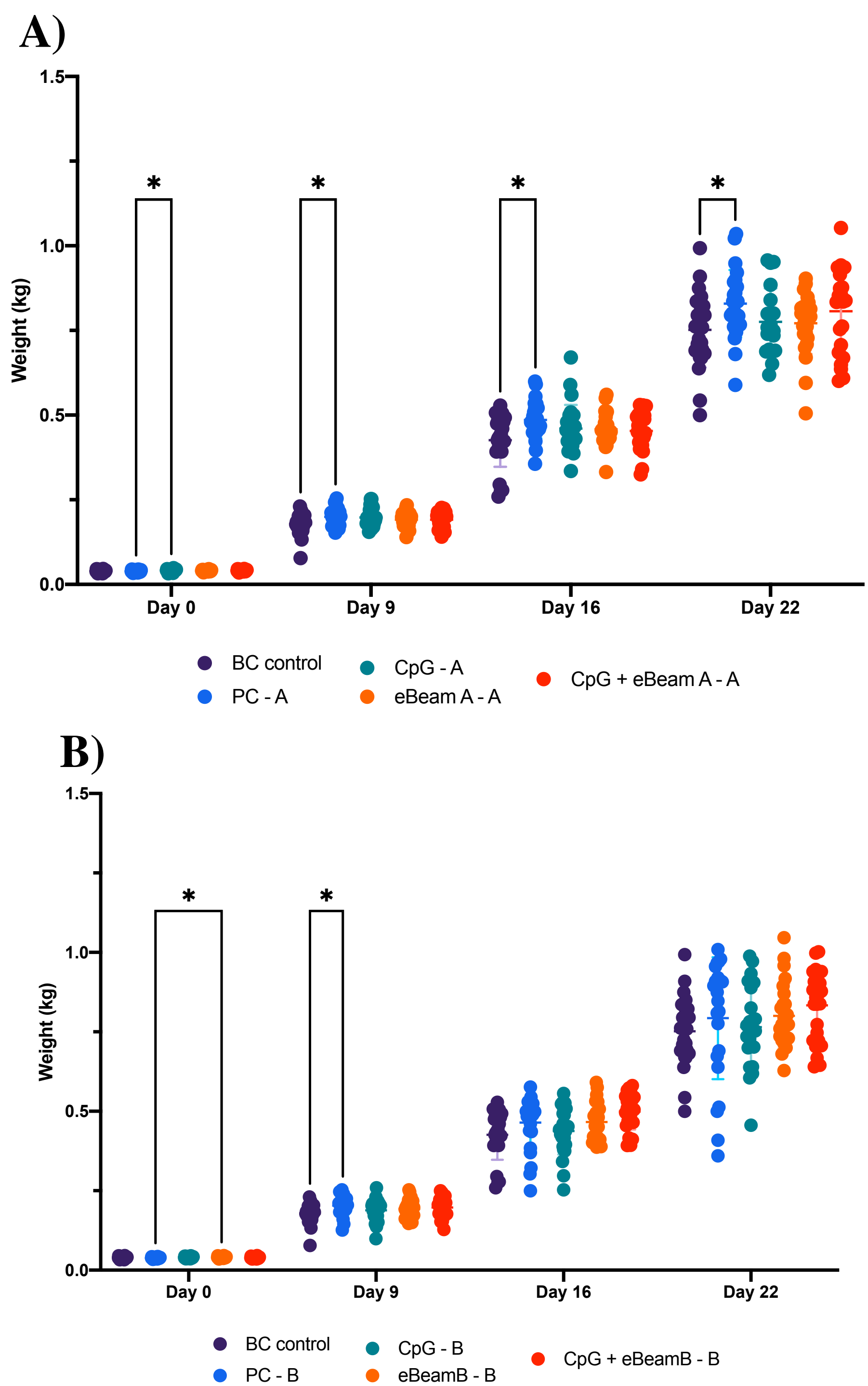


Figure 27. Body weights of birds vaccinated with EBCP and challenged with homologous live C. perfringens in Study #2 A) Birds challenged with live C. perfringens cocktail A B) Birds challenged with live C. perfringens cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Effect of vaccination on feed conversion

The feed conversion ratio (FCR) is a calculation of how efficiently a bird converts consumed feed into body mass and is a critical measure of efficiency in the poultry industry, as feed costs are an economic driver. Specifically, with subclinical necrotic enteritis that causes decreased efficiency in feed conversion, a vaccine that could prevent an increase in FCR (indicating decreased conversion efficiency) would be noteworthy. Thus, it was important to understand the effect of vaccination on the FCR of vaccinated birds. The differences in body weight before and after challenge did not always translate to changes in feed conversion ratio (Table 28). In Study #1, feed was weighed such that only a final day 21 FCR could be calculated (Figure 28). On day 21, the FCR of birds vaccinated with CpG alone or EBCP A with CpG were significantly higher than the FCR of the unvaccinated birds challenged with cocktail A (PCA). It is worth noting that challenge alone did not cause an increase in FCR, as indicated by the lack of statistically significant differences in FCR between unvaccinated and unchallenged (BC) and unvaccinated and challenged (PCA) birds. The same pattern was seen in birds vaccinated with adjuvanted EBCP cocktail B and challenged with cocktail B (Figure 28 B). Is interesting that in Study #1, upon challenge, rather than protecting the birds from NE-related nutrient deficiencies, vaccination with adjuvanted EBCP A or B appeared to exacerbate these issues.

Table 28. The day 21 Feed Conversion Ratio (FCR) of birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #1

	Avg. weight/bird (kg)				**Total Feed Consumption (kg)**	**Average # Birds**	**Avg. Feed intake/bird (kg)**	**Day 21 FCR**
	Day 0	**Day 9**	**Day 16**	**Day 21**				
NC	0.046	0.218	0.520	0.895	35.88	25.05	1.432	1.600
BC	0.046	0.219	0.545	0.861	32.67	22.77	1.434	1.666
PC - A	0.047	0.238	0.567	0.903	35.09	24.91	1.409	1.560
CpG - A	0.046	0.221	0.526	0.811	34.20	23.95	1.428	1.761
eBeamA - A	0.045	0.225	0.555	0.867	35.80	25.05	1.429	1.648
CpG + eBeamA - A	0.045	0.224	0.537	0.803	35.03	22.91	1.529	1.903
PC - B	0.046	0.237	0.563	0.896	33.72	24.50	1.376	1.536
CpG - B	0.046	0.216	0.528	0.863	34.65	24.77	1.399	1.621
eBeamB - B	0.046	0.226	0.549	0.898	35.36	24.82	1.425	1.587
CpG + eBeamB - B	0.045	0.216	0.518	0.807	34.64	23.41	1.480	1.834

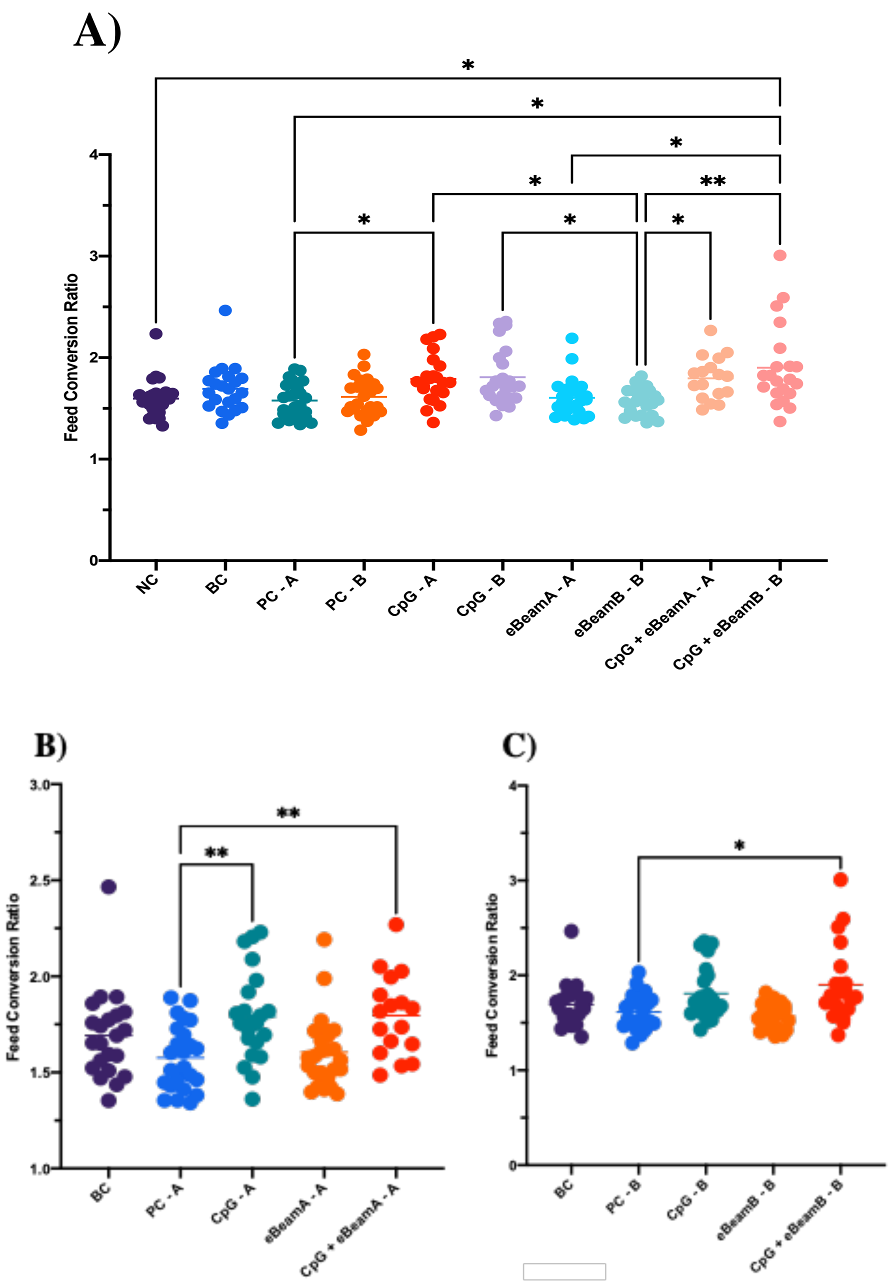


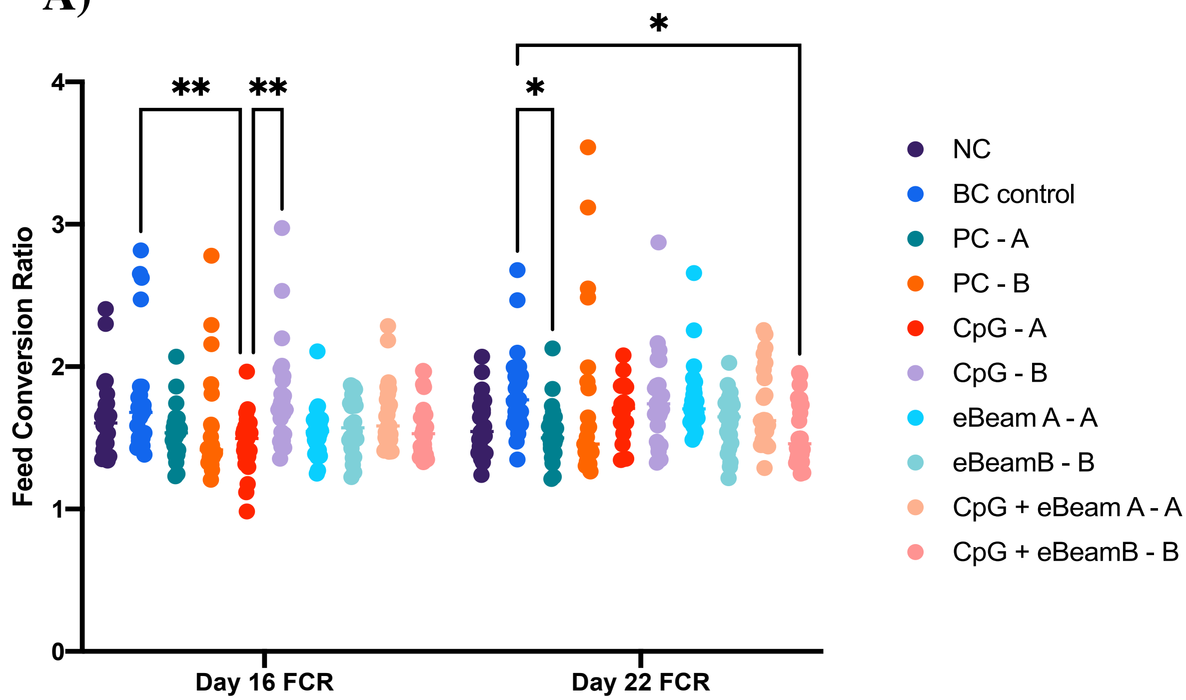
Figure 28. The FCR of birds vaccinated with EBCP and challenged with homologous live C. perfringens in Study #1 A) Birds challenged with live C. perfringens cocktail A B) Birds challenged with live C. perfringens cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

In order to better understand how vaccination would affect feed consumption and conversion prior to challenge, in Study #2, feed was measured such that the FCR could be calculated on day 16 prior to challenge and on day 22 post-challenge (Table 29). On day 16, prior to any *C. perfringens* challenge, birds that were vaccinated with only CpG ODN (CA) had a reduced FCR ($P < 0.01$) when compared to unvaccinated birds (BC) (Figure 29 A). At this point in the study, the two pens vaccinated with only CpG ODN were experimentally the same (CpG-A and CpG-B), but only the group that was to be challenged with *C. perfringens* cocktail A (CpG-A) demonstrated a reduced FCR. Furthermore, the FCRs of both of these groups were significantly different ($P < 0.01$) despite having received the same treatments. On day 22, NE induced by cocktail A resulted in a lowered FCR when compared to unchallenged birds ($P < 0.001$), although this difference was also present on day 16 prior to challenge ($P < 0.01$) (Figure 29 B). Furthermore, on day 22 the FCR of birds vaccinated with EBCP and adjuvanted EBCP was higher than the FCR of unvaccinated birds ($P< 0.01$ and $P < 0.05$, respectively). There were no statistically significant differences in FCR in birds challenged with *C. perfringens* cocktail B, before or after challenge (Figure 29 C).

Table 29. The day 16 and day 22 Feed Conversion Ratio (FCR) of birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #2

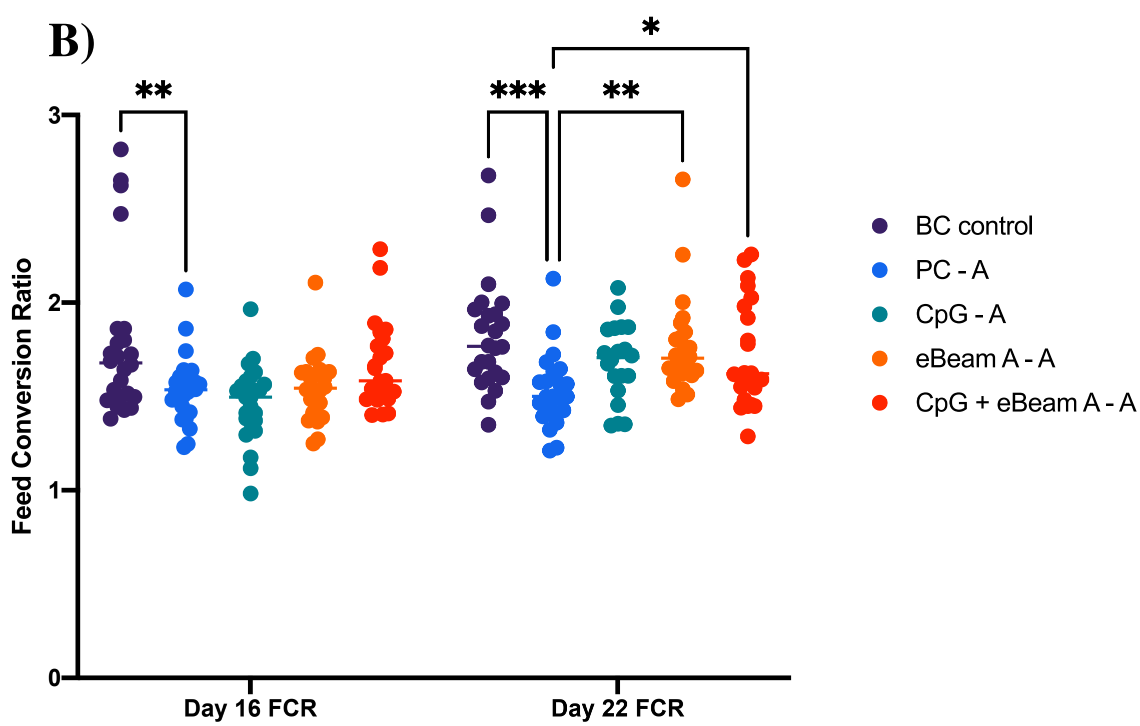
	Avg. weight/bird (kg)				Feed Consumption (kg)		Average # Birds		Avg. Feed intake/bird (kg)		Average FCR	
	Day 0	**Day 9**	**Day 16**	**Day 22**	**Day 16**	**Day 22**	**Day 16**	**Day 22**	**Day 16**	**Day 22**	**Day 16**	**Day 22**
NC	0.039	0.206	0.495	0.849	19.11	33.07	24.07	24.09	0.79	1.37	1.60	1.62
BC	0.040	0.178	0.426	0.752	17.56	32.26	24.07	24.09	0.73	1.34	1.71	1.78
PC - A	0.039	0.199	0.486	0.829	18.42	31.05	25.00	24.77	0.74	1.25	1.52	1.51
CpG - A	0.039	0.203	0.464	0.793	17.37	31.35	25.00	24.59	0.69	1.27	1.50	1.61
eBeamA	0.042	0.198	0.460	0.775	16.46	30.82	25.00	23.95	0.66	1.29	1.43	1.66
CpG + eBeamA	0.040	0.188	0.438	0.765	18.81	32.03	25.00	24.45	0.75	1.31	1.72	1.71
PC - B	0.041	0.191	0.458	0.771	17.49	33.18	25.00	24.73	0.70	1.34	1.53	1.74
CpG	0.041	0.192	0.466	0.800	17.55	30.84	24.27	24.23	0.72	1.27	1.55	1.59
eBeamB	0.041	0.191	0.453	0.807	17.28	31.32	23.27	23.09	0.74	1.36	1.64	1.68
CpG + eBeamB	0.039	0.197	0.499	0.833	19.32	31.30	25.00	25.00	0.77	1.25	1.55	1.50

A)



Feed Conversion Ratio
4
3
2
1
0
**
**
*
*
Day 16 FCR
Day 22 FCR
NC
BC control
PC - A
PC - B
CpG - A
CpG - B
eBeam A - A
eBeamB - B
CpG + eBeam A - A
CpG + eBeamB - B

B)



Feed Conversion Ratio
3
2
1
0
**
***
**
*
Day 16 FCR
Day 22 FCR
BC control
PC - A
CpG - A
eBeam A - A
CpG + eBeam A - A

Figure 29 Continued

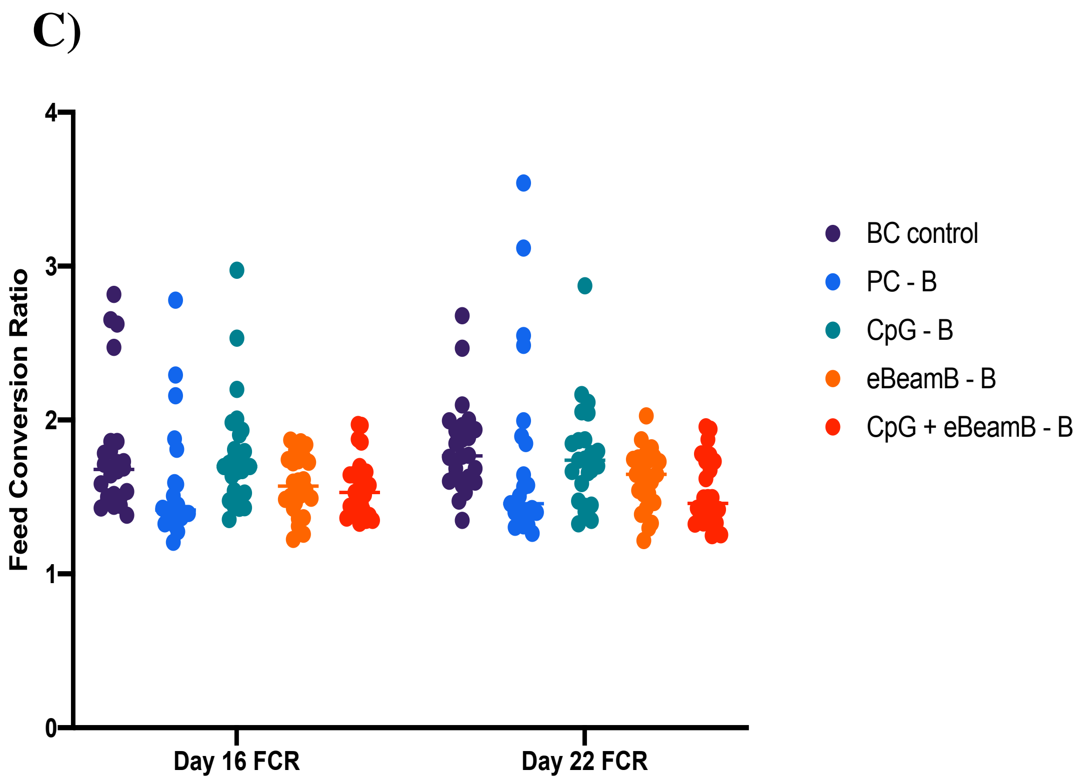


Figure 29. The FCR of birds vaccinated with EBCP and challenged with homologous live C. perfringens in Study #2 A) Birds challenged with live C. perfringens cocktail A B) Birds challenged with live C. perfringens cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

6.4.4. Effect of vaccination on intestinal *C. perfringens* colonization

To investigate the effect of vaccination on the colonization of *C. perfringens,* upon necropsy, intestinal samples were collected, stomached, and plated on SFP agar, a growth media that is selective and differential agar *C. perfringens* (Figure 30 and Figure 31). In Study #1, there were no differences in colonization between unvaccinated birds and vaccinated birds that were challenged with *C. perfringens* cocktail A or cocktail B (Figure 30 B and C). Challenge with both cocktail A (PC-A) and cocktail B (PC-B) resulted in significantly higher *C. perfringens* colonization levels when compared to unchallenged controls (BC) ($P < 0.05$ and $P < 0.01$), respectively. In Study #2, there were no differences in colonization between any of the ten treatment groups, including the NC ($P > 0.05$) (Figure 31 A). Interestingly, the NC group that did not receive any vaccination, immunosuppressants, or challenge, was colonized with similar levels of *C. perfringens* to the positive control groups that received immunosuppressants and were challenged with *C. perfringens* for two days. Compared to the NC and BC groups in Study #1, there was higher levels of *C. perfringens* colonization in Study #2 even in unchallenged groups.

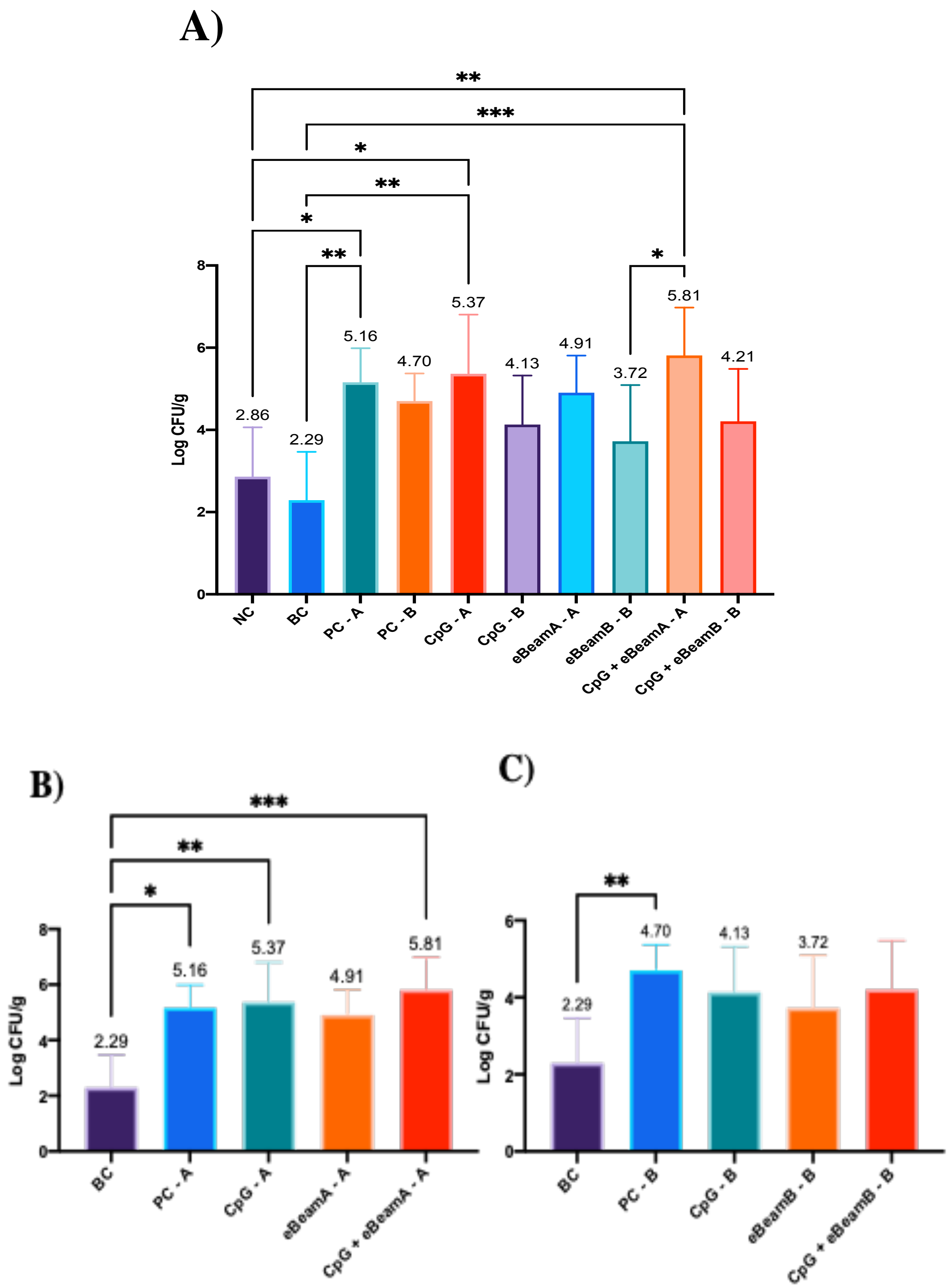


Figure 30. *C. perfringens* colonization in birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #1. A) All treatment groups B) Birds challenged with *C. perfringens* cocktail A C) Birds challenged with *C. perfringens* cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

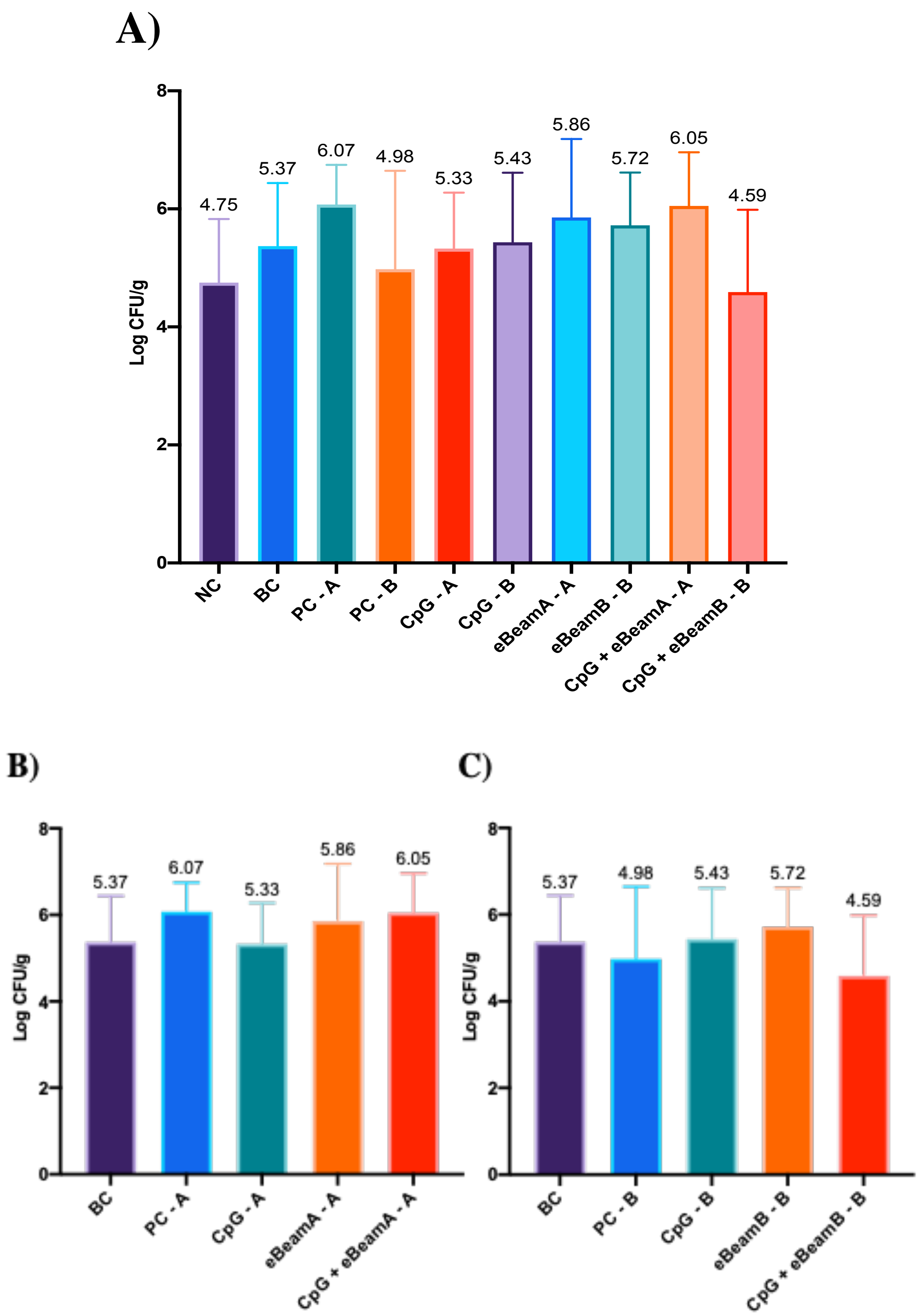


Figure 31. *C. perfringens* colonization in birds vaccinated with EBCP and challenged with homologous live *C. perfringens* in Study #2. A) All treatment groups B) Birds challenged with *C. perfringens* cocktail A C) Birds challenged with *C. perfringens* cocktail B. *P< 0.05, **P<0.01, ***P<0.001, ****P<0.0001. Vaccination did not protect chicks from NE

Challenge studies were conducted in order to assess the ability of eBeam-inactivated *C. perfringens* cocktails to protect against homologous *C. perfringens* challenge in an NE challenge model. Furthermore, the addition of an adjuvant to the vaccine cocktails was explored. EBCP cocktail A or B along with their adjuvanted counterparts failed to protect against a clinical NE challenge, as measured by the inability to prevent necrotic lesions, *C. perfringens* colonization, or reduced feed conversion caused by NE. Surprisingly, vaccination with the adjuvant alone or adjuvanted EBCP caused birds to develop NE that by many measures was worse than the infection in birds that were not vaccinated at all.

For a vaccine formulation to be effective upon challenge, it must be able to induce a prolonged and protective immune response. Live attenuated vaccines that retain their ability to replicate with a host, naturally illicit a strong CD8+ and CD4+ T cell response, as well as a strong humoral response, while inactivated vaccines often require the assistance of an adjuvant to help the vaccine elicit a stronger immune response in the host. An adjuvant is technically defined as a component that is added to vaccine to enhance an immune response, and typically provides the benefits of increased antibody titers and an increased speed, breadth, and duration of an immune response. Because MAyNC microorganisms are inactivated and unable to replicate within a host, their ability to act as a vaccine may be benefitted by the addition of an adjuvant.

The adjuvant used in this study was a segment of unmethylated single-stranded DNA containing cytosine-phospho-guanine (CpG) motifs, referred to as a CpG oligodeoxynucleotides (CpG ODNs). These synthetic motifs mimic bacterial and viral genetic material and are Toll-like receptor (TLR) 9 agonists that triggers innate immune responses characterized by the production of Th1 and proinflammatory cytokines (Krieg, 2007; Bode et al., 2011). As an adjuvant, CpG ODNs stimulate antigen presentation by antigen presenting cells and increases the production of humoral and cellular immune responses. Although chickens lack a TLR9, the immunostimulatory effects of CpG motifs is well documented in chickens, where TLR21 is the main cellular receptor for CpG motifs, although chicken heterophils have been demonstrated to respond to these motifs in a TLR-independent fashion (He and Kogut, 2003; He et al., 2005, 2011, 2011; Linghua et al., 2007; Fang et al., 2019; Chuang et al., 2020; Lin et al., 2020). The specific CpG ODN used in this study has been previously characterized for use as an adjuvant in poultry vaccines (He et al., 2003, 2005, 2005, 2007; Miyoshi et al., 2011; Kogut et al., 2012). For example, the GTCGTT containing CpG motif used in this study has also been demonstrated to reduce *Salmonella enteriditis* organ invasion when administered at a dose of 50 µg/chicken (He et al., 2005). Despite significant research into this adjuvant, it did not protect birds against challenge with *C. perfringens.* The addition of CpG ODN in this experiment yielded mixed results leading to decreased lesion scores when administered alone but had deleterious effects with significantly increased mortality and FCR when administered along with EBCP A, compared to unvaccinated controls.

It is important to note that when administered alone, EBCP A and EBCP B were not protective, but also did not have any negative effects on the birds. These results suggest that CpG ODN may not be suitable for use with an eBeam-inactivated vaccine. Future studies would benefit from the exploration of the addition of other adjuvants to a vaccine formulation including eBeam-inactivated *C. perfringens*.

6.4.5. The NE challenge model is inconsistent

A secondary outcome of this study was the observation that the challenge model used in these studies yields a very virulent, but inconsistent challenge that may not be suitable for vaccine studies. For example, the first time this challenge model was used, mortality after two days of challenge caused the study to be terminated early (Table 17). The second time this study was conducted, the study was able to be conducted to completion with significant mortality observed on day 21 (Figure 22). The third time this challenge model was used, there was significant mortality even prior to challenge (Figure 23). This demonstrates that although this challenge model overcomes the initial challenge of not being able to induce NE, it may be too virulent as mortality is not desirable due to experimental and ethical reasons. This challenge model uses a combination of multiple factors to induce NE – birds are reared on used litter, fed a 55% wheat diet, administered two immunosuppressants, and challenged for three days with a high titer of pathogenic *C. perfringens*. The immunosuppressants and challenge titers use in this study were consistent every time this study was used, leaving the litter and wheat diet as the variables that could potential cause inconsistencies. In each study, the litter was sourced from the same local poultry farm and the bioburden of culturable anaerobic bacteria, aerobic bacteria, *Salmonella* spp., and *C. perfringens*, was determined. Despite very small differences in the bioburden, there were large discrepancies in the severity of the challenge, that may be explained by pathogens in the litter that were not explicitly detected or quantified by plating (Wang et al., 2016; Bucher et al., 2020). For example, when a metagenomics analysis of the litter from the initial study establishing the challenge model (Chapter V) was conducted, *Corynebacterium, Brachybacterium, Lactobacillus, Streptomyces, Sphingobacterium, Staphylococcus, Escherichia, Pseudomonas, Acinetobacter, and Strenotrophomonas* were the ten genuses found in the highest concentrations (data not included). It is noteworthy that organisms in these genuses are both commonly found in the chicken gut microbiome and can also function as opportunistic pathogens (Wei et al., 2013; Wang et al., 2016; Huang et al., 2018; Shang et al., 2018). Furthermore, because the gut microbiome is heavily affected by the litter on which a chicken is reared, and also plays a key role in the immune system, the bioburden litter may indirectly play a role on the immune response to challenge (Wang et al., 2016; Borda-Molina et al., 2018; Bucher et al., 2020). Eliminating used litter as a predisposing factor in this challenge model in future studies may be worthy of investigation.

While other investigators have utilized these methods to induce NE, as is discussed in Chapter V, there is rarely a case where NE predisposing factors are stacked to the extent they were in this model (Chasser et al., 2019). For the purpose of determining efficacy of a vaccine, a less virulent challenge model would be more appropriate, as it would allow small differences in vaccine efficacy to be observed. Specifically, while a vaccine formulation such as EBCP is still being optimized, a less virulent challenge model would be beneficial to elucidate small differences in protective abilities. Alternatively, the extremely virulent, clinical challenge model used in this study is still valuable, as it mimics a worst-case scenario and could help demonstrate a vaccine's efficacy in preventing a clinical challenge.

7. CONCLUSIONS

7.1. Summary

In order to meet a growing global population, there is a need for safe, efficient, low-cost protein sources, such as poultry. Necrotic enteritis (NE) is an enteric disease of poultry that is caused by the bacteria *Clostridium perfringens* and its toxins. This disease can inflict devastating losses to the poultry producers with up to 1% flock mortality per day in severe clinical outbreaks as well as economic costs estimated at $5-6 billion annually (Wade and Keyburn, 2015). In these studies, a vaccine was proposed as a preventative control mechanism against Necrotic Enteritis. Electron Beam (eBeam) technology was used to inactivate a cocktail of *C. perfringens* (EBCP) strains and the Metabolically Active, yet Non-culturable (MAyNC) state and protective nature of these inactivated strains was explored.

1. *C. perfringens* strains were irreversibly inactivated at 10 kGy. The cells were non-culturable under the most favorable *in vitro* conditions and when administered in a NE challenge model in broiler chickens, did not cause disease *in vivo*.
2. The eBeam-inactivated *C. perfringens* (EBCP) were in a MAyNC state, as demonstrated by metabolic activity and membrane integrity assays.

Furthermore, metabolic activity was sustained for prolonged periods after inactivation

3. Metabolomic studies demonstrated that the metabolomic state of untreated control *C. perfringens* was mostly unaffected by growth in different anaerobic conditions. When exposed to eBeam, *C. perfringens* produced key cell-signaling metabolites immediately after inactivation.
4. A robust NE challenge model was established that uses a combination of used litter, a 55% high wheat diet, two immunosuppressants, and a feed withdrawal 12 hours prior to challenge with a high titer of a cocktail of *C. perfringens* for three consecutive days. This model was able to reproducibly induce disease and mortality in broiler chickens, but may be too virulent of a challenge to detect differences in vaccine efficacy.
5. Ten *C. perfringens* cocktails were combined into two cocktails of *C. perfringens* were composed to include strains with the genes to produce a broad spectrum of key toxins. The protective ability of these cocktails, alone or adjuvanted, was tested using the NE challenge model previously established. EBCP alone was not protective upon challenge and adjuvanted EBCP induced a greater severity of disease than unvaccinated controls.

7.2. Novelty of the research

These studies represent the first research into the MAyNC state of eBeam-inactivated *C. perfringens*. Furthermore, these studies are the first to investigate the metabolomic and genomic markers of eBeam inactivation in *C. perfringens*. Furthermore the use of a cocktail of strains to produce a whole-cell inactivated vaccine is novel to the field of eBeam-based vaccines and *C. perfringens*-based NE vaccines. The results of these studies prove that the MAyNC state induced by eBeam-inactivation is a versatile state that can be used to produce vaccine candidates against infectious diseases, although optimization of the vaccine candidates is still needed. Ultimately, these studies allow for an increased understanding of the MAyNC state and its application as a vaccine platform technology that can be used to tailor vaccines for human and animal infectious diseases, moving towards the goal of preventing and controlling disease without the use of antimicrobials.

8. FUTURE RESEARCH

Clostridium perfringens exposed to lethal doses of eBeam-treatment were rendered inactivated and were unable to replicate, even when enriched for 10 days. Despite being unable to replicate, EBCP had intact bacterial membranes and were metabolically active. When examined using molecular techniques such as whole-genome sequencing and metabolomics, the cells exhibited different characteristics, even immediately after inactivation. When incubated at growth conditions for 24 hours, the molecular markers were completely different, indicating that even 24 hours after eBeam, cells continue to attempt to experience shifts in cellular state. This metabolically active, yet non-culturable state, questions the paradigm of how bacterial cell “death” is defined.

When applied as a potential vaccine in poultry, MAyNC *C. perfringens* was not protective against challenge with homologous challenge with live *C. perfringens*. Furthermore, when combined with CpG oligodeoxynucleotides as an adjuvant, vaccination with EBCP not only did not protect birds from challenge, but led to increased *C. perfringens* colonization, decreased bird weight, and increased FCR, when compared to unvaccinated birds. Therefore, there is a need for increased research into optimization methods for this potential vaccine.

Therefore, the avenues for future research are as follows:

1. There is a need to understand the extent of metabolic activity in MAyNC cells: Are the metabolomic fluxes after inactivation a function of residual gene expression or are they simply abiotic in nature? Are these cells capable of secreting toxins? Are MAyNC cells still competent? Is metabolic activity after inactivation of function of eBeam dose?
2. The molecular markers of eBeam inactivation require further investigation: How does gene expression change over time after eBeam inactivation? At what stage is cell replication is replication of eBeam-inactivated cells halted?
3. The NE challenge model used in these studies was inconsistent. Could some of the predisposing factors be eliminated from the challenge model to make it more consistent?
4. Other adjuvants and vaccination routes need to be explored: Would a different adjuvant allow for greater antigen recognition? Would a different vaccination route (*in ovo* or maternal hen vaccination) or a different vaccination regimen (prime-boost) provide more protection upon challenge? Preliminary studies have demonstrated that *in ovo* vaccination with EBCP reduces colonization of *C. perfringens* (unpublished data).

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APPENDIX A

Metabolite	***C. perfringens* JGS 1235**											
	Anaerobic jar						**Anaerobic chamber**					
xylulose NIST	5764	8543	6820	8949	6795	7347	6078	6159	5735	8877	6318	6008
xylose	820	568	634	411	678	321	558	459	567	617	776	521
xylitol	4179	4276	3214	3881	3009	3369	2437	2179	2935	3408	1043	2568
valine	4681996	4144119	3988449	4964208	4218206	3769315	3985592	4280331	4115275	2109787	4553056	3898621
uridine-5'-monophosphate	921	1257	1292	1241	844	1347	837	720	832	720	3911	769
uridine	750	725	280	787	765	626	616	481	551	695	2674	560
urea	871	1487	7989	11211	7984	8091	8353	5573	8010	9224	17472	8711
uracil	21171	22513	19717	26242	17889	21479	16166	11371	17966	20444	6461	15076
UDP-N-acetylglucosamine	697	689	748	693	771	499	261	620	520	551	811	218
tyrosol	4443	4978	3577	4733	3471	3921	3294	2535	3348	3849	4412	3230
tyrosine	2764105	2817833	2432372	3028565	2374162	2639206	2198398	1633697	2299837	2491557	2288509	2030409
tryptophol	3842	4441	3160	5389	3184	4629	3016	2209	3028	3442	4172	2945
tryptophan	3039088	3209787	2744547	3174119	2672254	2903391	2446542	1856262	2509243	2784712	3163141	2333207
trehalose-6-phosphate	227	237	291	312	217	277	238	328	218	305	601	235
thymine	780	931	629	865	607	708	534	732	556	722	4855	789
threose	4149	4403	1894	4364	2403	3273	2240	1699	1721	2436	3015	2224
threonine	1326617	1247332	1304398	1426849	1265449	1312196	1100468	864758	1222893	1309937	1175614	988056
threonic acid	1279	1495	1008	1484	1057	1090	963	797	1038	1175	1273	924
threitol	1221	1255	416	1703	1289	717	438	881	837	475	905	1380
sucrose	635	628	518	265	556	533	677	197	557	603	3262	635
succinic acid	43124	49727	30958	47150	33373	37458	33646	24012	28881	33733	44515	27565
stearic acid	237690	192167	182222	329716	207911	201120	228901	253851	193937	275311	348384	271641

spermidine	50074	44614	31602	49691	28511	32275	29246	17688	26042	30564	51465	27337
sophorose	1026	1168	833	950	952	853	1124	1068	689	821	1017	1169
serotonin	7709	9379	7024	9083	8239	10603	7123	5067	6104	10860	3114	6806
serine	1047789	1201511	845816	1125830	852101	966058	827912	598034	866944	941247	1062362	783993
salicylaldehyde	2832	3098	2051	3530	2254	2389	2465	2304	1812	2496	2125	1841
saccharopine	2522	3137	2206	2628	2220	2410	2195	1456	1878	2293	2861	2299
ribose	15610	16199	15736	21504	13765	19512	12069	7325	13779	16138	31951	12411
ribonic acid	7839	8568	5866	8196	6173	7001	5659	5719	6062	8500	10097	5511
ribitol	5276	6192	4382	6242	4579	4926	4226	3372	4604	4915	5914	4121
raffinose	863	1070	941	1079	949	959	717	609	573	920	934	1027
pyruvic acid	33941	38480	39804	46405	37524	40770	24831	27311	35607	27476	84449	23435
pyrophosphate	31907	32968	21866	46658	16229	35907	28936	11221	19363	30332	51348	23219
putrescine	58650	68125	52906	65929	51595	59360	48407	41140	52465	53105	65721	48483
pseudo uridine	5288	6150	4617	3968	4421	5189	3890	2669	3995	4391	3016	1979
propane-1,3-diol NIST	108638	129925	105734	139689	106124	101723	105527	92059	107833	79073	96341	86501
proline	4942404	3637753	4959552	5235064	4727294	4529098	3657833	3240043	4131004	4964605	3440390	4112394
pipecolinic acid	14854	12447	10428	9485	11844	10063	6353	6144	8583	10987	10046	9217
phosphoethanolamine	13148	21874	14876	27372	14561	24097	18212	18899	14006	26065	5587	32358
phosphoenolpyruvate	1548	1620	1259	2041	1722	2051	1043	1497	1355	2003	2071	1426
phosphate	444864	326565	368755	272747	484693	477698	462582	338214	501483	564512	605817	515477
phenylalanine	1653279	683550	2919158	986248	2520095	2619031	2639632	2630091	2695594	3132885	3523751	2718325
pelargonic acid	12235	13352	11146	17309	10156	15230	12798	13124	9399	16235	14462	15019
pantothenic acid	970	1928	1043	1382	918	951	823	740	836	1098	1117	1159
palmitic acid	30870	24725	22998	39720	26478	26379	29670	34579	25094	36161	41637	33880
oxoproline	6482071	6006462	3403890	3216033	4760549	4786386	6215413	5789319	4183219	5014012	3996613	6530336
oxalic acid	1936	7657	1295	2014	1512	1413	5501	1176	1320	1495	2143	1577
orotic acid	2829	3852	1989	2928	2227	2285	1899	1968	2607	2966	5616	1783

ornithine	3018150	4661670	5090946	4615279	4720269	4824280	4441661	4642857	4807855	5261289	1339534	4507190
O-acetylserine	6565	8043	5972	7184	5753	6540	5501	4579	6065	6058	4098	5401
norvaline	2205	2208	2023	1745	2244	2119	1765	5248	1907	1925	1346	2061
nicotinic acid	83265	97855	67832	90628	65372	75504	63669	46363	63663	70703	88732	60452
nicotinamide	1921	2001	1754	3201	1366	2173	1376	1221	1492	1941	3018	1576
n-epsilon-trimethyllysine	1273	1337	1335	1251	1474	1257	942	984	528	988	1119	1130
N-carbamylglutamate	2868	3453	2256	2484	2321	3094	2301	1148	2033	2733	4303	1983
N-carbamoylaspartate	5735	5028	4882	4844	5459	3551	4483	3201	2592	3296	3617	4126
N-acetylornithine	15690	828	1734	1846	1654	1712	1327	1389	1500	1584	1275	1616
n-acetyl-d-hexosamine	2468	2702	1865	2486	2175	2067	2524	1524	1802	2294	1939	1782
N-acetylaspartic acid	6713	6223	2447	7992	5653	3300	5033	3600	2777	6790	3562	4200
myristic acid	4042	4381	3715	4243	4055	3128	4744	5071	4226	5715	2891	4911
myo-inositol	39931	43339	33089	40723	32666	35285	29697	23573	31697	34304	39328	28382
methionine sulfoxide	1444642	1648665	1266507	1589142	1218121	1399623	1137894	864528	1197515	1286155	1422937	1080597
methionine	953665	1033150	687656	1007511	713987	771861	627967	468055	685519	791316	608203	578317
mannose	2718	3106	2367	2829	2352	2558	2056	1708	286	2374	21338	2035
maltotriose	163	165	198	297	208	212	210	325	231	277	309	163
maltitol	755	733	696	1192	448	863	588	515	701	502	1147	764
malic acid	5358	6111	4205	6416	4308	4782	4066	3136	4254	4736	5413	4032
maleimide	71260	64166	55680	79298	58132	72575	48936	36479	35378	72023	149208	51989
lyxitol	4746	3529	2791	2399	3013	4588	3576	2080	2841	4272	5356	3519
lysine	624482	2257248	3174667	1167497	2418718	1713048	2683540	2736140	2872697	1828039	934848	1751308
levoglucosan	3996	4566	3269	3607	2517	2923	2547	2585	3370	2874	3734	3117
leucine	4166863	2220817	434688	284113	3355977	1866574	3025025	6021514	2126567	434341	4129159	3145627
lauric acid	14858	20155	20228	13001	12719	24818	14615	11100	19310	15682	4990	12608
lactic acid	1035501	272325	1073270	1159154	1007800	1230029	1007860	1182654	1095246	1037247	699830	1058025
lactamide	1012	1430	1691	1563	1461	1799	1197	1511	1334	1220	877	1533

ketohexose	1206	802	655	883	765	1204	589	1008	748	956	1897	522
keto-hexonic acid	385	418	472	507	373	453	235	463	437	484	572	204
isorhamnose	7942	8788	6336	8795	6240	5287	5730	3345	4451	6786	8874	5724
isoleucine	4302181	4164545	5152219	4703667	3916766	4369632	4722507	4178162	4842178	5336064	5724557	4596521
isocitric acid	2385	2680	1833	2549	1983	2119	1493	1512	1921	2434	1826	1761
hydroxylamine	66557	102670	91067	119309	89556	88142	107313	147968	83172	110963	144023	116311
hydroxycarbamate NIST	12354	21619	15369	19319	13377	19361	22521	18944	17932	22401	26037	25297
homoserine	11394	12779	8900	12619	8901	10304	8149	6119	8936	9475	10686	7939
histidine	201211	178604	240606	225248	215951	210545	206002	309172	224583	250889	301318	229164
hexose	5221	1516	4672	7565	4424	5023	4223	3548	4184	5182	10167	4101
hexitol	1230	1350	986	1292	483	1131	891	684	925	1053	1055	717
guanosine	3425	3648	3359	4419	3492	4744	3148	2244	2562	3414	2563	3090
guanine	8611	7863	6996	8411	6727	7307	6168	4344	6512	7477	9705	5282
guanidinosuccinate	1043	1527	1110	1321	1096	1396	1082	873	1104	1350	2620	1046
glycyl-proline	4548	8916	8702	15884	8268	6882	5764	6996	9355	6083	7520	8080
glycyl tyrosine	8027	11044	7372	7453	7702	7992	8088	4745	5318	6615	9015	6637
glycolic acid	6426	4869	5950	6453	5743	6226	4854	5472	5482	5787	5936	4470
glycine	676022	717058	639505	758602	610702	618175	536840	362682	551189	588133	602338	514752
glycerol-alpha-phosphate	5474	5987	5280	4844	5104	4331	5625	4369	4732	5250	28685	5138
glyceric acid	6288	6824	5070	6438	5113	5615	4887	3715	5242	5543	7266	4754
glutaric acid	299	350	246	376	267	332	234	200	228	528	363	496
glutamine	1526	1444	1045	1334	789	1312	797	555	1046	960	1085	1074
glutamic acid	20910	20900	14767	21720	15298	15527	16221	12789	16593	18023	18424	16291
glucose-6-phosphate	482	434	412	582	430	439	473	585	1622	401	1269	1157
glucose-1-phosphate	3436	3731	1898	3570	2767	2930	2583	1475	2616	2960	3185	2428
glucose	12751	13581	9987	13122	10360	10887	9503	7609	10043	10906	57321	8905
gluconic acid lactone	919	1629	1439	1100	854	1148	951	1320	959	1087	1945	985

gluconic acid	12976	14166	10010	12969	9977	11309	9032	6269	9657	10318	11368	8137
galactose-6-phosphate	544	524	509	618	487	530	459	467	465	572	790	573
galactinol	9263	11897	8822	10719	9123	9905	8327	5749	6966	8722	11050	8963
fumaric acid	53534	45055	42458	50544	45617	33970	43882	39609	37751	54296	28399	39467
fructose-1-phosphate	3178	838	2452	809	2352	895	664	560	2257	939	1101	646
ethanolamine	50026	54405	53632	65466	44919	55953	40047	52097	47444	46583	67802	41737
ethanol phosphate NIST	1265	1771	1079	1536	1161	1384	1095	1044	1080	1375	691	1235
erythrose	13228	15038	9376	14038	10561	10912	9506	7833	9141	11097	13640	9829
erythritol	3324	3523	2532	3689	2541	2939	2206	1913	2508	2984	3316	2292
dihydroxyacetone	1950	1817	1540	1999	1189	1824	1038	1945	1591	1193	2806	2076
deoxypentitol	1889	2392	1549	2225	1639	1784	1382	1244	1521	1553	5120	1398
cytidine-5-monophosphate	34688	40076	27993	32347	27209	29363	28893	19205	25380	28855	16380	31730
cystine	193020	222614	166720	194674	164375	168576	153765	110377	153169	165799	158616	143550
cysteine	173569	162059	126624	158374	128394	126413	116371	73955	109142	116726	189516	109105
cystathionine nist	10370	12217	8229	11170	8759	9325	8071	5800	8101	8868	9976	7749
cyanoalanine	767	722	721	3598	737	1900	593	797	824	1254	1019	580
conduritol-beta-epoxide	2056	2037	1531	2036	1576	1777	1324	1632	1322	1394	1959	1817
citrulline	55360	39760	47492	53907	49851	46946	32323	39457	48085	48611	105693	35842
citric acid	88185	97760	68128	92332	69231	75120	64526	48803	69666	75914	77971	60861
cis-gondoic acid	561	892	854	857	901	819	1036	779	455	935	420	597
cholesterol	370	375	497	519	448	436	457	524	402	443	755	627
cellobiose	12125	15575	14731	13689	14472	15200	13286	10375	12666	14243	17797	14809
caprylic acid	8321	8527	8561	9413	7890	7672	8717	9163	7571	8823	7462	9034
capric acid	1504	1478	1202	1640	994	1266	1220	1292	1335	1507	1217	1102
butyrolactam NIST	178557	215140	165622	208908	151082	165759	148837	114128	149623	150077	179783	141125
biphenyl	1935	1001	918	1413	755	1154	1299	1188	1108	1055	935	844

beta-alanine	1446	1913	1133	2762	1312	1701	1215	976	1966	1530	1187	1166
benzoic acid	22508	27733	19585	29038	22550	25822	23393	21508	22441	24208	30590	22323
behenic acid	14656	18071	15437	16829	14735	14792	6355	13544	12007	16649	16412	17402
aspartic acid	1539196	1592884	1091729	1760583	1222415	1268828	1181546	848087	1326794	1420267	99790	1057918
asparagine	46081	51937	37464	57060	41798	53152	30922	26627	33485	46295	58558	36211
arachidic acid	105528	115369	114888	128602	115394	109603	136117	148021	116095	181759	118867	157468
aminomalonate	27321	34450	25233	34078	23705	33490	23536	14079	29959	27694	27075	23606
alpha-ketoglutarate	801	888	949	956	817	925	616	511	827	733	2652	893
alpha-aminoadipic acid	1165	1815	1260	1760	1231	1105	1192	1064	1296	1084	1430	890
alanine-alanine	38793	36933	30468	46374	29886	34101	25548	18940	27307	33838	97174	22429
alanine	5590501	6729166	7464338	7571059	7033707	6816831	6345044	6341163	6811007	7113960	8324946	6340424
adipic acid	633	679	227	647	487	859	586	856	552	578	931	713
adenosine-5-monophosphate	2948	4015	4817	6458	3674	4766	2869	2444	2315	4583	12409	5370
adenosine	9720	10564	9135	13080	8888	14930	9561	6507	8061	10501	14441	8832
adenine	35663	30377	28180	38874	23433	34040	21897	16901	26401	31163	55161	20768
aconitic acid	1236	1806	1353	1932	1416	1541	1439	699	1412	1530	1644	1282
5-methoxytryptamine	17680	21552	14800	19596	18889	19304	17535	13625	15064	17721	8881	14940
5'-deoxy-5'-methylthioadenosine	1471	1445	1697	2183	1147	1731	1062	668	1064	1216	1591	1179
4-aminobutyric acid	549966	1538069	1629629	1715666	580519	1599418	1518519	2068076	1955556	1935548	2060331	2151357
4-aminobenzoicacid	794	752	594	785	934	671	567	639	616	1087	586	545
3-phosphoglycerate	15785	17301	12774	17699	10606	14892	9611	5793	13073	14566	24229	9128
3-phenyllactic acid	7470	8206	5834	7918	5752	6575	5599	4405	5751	6532	6729	5535
3-hydroxypropionic acid	18035	17130	16432	17090	16188	16751	15173	10927	16505	16151	17193	14725
3-hydroxybutyric acid	9814	10231	8291	10433	7709	8248	7733	6529	8599	7624	12374	7038

3-hydroxy-3-methylglutaric acid	635	1212	517	1192	555	603	790	449	533	683	702	600
3-aminoisobutyric acid	137	132	131	187	124	128	105	116	98	141	216	3249
3'-adenylic acid	278	322	319	333	297	334	270	327	324	345	486	277
3,6-anhydro-D-galactose	47050	49325	40949	60022	40363	42805	39916	39325	40364	51658	58447	41814
2-picolinic acid	789	718	613	612	499	560	517	655	516	629	863	585
2-methylglyceric acid NIST	57394	64758	47513	64004	47339	51067	44888	34669	46182	50977	60800	42382
2-hydroxyhexanoic acid	56137	66470	44677	70220	64249	44827	43492	35060	41666	39782	36708	55197
2-hydroxyglutaric acid	29705	31298	18909	29108	21473	19935	21799	17575	21428	28105	15091	19241
2-hydroxybutanoic acid	136677	141978	102156	183531	110529	136160	105923	93289	119901	141203	50643	102735
2-deoxytetronic acid	8679	9692	3357	12218	6826	7501	8417	6740	6508	7338	8840	8387
2-deoxyerythritol	4413	4393	4036	4340	3873	2947	2802	2177	3671	3785	5148	3127
2-aminobutyric acid	52986	60025	42050	62583	42864	44952	39216	36387	39954	51960	24700	36262
2,6-diaminopimelic acid	20433	17935	20532	29908	16014	19478	11054	8291	16614	15726	18825	11212
2,5-dihydroxypyrazine NIST	1539	1755	1421	1988	1487	1653	1154	1360	1028	1528	1607	1370
1-monostearin	661	805	708	693	599	555	561	537	491	645	978	590
227437	975	1730	1022	1186	979	1102	970	628	611	968	1344	1003
227427	830	1146	541	1505	978	1019	501	349	420	804	989	477
227291	660	816	421	450	782	698	399	563	358	455	504	314
227280	778	1077	381	943	455	817	460	289	391	422	780	391
227272	619	959	432	597	790	887	394	713	369	288	588	501
227260	1072	1545	1222	1035	780	1383	847	489	888	780	715	821
227255	1078	863	816	1074	585	773	600	303	376	697	1229	544
227232	891	1402	1529	1231	905	691	985	431	874	683	1312	845
227229	767	1920	726	1831	820	1396	813	635	552	1217	2591	1448

227223	1197	1223	772	1711	915	1063	858	664	825	1021	1187	731
227216	1273	2130	1388	2012	1081	1319	1044	1100	999	1113	1240	943
227209	1185	1459	1558	1914	1575	1536	1206	1481	1442	1681	1401	1125
227200	2891	2793	2219	3153	2392	2214	2245	1633	2223	2526	3364	2175
227176	4907	4735	3698	5488	3975	4609	3367	3057	3404	4871	5482	4133
227159	15361	19291	10808	12979	15355	11207	11097	7587	9755	11147	5144	10637
227158	14049	17839	10580	16591	11798	12816	9734	5508	10042	10549	9810	8517
227094	352	395	444	466	372	562	411	400	434	659	436	514
226710	1112	1523	1711	1945	1071	1068	990	1320	1155	1702	1971	1661
226556	1006	727	600	767	572	663	487	444	566	592	782	525
226546	758	951	1087	1010	988	1019	1152	1229	997	1195	1382	753
226436	5205	6089	3726	5480	2173	4311	3945	3552	3816	4483	5254	4016
226431	3317	1522	2767	2506	3314	3219	2385	5884	3608	4544	1859	2938
226008	1360	1668	1385	1404	1176	1445	1222	980	1025	1372	1149	1240
225844	2153	2072	1606	2338	1774	2025	1386	712	1442	1619	3133	1184
225834	1975	2178	1559	2185	1515	1827	1412	792	1214	1665	1516	1653
225831	1623	1968	1337	1210	1457	1226	814	741	774	1052	2162	682
225797	1340	1506	1112	1455	1234	1304	1092	895	1126	1361	1263	1103
225783	615	492	734	621	653	752	415	844	684	943	1072	711
225773	5700	7302	3674	7115	4450	4669	4468	3607	5199	4844	5788	3569
225765	4775	6535	5461	5461	4669	4501	3632	3949	3688	5261	4164	2977
225748	19055	20767	16693	20087	15572	17064	14451	11232	15666	16865	19956	13821
225741	8488	8798	4756	7566	5378	6778	3294	1861	3503	3825	8973	2496
225526	506	324	220	354	232	267	405	423	437	451	348	182
225514	278	352	258	335	269	541	478	479	203	237	496	395
225320	1306	1566	1421	1099	1347	1136	1147	1232	1140	1147	1176	1592
225305	2324	2150	1806	1882	2001	1915	2570	2697	1592	1927	2674	2212
225303	1319	1506	1247	1395	1124	1274	1138	793	1025	1277	1215	1106
225279	3469	2809	2173	2788	2130	2213	2030	1676	2064	2389	2647	2488

225276	2436	2704	2403	3124	2121	2281	1930	1899	2127	2023	1640	1735
225272	4053	5132	3698	4365	3778	4231	3534	2625	3093	3853	5552	3524
225233	609	322	917	591	393	329	551	803	527	504	434	559
225226	361	390	303	721	323	381	343	499	623	628	762	362
225211	805	1126	971	1708	1062	1691	922	867	883	1657	1489	1458
225203	432	597	356	539	624	431	602	532	360	334	542	294
225193	579	720	559	691	571	549	800	420	560	536	692	450
225192	744	765	565	689	594	640	547	416	1052	682	1170	934
225185	469	608	359	538	391	452	684	256	698	428	556	561
225184	517	722	530	775	597	706	512	695	381	636	1407	581
225170	1032	1275	1327	1304	1175	1252	869	961	950	1800	1997	1324
225159	1041	1266	369	659	689	1224	567	1236	608	500	574	1467
225156	990	1089	778	924	903	767	691	219	982	618	1290	550
225149	753	968	685	930	810	736	688	992	581	755	1080	738
225148	835	1175	732	1083	764	808	655	339	601	883	2280	780
225146	849	939	783	1004	659	754	672	1117	675	775	869	612
225143	1454	1870	1306	1948	1171	1953	1411	1651	1303	1920	4541	1822
225134	957	1149	828	1195	880	913	811	1047	1108	888	1166	761
225130	910	1351	976	1003	895	1000	907	1149	824	1287	788	883
225117	1206	1296	979	1077	1016	1095	774	785	922	554	426	991
225114	1769	1789	1158	2388	1556	2006	1487	1515	1695	1908	2398	1720
225112	1791	1520	1881	1800	1702	2381	1862	2036	1627	1870	2091	1314
225103	595	1441	2007	1958	2372	1844	673	2988	3768	3095	5759	1015
225100	1792	2182	1602	1847	1864	1354	1262	1100	1030	1450	1148	1345
225096	1320	1133	1278	1646	1374	979	1086	601	774	935	1694	1065
225091	1720	1855	1203	2443	1517	1799	1323	1565	1213	2242	1458	1742
225090	1386	1473	1157	1757	1036	1235	873	1259	1065	956	911	835
225088	1143	1276	819	1148	1382	1039	928	1139	1194	1464	2450	1447
225081	1574	2030	1319	1679	1377	657	1301	872	1274	1459	1421	1332

225074	2058	2428	1963	2132	1476	1944	1635	1167	1481	2317	2452	1901
225070	1516	2136	1570	1518	1075	1462	1514	1024	1465	1318	1442	1202
225067	1633	2055	1803	1412	1393	1490	1843	1504	1302	2423	2994	952
225064	2766	3767	2073	2807	2221	2212	1865	1975	1997	2155	3239	1968
225063	2148	2510	2173	2049	3145	1683	2060	1736	1629	1810	933	2154
225048	3149	3868	2696	3520	2797	3062	2562	2717	2536	2480	3294	2656
225039	4289	4919	3240	4062	3641	3473	3369	2227	3058	3290	1586	3354
225035	4207	5869	2992	4956	3634	4727	3302	2624	3246	5294	8812	3971
225028	5077	3197	2624	4083	2817	1877	2984	2193	2574	3083	516	2961
225020	6178	8672	5650	8434	4799	6908	5672	5248	5902	6594	9020	2848
225017	6092	7171	4409	6078	4857	5410	3896	2500	4403	4454	5629	3761
225013	11734	9860	11546	17144	9730	18774	7337	4201	8788	8809	2022	5863
225012	15154	18561	9746	15339	11222	12760	7560	8981	16533	11739	16106	9101
225010	12476	13769	9728	13496	9882	11134	8752	7884	9535	9878	14527	8424
225006	15827	17091	13361	17592	12617	15233	12390	9713	13248	14925	13669	12366
224998	1708927	1999834	1510360	2226178	1425240	1822083	1270122	1046469	1376092	1619346	1057731	1274228
224866	375	504	482	591	181	580	468	408	309	376	645	582
224759	181	229	171	279	278	218	208	325	256	276	292	233
224462	1073	1088	1075	1741	1449	1282	935	1940	417	1807	1639	728
224435	2669	2942	2534	2763	2645	2337	2221	1433	1633	2557	877	2672
224427	1444	2632	1258	3594	1664	3889	1519	2525	1604	3913	976	1892
224423	1282	2551	1640	2247	1840	1999	1712	863	1605	1985	2138	1497
224377	144464	55715	106270	71045	81800	50373	61608	45944	69861	46128	31623	30098
224374	227	449	377	608	172	305	548	465	317	629	302	579
224359	961	979	824	1017	592	1080	758	511	658	740	976	933
224322	558	640	505	274	228	524	527	485	191	202	578	482
224312	659	833	556	858	642	1263	708	403	624	805	748	770
224308	469	579	535	631	471	504	481	472	476	574	585	394
224299	934	736	413	944	476	838	929	577	726	736	996	781

224298	766	1152	565	878	667	754	847	837	527	751	801	942
224287	1395	1170	788	1686	791	1038	772	529	713	1350	1847	726
224286	766	837	681	1435	674	959	670	859	648	782	1264	705
224273	1084	1385	1228	1384	1196	1560	1130	703	787	1459	2139	821
224272	963	1107	928	1030	846	876	870	561	760	992	978	959
224271	1152	1356	830	1179	964	1088	796	961	687	1301	1565	803
224265	1217	1351	1733	1427	1011	1121	1123	1235	1174	1495	1068	1043
224261	1246	1035	799	944	731	739	753	757	771	756	1066	654
224260	653	792	1157	1014	962	1061	898	1207	738	1041	1186	1338
224259	921	1088	692	607	630	1121	1034	1129	674	1125	1436	1373
224253	850	786	690	666	538	316	355	661	384	711	447	323
224250	1861	1662	2063	1624	1609	1503	1296	1695	2073	2075	161	594
224248	820	1012	1171	1107	976	1127	987	516	773	1328	901	1440
224220	1661	2234	1133	1655	1119	1347	1074	1168	1113	1616	1775	961
224218	629	526	495	449	519	545	334	280	483	462	489	291
224216	1010	1259	744	1067	619	862	584	859	758	871	1598	732
224215	1088	1477	292	1344	1207	909	979	388	995	919	1902	932
224214	599	732	418	1084	506	547	742	572	754	870	933	694
224211	1288	1499	2006	1774	1904	1881	1178	1009	892	2038	2771	2460
224205	903	1368	921	949	942	848	944	712	699	1254	1555	775
224201	2060	2281	1982	2198	1720	2334	1834	1829	1826	2194	2472	2529
224199	606	414	611	575	575	486	480	583	528	452	321	334
224198	797	565	514	735	498	553	480	577	699	771	782	545
224193	1003	1536	2026	1639	2252	1588	1916	3144	1330	3771	1134	5661
224191	3570	3745	2656	3721	3072	3396	2763	2200	3010	3207	3767	2575
224190	1689	1310	1588	1522	1637	3644	1552	1593	1322	2554	2159	3874
224183	2625	2203	1794	2243	1882	2023	1694	1841	1535	2042	2828	1657
224181	174	165	212	257	165	245	214	400	222	303	325	240
224176	342	277	373	495	273	312	275	361	278	370	429	354

224170	462	541	460	534	515	507	448	507	460	604	647	547
224167	1841	2128	1667	2266	1721	2005	1830	1727	1459	2848	2321	2049
224166	1116	1023	1633	1207	1421	1392	1070	2161	935	2283	1312	878
224157	590	753	479	594	504	519	604	611	438	608	934	601
224156	904	1495	652	1345	741	1234	765	1231	1049	1327	1200	797
224155	1206	756	735	622	900	546	820	496	856	832	838	590
224147	3320	3552	3105	3186	2862	2723	2667	1872	2378	2815	3282	2953
224143	3315	3495	2818	3180	2852	2982	2358	1568	2419	2457	3800	2564
224142	172	168	268	210	187	292	193	265	209	249	280	196
224140	2869	3306	2745	2819	2680	2916	2263	1716	1965	2113	5037	2416
224136	3284	2410	1758	2576	2750	2797	1750	1533	1092	2338	2160	3058
224131	3050	4253	2880	3358	2954	2893	2582	1732	2769	3218	3770	2805
224126	3001	3971	873	3660	1163	974	1648	2171	2346	3374	6895	2802
224124	735	754	1005	1065	1136	1092	838	1603	658	1429	1241	669
224123	761	868	618	780	691	634	717	536	628	678	1136	2078
224119	2604	1518	1153	1490	1065	1284	1079	825	985	1291	1535	871
224116	1257	1775	1216	1899	920	2575	793	661	932	873	1716	1187
224115	7815	8340	6934	9596	6503	7740	6561	867	6759	6774	2504	4990
224112	4550	3530	3495	2041	3403	1337	2007	929	2348	1553	4652	1608
224111	9303	9802	9823	11345	8279	9514	10297	12440	8772	11517	12438	6259
224110	9767	11120	8314	9393	8256	7701	6824	6532	7941	8087	12471	7786
224109	4574	6043	3709	4903	3829	4646	3818	2880	3539	4244	6106	4179
224107	5082	6174	5871	9088	6076	7428	4253	3095	2649	7475	14536	7484
224106	6189	7035	4886	6133	4824	5215	4325	3208	4729	1182	7202	4223
224105	4862	3496	4363	4609	3689	2580	4493	2872	4391	4105	571	4282
224103	7255	7728	5432	6414	1789	6309	5583	3509	5201	5448	6787	5265
224102	1101	1013	867	936	777	853	812	676	943	935	1699	676
224100	11635	12766	9771	12602	9859	10757	9436	8727	9656	10816	12697	9934
224099	330	310	335	332	316	323	276	325	277	452	343	237

224097	8055	10243	7109	7960	7068	7024	6288	3695	6112	6643	5506	5712
224096	9453	11266	9186	11045	8860	9739	8551	6876	8722	10083	11408	9033
224095	10585	12882	9877	10750	8084	3519	10188	8575	12350	11279	6872	10473
224093	3325	3787	2761	3615	2721	3498	2532	1793	3216	3207	3458	2616
224092	10353	12021	9252	11264	8741	10439	7686	8120	8648	8869	13341	7614
224087	815	484	329	520	653	529	374	393	581	731	365	499
224086	2468	1866	2021	3180	2096	1860	2068	1581	1602	2003	2270	2115
224083	16042	23562	23257	3223	24193	20085	24362	16183	19665	25939	28106	29607
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224076	534826	510641	567150	614922	511723	49171	502650	599735	488312	592790	710091	520135
224075	270663	376848	224749	326971	252021	262195	220232	168764	202841	267656	366510	218848
223946	148	222	131	416	173	333	398	436	295	158	223	196
223940	109	324	151	146	185	498	179	360	168	254	445	581
223771	837	1350	767	939	695	822	677	875	1000	1089	863	1070
223766	352	384	255	352	270	526	221	143	308	305	543	222
223753	640	750	668	491	620	403	371	475	251	791	898	589
223745	442	350	291	586	552	256	262	399	242	457	529	220
223738	345	559	396	136	486	395	461	331	170	393	750	383
223737	635	1011	357	868	375	833	419	487	471	602	751	503
223659	2603	3076	1604	2358	2119	2311	1969	963	986	1976	3035	1958
223624	1384	1506	1079	1572	1078	1349	1022	795	937	1216	1534	1121
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223555	571	686	529	573	728	615	466	297	652	520	1123	920
223542	1902	2236	1349	1569	1305	1149	1164	1349	1259	807	2368	351
223535	889	630	517	810	903	982	206	353	559	451	769	374
223522	748	837	531	774	550	636	510	781	553	638	669	471
223516	639	1408	849	898	835	1509	740	1003	746	1036	2510	919
223491	2037	2428	1915	2360	1797	1862	596	1488	1756	1793	2394	1986
223452	2948	3484	2305	3210	2434	2625	779	1639	2138	2529	2975	756

223421	598	474	503	764	312	391	287	519	218	590	820	519
223419	1305	1382	1100	1126	1110	868	1008	1001	881	944	674	961
223418	1074	1056	1283	1427	1015	1179	813	495	816	924	318	758
223415	937	1207	312	1073	752	892	630	1224	723	815	1200	628
223412	859	1113	1028	1230	571	1319	868	989	718	1342	1361	1301
223405	817	636	452	530	586	520	187	223	353	613	595	483
223398	677	493	400	832	449	404	569	175	416	328	622	459
223397	516	947	460	724	449	613	425	251	466	520	363	520
223394	524	496	267	584	456	479	382	343	339	672	531	384
223393	1659	1809	1791	2018	1787	1738	1562	1105	1152	1847	999	1966
223373	1649	1526	934	1030	1136	980	899	452	818	903	436	914
223367	623	743	581	190	604	658	502	591	496	526	506	422
223363	457	573	413	539	369	495	357	260	371	752	727	373
223359	794	759	669	788	520	656	576	792	578	995	673	663
223339	693	803	711	828	686	793	810	428	425	743	1053	1121
223337	1019	1222	780	1234	925	1097	874	661	703	988	1436	898
223335	871	937	650	891	720	588	629	415	570	672	709	630
223334	910	1080	682	943	832	802	711	464	661	694	873	617
223330	415	611	579	611	387	579	566	403	395	439	879	355
223327	990	1044	538	985	1089	1038	937	428	504	772	1767	661
223317	852	938	737	1096	878	864	1233	428	473	857	2798	955
223316	942	1200	712	1063	837	971	781	548	838	863	933	711
223315	615	775	782	1122	511	830	747	656	532	655	830	579
223310	1106	1259	784	1123	929	1001	813	603	928	944	961	770
223306	399	637	267	575	348	489	327	437	402	511	1059	448
223302	819	1466	1150	1188	963	1500	813	1020	1337	991	1150	777
223299	1019	1199	821	1141	865	987	802	632	852	976	1121	813
223298	1046	1090	541	1012	724	804	648	173	725	739	1228	587
223295	974	1231	906	1626	958	1357	791	944	493	1333	2152	1312

223293	1051	1179	796	639	813	1042	991	880	818	860	1380	909
223292	768	1650	1099	1158	1007	1267	866	685	785	1151	794	1396
223290	534	480	425	654	515	510	352	353	401	507	1541	350
223289	1917	2215	1553	3377	1634	1670	2122	1899	1627	1827	3100	1635
223284	699	718	1181	1180	1097	1121	462	811	691	1336	1331	919
223280	749	1072	886	1266	810	823	859	717	919	973	1772	857
223277	1394	1386	1244	1280	1164	1109	1065	569	1046	1087	982	942
223274	1143	1490	1077	1556	1167	1259	919	619	921	1313	1747	1097
223272	288	1029	725	765	653	925	637	381	384	756	1271	919
223271	2553	2414	1888	2364	2132	2244	1678	1272	1891	2232	2467	1942
223268	1673	1821	1228	2364	1047	1968	894	1639	993	913	3056	982
223266	1563	1906	1860	2912	1560	1868	888	1764	1148	2569	2905	1730
223263	1650	1800	1032	1441	933	2533	1244	528	1193	1628	1761	1575
223259	888	1839	738	1372	1117	1225	1045	645	760	1296	989	903
223253	1157	1246	1041	1101	1118	1072	912	591	874	961	1270	1012
223252	2313	2105	1478	2008	1442	1657	1645	1255	1487	1734	2615	1642
223251	1877	1964	1454	1886	1034	993	1460	1039	1481	1568	1574	1316
223250	1647	2034	1082	1470	1312	1304	1093	744	1297	1130	1903	982
223249	2016	2128	1511	2662	1484	1798	1315	1099	1511	2235	1788	2084
223248	1109	999	1355	1211	1254	1275	1098	511	1223	647	3185	710
223244	1211	799	1094	1126	1110	930	794	669	922	637	677	605
223243	1131	2026	1427	1764	1596	1773	1396	896	948	1599	4722	1777
223241	1342	1596	1057	1454	1085	1213	999	1048	1091	1217	1392	922
223237	988	1277	1206	1354	1148	1192	979	1303	762	1174	1546	1298
223234	1178	1828	1213	1587	1791	1295	1278	1339	1153	1308	1670	1142
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223226	1717	2324	847	2158	1217	1820	741	1119	939	1120	1736	1068
223223	980	1789	1174	1323	1402	1553	1140	1908	1679	1655	1131	1685

223222	1702	1927	1864	2102	1694	1882	2184	2339	2060	2424	2514	1430
223216	2027	1347	1408	1491	1272	1281	829	552	1016	1077	1290	645
223214	843	743	926	1064	888	803	747	707	687	1170	1977	1104
223211	1797	2775	2042	3486	2550	3121	1781	1833	1770	2044	2622	2049
223208	3286	3436	2693	2906	2460	2399	1627	1092	2312	1839	1203	1386
223204	3014	2773	1585	2818	642	2201	1757	2151	1913	1442	2871	2175
223203	2174	2402	2059	2066	2596	1907	1620	869	1303	1404	1168	1460
223202	1856	1693	1597	1999	1635	1692	1427	1120	1510	1736	1938	1465
223201	8323	12470	7359	12283	8837	10981	8884	5939	6378	9073	3289	8097
223200	1389	2062	1523	2140	1417	2462	1652	1252	1229	2006	3952	2226
223199	378	540	472	368	535	386	290	276	311	399	4266	402
223196	1705	1970	1305	1926	1346	2116	1484	1049	1389	1774	2981	2025
223195	2010	2297	1663	2058	1660	1804	1497	1035	1253	2178	1977	1470
223194	5547	4664	4194	2201	4053	4346	3241	4455	3121	4182	4858	3235
223192	2878	3383	3008	3592	2471	3741	2275	1941	2651	2442	3528	2648
223187	569	870	1000	1003	662	724	660	743	759	864	1869	656
223186	1284	880	985	1033	1142	524	96	345	517	495	2448	378
223185	1089	1311	753	1115	968	938	558	843	1280	861	2118	657
223184	2324	2788	2859	2858	2727	2752	2009	1675	1421	2928	2273	3281
223177	2938	3391	2441	3057	2471	2253	2426	1057	1991	1918	5589	1880
223175	2773	4934	2586	4462	2977	4613	3248	1976	3306	3872	3113	2483
223173	1345	1424	1365	1652	1898	2095	543	1144	1136	2446	1602	1607
223172	1320	3197	1788	2748	2187	2618	2196	1321	1455	2095	3765	2963
223171	6661	9807	6512	8453	6016	11656	6945	5099	6137	6455	2655	7783
223169	2099	2892	1801	2472	1976	2270	1800	1296	1730	2059	2537	1715
223167	2730	3376	2286	3167	2113	2508	1915	1125	1911	2067	2587	1724
223165	2192	2756	2044	2493	2068	2260	1921	1721	1705	2076	2652	1883
223164	3784	4434	3232	4365	3164	3337	3863	1699	3046	3297	2872	2259
223160	6068	4880	6227	5869	5261	4529	3890	2823	3916	3930	1952	3355

223159	5713	6783	6222	7156	6083	5732	5036	3328	3978	6164	3725	6818
223158	4215	4396	2883	5074	2881	3899	3776	2455	2821	4276	4229	3573
223156	2144	2478	2038	1969	1822	1721	1419	1071	1470	1755	2401	1750
223155	1544	4980	2437	3256	2142	1623	4395	2957	3076	2655	5007	4561
223154	3872	3731	2170	2864	2628	2525	1906	999	1707	1771	2225	1551
223153	910	933	655	1087	714	833	534	480	812	601	1872	550
223152	3282	2478	2607	2951	1911	2918	2130	1861	2170	1542	3686	2375
223150	3832	3257	3602	3265	3255	2394	3791	1936	3379	2349	3552	2444
223149	4774	5537	3737	4687	4080	4340	3820	2573	3548	3744	4865	3777
223148	4114	4658	3570	4652	3430	4044	3318	2507	3501	3771	4516	3242
223146	6876	7876	7202	6936	7271	6441	6108	4564	4400	6917	3206	7651
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223135	7883	7107	5376	7275	6748	5230	5058	3484	5161	5433	5113	4313
223134	2965	3831	2856	3600	2630	3032	3120	2548	2415	3418	4173	3211
223132	3568	4228	3031	3347	2664	4967	2980	2372	3789	2836	3258	2461
223129	6816	9550	6872	10766	7159	10578	6759	4781	7537	8729	5313	7463
223126	6259	5948	4396	4484	2891	4857	4202	2665	4947	4791	5851	3940
223125	4139	3487	4173	3397	3516	3019	3078	2272	4235	3362	4475	3087
223122	4883	10968	4588	4419	4495	7966	5076	3401	4627	6543	9211	5784
223120	7074	6995	3807	5962	4210	5311	2560	1387	2904	2923	7238	1870
223117	5034	5638	3674	5350	3946	4363	3631	2749	3664	4251	5179	3463
223116	1593	1346	1466	1727	1064	1891	1512	1455	1087	2124	4650	1177
223113	3915	5884	5451	6615	5144	5062	5395	3623	4903	5719	9705	5035
223109	10216	9702	8380	11702	8481	9979	7979	5520	5529	9404	12173	8549
223108	17344	19951	14853	19319	14324	16858	13605	11036	14247	16098	17447	13086
223107	6767	7704	5217	6461	4961	5929	4875	3661	4977	5729	6548	4674
223106	9226	9989	6246	8980	6478	7352	6087	4227	6120	6595	7225	5085
223100	9836	10349	7838	11309	5776	9136	5537	3736	6140	8538	17248	6625
223099	8599	7332	4644	8030	5371	5495	4801	4749	5506	5104	5114	4691

223098	6225	12088	4682	9621	7443	8977	7945	6349	6453	8400	13041	10110
223097	6890	7614	5317	7230	5570	6074	4868	3735	4961	5797	7157	4570
223096	4567	6199	5259	6398	5480	5961	5191	4847	3763	5984	12476	7320
223095	6446	7715	6396	7054	5994	6507	5317	4527	4083	7185	12573	7557
223093	8869	10580	10549	12101	10402	11296	8060	6063	5748	10919	12536	11826
223092	10620	11064	8758	10166	9118	8824	7839	4167	6530	7315	10929	7570
223090	30867	23154	21629	24815	21124	17090	19106	10367	15439	18862	21335	18305
223087	24652	28211	23032	28684	21984	24177	21455	17511	21567	23561	28777	21799
223085	23402	34808	23066	32771	26038	29799	27042	20851	21895	29344	33556	28844
223084	17926	24006	14983	21590	15533	17408	16460	9765	12294	15547	27317	16204
223083	19719	26374	16668	23850	17105	19454	17364	11600	13417	18151	26348	17877
223080	58018	93569	51247	65433	54221	58491	51833	37712	48136	59251	70179	44515
223078	48960	55555	39402	47887	40923	40700	36840	32012	39783	44389	37579	40448
223077	34221	51207	30743	41698	31442	43529	30512	17099	35427	39160	45767	31316
223076	705026	644467	505153	655782	536219	540391	481422	383574	487062	475400	770433	412215
223072	425317	532182	364682	439119	365892	386093	370311	275175	361710	405941	331198	363832
223071	993353	1026325	736795	1017138	729593	786823	693197	487863	749677	798600	827104	637472
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222267	596	575	379	598	566	504	514	565	637	465	1604	531
219394	620	753	693	1064	646	826	509	827	715	871	773	552
219374	8499	7070	5290	6872	5434	5479	5591	4475	6037	5519	6163	5090
217691	15476	19315	14081	20108	13949	14346	13138	13379	14125	13614	19768	12676
217651	3268	3795	2891	3340	3053	3215	3092	2688	2817	3065	3876	2665
216193	2518	2914	1324	2859	1439	1881	1207	1489	2174	2231	2747	1413
215896	6545	7104	5159	5244	5270	5440	3589	3516	3926	5282	4213	3197
215857	47636	51459	38881	50046	38926	44007	34827	27489	38017	40069	45510	34759
215855	733	679	365	634	717	595	531	439	341	667	586	589
215853	29122	34805	27102	43672	28477	35870	28393	21055	18783	40501	81921	35555
215800	1286	1399	1250	1863	1030	1522	962	988	1303	1203	1816	993

215769	1341	1700	1094	1709	1159	1376	1094	855	1037	1361	1751	1068
215691	10617	8816	3945	6555	5890	8858	3631	2739	6230	5049	9473	4310
215683	203	189	193	225	189	175	238	357	221	265	270	254
215676	5100	6497	4958	4649	4751	4323	3335	2952	2964	3791	4753	3974
215671	668	1182	1270	1739	1291	1317	597	1075	521	1040	6409	942
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210996	2153	2072	1606	2338	1774	2025	1386	1119	1442	1619	2345	1184
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210885	2026	2219	1560	2558	1651	2089	1499	1324	1533	1875	2391	1451
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210697	2396	2703	1719	2629	1892	1961	1745	1455	1701	2072	2640	1844
210403	1172	1024	1173	1297	988	1183	965	623	688	646	863	732
210321	1781	1845	2160	2269	1861	1996	2318	2575	2017	2262	2835	2646
210316	1752	1853	2216	2154	1853	1495	2061	2049	1870	2191	2604	2102
210297	9220	9961	4060	8900	1937	8236	2944	5603	6835	2985	1837	8165
210272	457	422	369	582	403	397	418	516	431	448	1114	441
210262	774	544	1570	587	523	790	480	491	548	1816	940	496
209690	287	271	297	421	330	406	300	431	286	452	537	269

208761	6885	5865	4579	5547	6051	5978	3655	4669	4181	6389	4409	4452
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187055	639	413	492	627	539	518	269	456	454	479	645	481
187054	1816	3384	2525	1489	2084	1787	1091	1823	2008	1530	3079	2007
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183117	1827	1427	1528	2707	1650	1968	1770	1328	1650	2445	3511	2681
183107	1388	1420	1027	1841	1218	1207	1343	1060	1080	1482	2111	1786
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171302	5661	6883	5656	6489	5643	5705	5273	4255	5071	5936	7041	5451
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171045	1273	2029	1200	1750	1273	1825	1330	1221	1053	1753	2245	1547
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170977	1552	1427	1057	1393	974	1583	1038	1175	1080	1084	1103	1252
170974	8754	10894	7239	10444	9595	10825	8817	6533	6912	8264	12390	8620
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170512	724	753	831	830	623	654	854	309	487	588	831	486
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130801	4899	6167	4768	5891	4748	5488	4619	3561	3912	5215	7409	4555

130800	5448	6121	4747	5927	4775	5007	4718	3391	4092	7043	6010	4943
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117141	1719	4639	2481	2435	2538	2291	5888	2911	2594	2830	2562	855
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104123	1666	2696	1374	2080	1865	1598	1865	1475	1291	1819	2187	1655
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94582	265	232	242	303	209	228	241	301	162	293	481	230
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18242	1321	2346	1449	2238	1450	1737	1581	1256	1437	1878	4143	944
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	4937	343	359	273	204	251	272	414	301	382	613	567	732
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	3781	1449	1874	1564	1770	2367	1646	4020	4208	1726	2274	1673598	2430
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2673	5824	5807	3553	5284	3999	4259	3481	2807	3811	5546	8542	5046
2667	4253	5239	2968	4698	2880	4160	2841	2019	3179	3204	6836	2760
2665	857	1973	1323	1325	1369	1974	1081	936	1107	1959	4022	2082
2662	945	918	1080	819	823	944	838	623	652	834	8231	840
2659	1405	2140	1017	1356	2080	1227	1161	1400	1535	1486	1956	2225
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2018	25875	17428	18035	25694	13352	12775	19774	11587	19435	13664	3137	17293
2017	16939	24422	13078	12332	12464	16996	11256	10567	11625	8789	7945	18139
1996	39921	47117	37712	46255	37382	41002	34109	28923	35063	40076	54095	36845
1981	115167	117377	93555	116679	90718	96831	84291	71040	88486	96931	118468	83134
1970	59723	60939	46983	65989	46584	54149	43947	37104	47868	54598	66407	45390
1941	8634	8367	8715	10681	8140	8771	9519	11828	8610	11011	10486	8878
1925	1470	3344	2495	3139	2436	2776	3076	4457	2600	3795	5625	3942
1912	2813	3219	3016	4344	3959	2994	3567	4000	2877	5472	3565	4155
1875	4888	4385	4275	4952	2983	4103	3238	5744	3966	4690	6090	3550
1872	3792	3851	2803	4304	2595	3344	3363	4343	3192	3576	5304	3210
1846	16244	10620	9392	14071	11201	9545	10722	7369	10114	10042	4499	10224
1840	9925	6531	5731	6967	5895	6460	7192	3555	6665	7076	13935	4785
1833	25087	29263	21009	26988	21166	23615	19837	13735	18726	22233	21976	18126
1832	1547	1674	1506	2001	1385	1521	1244	1611	1312	1624	1960	1500
1831	34715	41492	26811	38432	28958	33093	25738	20624	25626	29944	35454	26418
1830	35030	45889	31950	45913	32494	42524	29563	21601	21719	38432	61326	38111
1829	49678	46414	35783	45176	36665	41379	35339	30013	30742	48844	71472	39247
1805	2378	1289	359	1409	1146	1003	964	860	1233	800	1267	859
1801	10116	10681	7869	10778	7847	9270	8112	5608	7990	9441	10225	8158
1799	5941	4520	13594	1715	13666	16012	12603	9956	13341	14372	13317	2066
1781	90250	104694	74234	96541	59746	76827	60382	49015	62765	84926	29492	59657
1767	35071	39609	30428	35590	25689	33221	27856	21124	27613	32528	30591	25752
1751	1473	1910	1390	1784	1420	1447	1536	1588	1520	2099	2557	1801
1750	42791	48693	34772	55415	36071	45591	39243	24035	28611	48250	57268	46617
1746	7198	18994	10504	14626	11650	17840	10725	8048	7900	13940	33449	15653
1740	27768	33031	23268	29564	22139	27333	22905	16687	20969	24822	12999	23036
1737	82733	103271	77939	99228	79929	81673	74250	49077	55979	83117	85446	90561
1735	9394	18277	12315	17227	13061	19368	11511	8669	9319	14562	35559	15435
1732	50178	58506	42493	52407	39051	48337	39839	29923	40670	47536	40981	39370

1725	2536	2527	2817	3866	2381	2390	2034	2859	2374	3000	3648	3152
1721	50704	79490	52058	64849	51117	64318	51844	35341	38350	58967	130421	64803
1717	44410	76232	50619	61258	51047	73536	49851	33621	35871	63295	95532	60726
1713	83389	119581	82273	127473	85100	111037	83408	57308	58354	103522	226984	103472
1711	589	710	513	878	535	763	383	676	633	581	1423	571
1708	27616	61014	33763	48127	31794	48116	31125	24267	27487	47698	98739	44206
1705	370967	437555	336500	393950	333994	354107	306640	206924	271373	325705	416399	303868
1702	16725	20665	10356	23722	15180	25874	13465	8164	16209	15546	64274	27908
1700	1694	1984	1535	2151	2229	2471	1888	2109	2286	2053	2368	9914
1684	801	852	692	875	701	749	759	623	637	992	822	608
1661	35610	29248	25733	42315	20059	27593	17018	10004	23804	27200	7706	15372
1380	50206	94413	54349	149409	30524	81365	122167	34496	69678	59903	55367	35185
892	2976	2650	1880	2938	1883	5419	1623	6037	5274	3988	7589	4881
782	311	413	400	390	304	375	313	384	337	373	583	360
657	13214	11660	15263	14059	14003	14334	15039	17401	14738	12671	15480	14761
592	20938	42851	19033	54720	18065	31848	24043	15137	22606	34735	30019	28600
453	1254725	1918649	1190081	2500995	1041761	1597516	2242299	1328982	1631118	1405769	1809908	1191542
307	62477	66250	70317	64432	61268	53354	69032	37356	48911	53845	62856	58657
299	9596	19606	12610	15438	10594	9058	23398	15757	9668	15346	29480	18266
168	504	470	492	699	814	1027	485	444	435	779	576	483
137	41171	46444	55703	56583	48485	46653	60483	61220	50973	63118	71938	53641
134	7166	8090	5424	4073	15725	7645	6488	6384	5395	10510	6892	7084
110	6242	8069	5849	8272	5547	6055	6618	7277	6075	7580	8216	6137
91	3082	2571	2208	6680	2823	3405	3605	3733	794	3553	2761	2922
47	4733	6203	4949	5791	3861	6549	5356	6536	4718	5263	4981	7456
39	5426	6429	5594	7020	5140	5724	6054	6631	5272	7078	7822	6288

APPENDIX B

Metabolite	C. perfringens JGS 1473										
	Anaerobic jar						Anaerobic chamber				
xylulose NIST	9558	10539	6089	13415	16995	8638	6496	10431	10666	14369	9216
xylose	692	724	928	878	1035	633	871	885	1063	332	724
xylitol	1121	1925	1295	1731	1891	858	1692	2024	1430	1892	1130
valine	4504583	5043191	3907132	4716890	4261995	4032739	4754136	4812426	4139162	5078237	4278203
uridine-5'-monophosphate	1312	2028	2911	4174	4431	1932	2467	3116	1998	6342	1138
uridine	1722	2330	2060	3071	3886	2022	2230	2241	1794	2314	1791
urea	13966	14553	13873	18205	2247	13660	17008	18777	18086	21502	12443
uracil	9468	10808	9452	7755	11038	8922	9076	15433	10564	14983	8315
UDP-N-acetylglucosamine	446	798	620	987	1455	913	567	1001	765	1229	660
tyrosol	4273	4971	4295	5598	7697	3911	5326	5182	4429	5219	4291
tyrosine	2318589	2706993	2284733	2910426	2596113	2102525	2871731	2920790	2377478	2901869	2250945
tryptophol	3694	5201	3851	5287	7060	3535	5935	5946	4369	4501	3908
tryptophan	3034288	3437941	3236438	3841865	383310	2876694	3744815	3690703	3238797	660866	2930629
trehalose-6-phosphate	409	771	629	1316	2035	399	1171	634	380	675	354
thymine	3760	4887	4362	5957	8387	3074	5332	4366	4826	5344	3481
threose	2498	2955	2760	3208	6103	2197	2980	3276	2376	3910	2444
threonine	1297625	1430934	1240178	1433003	1321633	1299501	1519047	1528549	1418348	1405163	1342430
threonic acid	1279	1453	1260	1656	2229	1204	1670	1517	1282	1856	1248
threitol	852	1654	811	789	4332	799	2285	1132	1037	472	944
sucrose	2799	3732	3874	4371	6328	3374	3896	4162	2668	3885	2987
succinic acid	42513	51365	41572	55855	74630	38400	53558	54887	42780	60617	42723
stearic acid	204803	184436	197927	216497	309430	206236	185995	247452	211810	337038	209010

spermidine	53360	77211	42195	77337	151055	45541	72647	80215	51169	87916	51283
sophorose	783	1149	1169	1395	2383	873	1310	1578	1026	2328	927
serotonin	2406	2546	2479	3875	5176	2897	3639	3141	3168	3957	2988
serine	977422	1147862	814541	1362809	1649076	762560	1387212	1279054	1089166	1650349	957959
salicylaldehyde	2478	3168	2423	3054	4742	2221	2704	3716	3074	2995	2377
saccharopine	2172	2878	2677	3683	5536	2290	3200	3103	2376	4034	2284
ribose	25861	26039	31507	37222	38648	27894	31452	38031	29594	39082	27675
ribonic acid	7928	9935	7600	10767	15864	7145	10619	10044	8275	12315	8102
ribitol	5579	6855	5345	7347	9961	5190	7651	6702	5858	8021	5361
raffinose	666	899	1147	1310	1812	1068	1073	1266	693	1522	852
pyruvic acid	81552	64071	61268	227071	208275	61500	193387	59772	82764	182511	43204
pyrophosphate	69472	75917	65862	92939	92827	46124	66614	89133	94071	107017	46214
putrescine	65025	76031	62896	88659	116133	58815	85509	77250	32531	27137	63152
pseudo uridine	5153	6139	3076	8296	10908	4608	6721	6254	996	7156	4393
propane-1,3-diol NIST	112841	122760	119007	136020	164040	104674	129142	138474	116096	123533	105681
proline	3980381	4368152	3228959	4122597	4563574	3700584	4392626	4235147	3672556	3534216	4237768
pipecolinic acid	13545	19133	5223	17054	19650	10083	20086	10874	13663	14104	15172
phosphoethanolamine	3321	3622	4012	6013	7283	3779	5532	6276	3139	6271	3070
phosphoenolpyruvate	1889	2613	1898	3261	4968	1705	2875	2351	1741	3889	1582
phosphate	553888	366932	608577	493704	211595	606453	527021	188850	579451	296054	492131
phenylalanine	3140160	3315906	3144118	3499195	1973755	3092778	2254335	1266262	3346265	2395072	1268366
pelargonic acid	8593	8619	16312	12347	11007	11492	10048	23494	16170	11943	9580
pantothenic acid	1414	1793	1198	947	3347	1283	1371	2759	1322	1940	1375
palmitic acid	24004	22826	27429	29724	32371	26764	24423	32834	29236	34261	26023
oxoproline	6421136	3944055	5638558	4665060	4611467	3953027	4619076	4837291	2925280	7169958	5500289
oxalic acid	1609	1738	2130	2292	3736	1507	1680	2111	2083	2706	1765
orotic acid	4822	6378	5090	7151	10168	4342	5829	6101	5290	5817	5059

ornithine	1701784	2360562	1354375	2331042	3712185	1791194	2350664	2093318	1673965	2100276	2022400
O-acetylserine	4063	4683	3751	5804	7475	3526	5673	5312	4935	5227	4335
norvaline	2367	2129	2294	3273	3702	7278	3306	2196	2808	3516	2035
nicotinic acid	83530	97850	81416	113483	158263	72543	105267	106167	86928	127965	82638
nicotinamide	3074	4119	2944	3099	4248	3267	3595	3902	3838	3224	2907
n-epsilon-trimethyllysine	942	1336	954	953	1338	1027	1836	1395	1171	1599	1411
N-carbamylglutamate	2343	3148	2844	3764	7867	2688	4166	4273	3065	8393	2832
N-carbamoylaspartate	3530	5168	5312	5366	8864	3750	5226	4307	5337	2925	4058
N-acetylornithine	1633	1577	1628	1724	23929	1318	1624	1783	982	858	1109
n-acetyl-d-hexosamine	1646	2161	1779	2545	3322	2256	2390	2159	1922	2808	1718
N-acetylaspartic acid	3887	4770	2641	4566	5443	3969	4108	4969	2610	4013	4054
myristic acid	1837	1708	2371	2570	2705	2004	2052	2298	2332	2670	2112
myo-inositol	38513	46483	36610	51302	73863	36008	51462	46344	40476	57427	38368
methionine sulfoxide	1424455	1692151	1329338	1985538	2768798	1280911	1823296	1743140	1488414	2146344	1342690
methionine	667373	823348	510842	826742	1288427	563015	860440	850284	661016	905744	658610
mannose	3849	3382	2902	32732	27320	2121	13137	3347	7683	65495	2963
maltotriose	277	254	89	338	122	310	332	362	255	101	278
maltitol	540	883	1034	1350	1703	942	1247	1251	1008	1329	531
malic acid	5366	6471	5060	6908	9689	4757	6873	6540	5427	7641	5176
maleimide	46313	74357	46523	83253	185588	58096	106125	107232	60826	105356	78659
lyxitol	5327	5035	4947	7427	10386	4662	7473	6151	5291	7857	4741
lysine	2272107	1648304	1463923	2739196	2742106	3569173	1492786	1539224	2749499	2480721	3260425
levoglucosan	3237	4006	3386	6133	7960	3020	5650	5632	3627	6738	3482
leucine	3608815	3571610	3805789	2835997	4444976	3412848	4029896	3711135	4003480	394806	3816438
lauric acid	10988	15934	28035	7752	20985	6168	17606	8356	11756	17219	12403
lactic acid	975245	832116	540241	1044141	1067898	726208	1072063	1034351	1108210	886406	872660
lactamide	1140	507	1044	1552	1262	868	1491	1649	1355	1177	1284

ketohexose	1128	1579	1197	1636	2173	1469	1561	1309	1770	3126	1396
keto-hexonic acid	486	504	218	612	620	148	597	608	555	573	486
isorhamnose	8466	9754	7868	11831	18215	7701	11277	9786	8154	12430	8047
isoleucine	2313328	4551817	5636477	3861122	2563194	5348406	3825331	4834432	5672659	4921738	4426226
isocitric acid	2484	3006	1849	2782	5134	2194	1945	2972	2431	3024	1862
hydroxylamine	98262	73425	146683	104645	88182	98938	98987	146122	89182	129091	65125
hydroxycarbamate NIST	12525	11941	21757	16022	15606	18437	15768	23145	18050	22856	14642
homoserine	11528	13575	8443	14264	20143	9691	14944	14254	11716	14639	11339
histidine	396118	379345	376941	188426	126781	357319	238172	646313	394589	827064	238492
hexose	9656	10790	9180	15244	17849	8471	13860	11810	10770	16472	9059
hexitol	1104	1222	1022	1464	1890	997	1494	1240	1145	1946	1132
guanosine	1368	1910	1979	3001	3611	1687	2646	2563	1932	5811	1829
guanine	8642	10403	8521	16979	15541	6153	11651	11618	12661	17423	6644
guanidinosuccinate	1288	1767	1274	1508	3160	1364	1375	2272	1577	1403	1192
glycyl-proline	6785	10687	7954	11746	7378	12474	9709	11172	8466	12230	10216
glycyl tyrosine	6610	10061	8164	12436	17270	8850	11625	11397	6305	13942	8323
glycolic acid	6913	7021	6566	6802	7972	6875	8299	8152	7161	7871	6619
glycine	654784	688270	572619	756182	827724	551947	752587	746437	617826	764971	621737
glycerol-alpha-phosphate	15535	19279	21122	26479	38262	14863	24967	21184	19862	34161	16392
glyceric acid	6301	7204	5916	7763	11301	5308	7678	8691	6925	17232	6164
glutaric acid	289	702	377	410	549	627	676	753	654	448	313
glutamine	1427	2170	893	2226	1983	1092	2649	2066	1529	2381	1518
glutamic acid	17020	22787	16429	24557	39250	14729	29847	23377	20304	59652	17690
glucose-6-phosphate	616	671	2270	1267	1168	521	848	1018	663	21642	623
glucose-1-phosphate	2972	3602	3094	3457	5681	2814	3994	3999	3120	4793	3075
glucose	13049	15954	11812	22226	26695	11567	19655	15212	13962	1178267	12491
gluconic acid lactone	1264	1384	1344	2359	2258	1165	2139	1622	2106	2518	1156

gluconic acid	11906	15282	10740	16079	25489	10393	16610	15253	12042	18967	12403
galactose-6-phosphate	674	702	661	766	907	623	760	622	651	1183	529
galactinol	8590	11068	12368	14745	20463	10484	14014	13396	8488	17631	9718
fumaric acid	17461	18091	25535	18338	25863	22735	21826	27429	19802	22194	22026
fructose-1-phosphate	1126	3486	1176	1287	1624	2516	3144	1930	3662	1653	3356
ethanolamine	61715	66674	52048	69465	98329	46781	62702	82801	23523	75404	62667
ethanol phosphate NIST	877	1038	804	1012	1358	781	923	988	826	869	683
erythrose	12563	14189	12179	15804	21501	10858	14479	16465	14659	20724	12407
erythritol	3297	3869	3086	4338	5949	3073	4377	4200	3603	4876	3294
dihydroxyacetone	2299	2125	2359	2667	3301	2355	2361	3063	2942	3097	2134
deoxypentitol	2770	2596	2075	11765	22036	2313	14138	3100	3778	4126	1955
cytidine-5-monophosphate	11845	13638	16110	17926	22534	13810	15740	16300	11108	39141	11776
cystine	153190	210730	163104	231228	350465	155708	216233	199796	158528	257135	161711
cysteine	119941	164007	112091	171704	345938	109259	189317	185816	133125	222259	146394
cystathionine nist	9964	12678	10383	14976	20706	9307	13469	12908	10125	15029	9616
cyanoalanine	711	777	707	1010	1288	946	966	2909	1839	627	1489
conduritol-beta-epoxide	2512	1745	1823	2120	4009	1558	2319	2120	2584	3580	2330
citrulline	134470	172797	105903	154853	220162	168420	156375	186596	144276	134265	150447
citric acid	82089	102608	76658	113809	174108	72838	108033	100647	84682	127599	79802
cis-gondoic acid	365	343	458	379	495	520	467	629	415	432	392
cholesterol	801	634	429	755	952	414	778	555	691	1040	702
cellobiose	14367	14871	17767	17376	19942	11995	15163	13361	15173	18287	14671
caprylic acid	6984	6442	8353	8967	9477	6737	6394	6780	9787	8133	5942
capric acid	991	1282	1225	1235	1743	1161	1381	1188	1486	1173	1316
butyrolactam NIST	180406	204512	174337	221845	314827	156763	190273	214438	190757	208472	176909
biphenyl	1503	2142	1482	1207	2895	1658	1659	2387	2439	1467	2528

beta-alanine	937	1174	969	960	1820	1020	1026	2007	278	926	173
benzoic acid	26329	28354	29317	32112	40254	25225	31877	34617	29539	35780	26590
behenic acid	10588	14061	15574	18476	25251	13281	16288	17524	13321	20921	12164
aspartic acid	141455	138662	103044	161643	246371	106176	217254	269937	129414	576471	98574
asparagine	44932	70775	30083	73248	121425	42013	78548	58957	46720	75314	51642
arachidic acid	64646	75887	110875	84603	104373	104024	61051	108133	94083	68396	80157
aminomalonate	20417	29717	23089	29629	46043	27941	29430	29807	30734	35545	32058
alpha-ketoglutarate	2529	3241	3072	3356	4716	2390	4160	3529	2405	3925	2640
alpha-aminoadipic acid	1321	2099	1219	2547	3643	1562	2512	1082	1351	2840	1134
alanine-alanine	75846	91460	73120	130135	148429	59953	131407	120496	136948	176266	70607
alanine	8538199	7358400	8555331	4260275	5394732	8582780	5449388	5678419	6964616	5272247	7961012
adipic acid	741	805	931	695	792	699	707	927	986	844	801
adenosine-5-monophosphate	5100	7178	10747	10167	10168	11203	7963	11653	7247	9092	5959
adenosine	8199	10460	11002	14586	15794	9674	11705	15032	11381	32771	8597
adenine	50397	53003	42211	71565	78529	37761	63728	69389	70603	102972	35684
aconitic acid	1743	1487	1724	1594	2839	1528	2104	1920	1844	2368	1671
5-methoxytryptamine	7180	11032	4251	10868	12997	6948	10873	10448	8233	11034	7996
5'-deoxy-5'-methylthioadenosine	1163	1716	1427	1903	2278	1554	1816	2070	1751	1443	1428
4-aminobutyric acid	1712125	387464	2244243	1559283	1438189	2010905	1649393	1833805	2146881	1604375	2057023
4-aminobenzoicacid	748	1312	1096	880	1374	944	1432	861	1199	1097	748
3-phosphoglycerate	21514	24504	16567	29694	35124	17645	26478	20651	19098	49654	18937
3-phenyllactic acid	6677	7850	6486	8843	12198	5960	8519	8310	7013	8748	6589
3-hydroxypropionic acid	19658	20377	21190	22197	23897	15520	23238	24871	20664	25428	20184
3-hydroxybutyric acid	12329	14604	12160	14456	19847	9690	14826	14895	13058	13805	10527

3-hydroxy-3-methylglutaric acid	636	1326	578	1505	2009	579	920	761	708	1140	765
3-aminoisobutyric acid	57	162	78	176	6446	140	228	188	4179	195	102
3'-adenylic acid	423	210	503	275	359	352	484	153	390	489	88
3,6-anhydro-D-galactose	53198	57733	63161	84969	82068	57267	67520	71885	61684	89739	56623
2-picolinic acid	698	697	435	841	1022	729	714	886	806	1034	673
2-methylglyceric acid NIST	61574	70429	57277	76722	102374	54167	75062	73563	61067	84971	58532
2-hydroxyhexanoic acid	49763	46208	40328	51288	77560	33277	57530	54754	49613	47068	39268
2-hydroxyglutaric acid	11459	16216	12093	19284	25469	11617	16135	16597	15671	18479	13090
2-hydroxybutanoic acid	59466	59901	51596	59133	72019	57185	58899	68404	54618	58177	57446
2-deoxytetronic acid	8570	10125	8129	11093	15127	7572	10830	10633	8656	12521	8355
2-deoxyerythritol	4471	2416	5301	6204	7196	4310	4261	3500	7071	2748	3437
2-aminobutyric acid	26504	31421	23903	27628	39564	25037	28191	29389	26893	28171	25611
2,6-diaminopimelic acid	22100	33718	24032	23249	41841	26689	20205	29267	23549	23248	25293
2,5-dihydroxypyrazine NIST	1085	1698	1145	2083	3264	1295	1979	1999	1415	2764	1498
1-monostearin	781	897	789	1172	1708	791	1144	1019	805	1720	674
227437	924	1691	2199	1145	1575	1393	1563	1323	926	2106	1590
227427	697	1164	759	1144	2236	832	1293	1201	910	1208	1125
227291	690	800	788	1008	1175	443	583	807	470	1009	745
227280	743	1006	763	1144	1928	505	796	789	807	1536	840
227272	603	619	510	1136	1075	309	1080	714	627	1764	606
227260	755	642	684	753	1243	610	735	838	653	1018	679
227255	626	1168	494	1074	2582	501	1047	916	457	1657	749
227232	1772	1388	1992	1772	2889	1243	1203	1543	1537	1788	1629
227229	1223	1523	1119	2104	2782	853	1182	2478	641	2361	1170

227223	1120	1085	504	1650	2135	673	1319	1071	1351	1465	805
227216	1556	1464	946	1679	2187	878	2027	1355	781	1804	1428
227209	1447	1464	1460	2141	1536	1426	1921	2018	1553	2025	1284
227200	2316	3231	2202	3459	6044	2265	4431	3416	2838	3945	2540
227176	3526	4979	2832	5866	9658	3822	5931	5994	4351	6350	4714
227159	4336	6049	5895	6209	9017	5548	5775	6860	4161	7493	5062
227158	8634	12533	8884	13223	19853	7389	12831	12891	9071	14542	9837
227094	198	218	273	305	568	240	319	271	117	555	200
226710	487	969	1055	1293	2167	1553	1127	1083	582	1207	757
226556	1043	1167	935	1046	1235	608	870	952	660	1020	645
226546	828	798	1289	1233	1041	1167	990	1089	1083	1127	759
226436	4640	6035	4227	6564	11662	4109	5722	3111	3502	9485	4548
226431	4019	3520	2459	2920	1792	6128	4088	4047	5058	581	5241
226008	1094	1436	1423	1859	492	1502	417	1593	312	444	1193
225844	3524	3491	2611	5415	5943	1760	3691	3296	3227	4002	2608
225834	1920	2279	1942	2641	3368	1672	2632	2361	2105	2991	2030
225831	741	1209	1290	2250	4559	1070	1348	1696	721	2447	939
225797	1346	1443	1437	1790	2559	1330	1659	1575	1429	1886	1323
225783	493	754	478	1726	1045	585	859	1074	563	996	585
225773	6502	10442	6592	8611	12822	7564	8099	8764	5473	7049	5972
225765	4634	6748	4186	6489	9342	4605	7029	6236	4090	5806	4948
225748	18954	22335	17745	24612	35152	17649	24771	22584	19938	27989	18338
225741	14970	14605	6918	22736	56558	6856	22652	10479	7752	21862	8246
225526	331	351	614	390	604	535	705	601	637	772	487
225514	291	346	306	315	961	536	435	672	269	415	222
225320	1323	1112	2606	1676	2037	1398	1437	1438	976	1779	862
225305	1525	1391	1977	2055	2369	1503	1074	2303	2051	2360	1573
225303	1350	798	1130	1821	1776	1158	1560	1342	1360	1494	1078
225279	2736	3021	3322	3335	4465	2466	4172	3187	2854	4616	3131

225276	2558	2877	1826	2777	3539	2375	2982	2852	1959	2853	2360
225272	5148	6141	6748	6371	9439	4766	6916	7254	4870	8002	5364
225233	306	656	458	550	942	579	517	622	406	546	385
225226	588	405	643	580	653	393	397	861	726	479	310
225211	921	1093	637	1570	2796	1468	1603	2074	1267	1648	1206
225203	484	526	226	708	657	450	349	608	471	584	377
225193	924	750	1051	1167	1507	586	872	873	712	923	647
225192	708	693	732	1354	1887	613	854	1294	675	1541	599
225185	492	625	488	728	1191	523	755	656	607	805	584
225184	518	1024	552	1384	2234	767	1343	918	672	1460	846
225170	665	959	1594	1879	1283	1393	1395	1758	798	1787	936
225159	1023	412	769	490	1522	1213	1231	1456	1296	643	570
225156	806	825	696	643	1143	981	962	1403	799	821	712
225149	804	1008	950	1230	1669	870	1131	1113	698	1026	788
225148	1182	1700	2040	2868	4136	1622	1901	2427	1566	2698	1431
225146	1108	1181	1516	1189	1476	850	1316	1180	886	1963	785
225143	2037	3462	4106	3235	5780	2466	2593	5311	4518	28130	2194
225134	1117	1537	1242	1137	1341	1009	1134	1369	1313	1598	988
225130	737	916	1232	1466	1300	1411	1382	1153	880	2017	793
225117	697	1080	429	702	1199	447	986	456	699	801	677
225114	2605	1514	2146	2855	1066	1785	1274	2153	1776	3565	1787
225112	2221	1941	2940	3719	2436	2087	1713	3405	3538	1989	1519
225103	5142	8012	1687	9092	11748	2865	7313	3962	4768	3050	5062
225100	811	1070	1214	1071	1374	1168	997	927	580	1023	594
225096	751	826	2281	1156	1984	800	987	1162	827	3210	973
225091	2102	1762	702	1902	3166	1990	2363	2225	1690	2067	1887
225090	1167	736	556	1547	2871	954	1701	1402	774	2359	1169
225088	1998	2650	2364	3566	4619	2155	2952	2210	2304	4272	1875
225081	1284	1632	1456	2509	3263	1215	2660	1723	1270	2610	1289

225074	1774	2301	2498	4211	4530	2203	2647	2699	2192	3481	1973
225070	1464	1350	1937	2195	3108	1684	1521	1428	1740	2627	1215
225067	1353	1516	2460	218	2503	881	1948	1629	1302	2222	1087
225064	2040	2596	2286	3132	5967	2103	3023	2782	2172	3259	2094
225063	771	856	1201	1059	686	937	827	553	698	1464	663
225048	3062	3943	3397	4499	6976	2489	4133	3234	2994	5008	2558
225039	1001	1302	1581	1905	2179	1316	1674	1824	1335	2556	1088
225035	1209	9083	6310	14048	19911	6392	11944	6976	4591	4155	6008
225028	485	452	510	491	739	501	424	527	415	569	437
225020	6879	8080	7264	9392	14479	7278	9062	11583	5967	12776	6528
225017	5791	8021	5405	8856	13730	4396	7172	6313	6232	8638	4802
225013	1669	2488	1919	2272	3835	1539	1976	5153	2381	2857	2658
225012	17933	19788	8417	19991	40140	11982	22943	19554	15652	24323	13774
225010	14163	17622	13091	19859	24024	12848	16306	18103	14748	21005	14174
225006	12631	15808	12636	17697	24545	12165	16832	17466	13752	19198	13336
224998	806694	948050	679438	1327828	1733899	888023	1240412	944484	632051	136321	911895
224866	272	464	267	523	723	247	301	682	605	576	498
224759	229	179	249	250	191	292	191	209	252	242	203
224462	1114	1366	1030	1312	2050	1381	1315	1543	994	1599	1119
224435	710	838	1110	786	485	755	877	1107	644	939	881
224427	1592	2501	698	2011	3008	1582	2296	2540	1716	2918	1505
224423	1598	4006	2299	4424	4023	2075	2348	2655	2405	3246	1697
224377	46422	144508	57008	44046	119699	82619	110887	102989	129372	67987	120974
224374	232	700	369	323	1156	591	723	308	353	364	620
224359	826	1079	999	1096	1300	981	838	1138	955	823	540
224322	702	626	594	690	427	454	740	656	245	676	563
224312	708	977	765	891	2120	573	964	833	707	1296	599
224308	522	596	555	628	833	584	636	615	584	677	565
224299	384	1104	905	1249	1180	783	1156	1104	947	1239	780

224298	514	845	709	1502	2058	760	963	1408	798	1632	659
224287	901	1272	876	2028	2489	973	1796	1730	1258	1930	1607
224286	804	953	1011	1774	1898	844	1030	1226	855	1525	894
224273	900	1686	909	2337	4480	691	2191	1842	1165	3247	1386
224272	937	1024	742	1219	1738	731	1113	1152	1095	1348	849
224271	1377	1186	972	1787	2454	1298	1421	1897	983	1598	1196
224265	1284	1136	1476	1859	1528	1349	1611	1122	1165	1976	1454
224261	1266	1697	968	1603	1987	765	1505	1240	777	2270	725
224260	859	1028	1567	1394	2020	1481	1194	1554	919	1762	897
224259	1025	1371	1648	2243	3098	1086	1929	1610	1057	1582	1028
224253	1010	1121	908	832	1811	827	875	1217	860	1815	679
224250	1558	2111	1621	2199	3586	1438	2463	2063	163	2446	1552
224248	559	547	632	578	820	729	753	1175	561	675	610
224220	1388	1820	1771	1997	4160	1584	1835	1920	1475	2841	1332
224218	352	430	612	632	750	579	599	610	682	892	388
224216	1685	2031	1370	2139	3534	1375	3233	2570	1549	2377	1465
224215	226	1360	1212	962	3163	874	1648	2089	1312	1879	702
224214	956	1069	1084	1307	1615	891	1159	1166	978	1261	876
224211	1134	1571	2010	2063	3047	2635	2330	2698	1192	2336	1656
224205	609	891	998	1576	1895	624	1154	803	620	1032	951
224201	2036	1995	2101	2892	3400	1954	2212	2495	2394	3299	2073
224199	396	357	369	306	346	360	356	545	302	357	291
224198	627	545	541	1276	786	745	921	1015	696	1098	798
224193	436	409	837	726	587	737	491	619	574	641	252
224191	3957	4159	3786	4960	6251	3477	4661	4699	3605	4572	3342
224190	2631	3431	2093	1921	3118	1185	994	2102	1685	3437	1363
224183	1449	2902	1940	3881	3782	1949	2637	2905	2266	5721	2016
224181	235	161	345	208	278	268	266	210	385	281	268
224176	439	474	477	365	466	483	543	379	425	360	382

224170	336	561	697	666	605	516	610	506	650	573	546
224167	1298	1680	1400	2254	3777	1326	2009	2198	1449	2911	1030
224166	693	736	1607	558	554	798	858	572	648	784	930
224157	561	645	644	678	1044	637	886	832	571	872	592
224156	985	1447	1028	1579	2377	875	1607	1437	1302	1708	1219
224155	856	1064	895	1055	1334	847	905	915	937	1014	354
224147	2145	2572	3463	3385	5512	2411	3022	2954	2186	3960	2166
224143	2740	3158	3412	4629	6255	3023	3837	3958	2954	4843	2936
224142	244	245	284	180	222	293	289	195	278	224	268
224140	3871	5958	4544	6642	7720	3634	5706	4363	4464	6448	3617
224136	1490	1854	2202	2399	3276	1684	2099	1244	2027	3491	1550
224131	3179	4334	3156	5459	9364	3610	4432	4478	2800	5995	3246
224126	5376	7511	5640	4449	15763	3822	8305	1877	2156	11472	4841
224124	637	696	820	5625	3866	702	2704	704	837	3692	508
224123	939	1073	1165	1992	2092	834	1229	1097	892	1806	762
224119	1419	1680	1097	2343	3087	1277	2179	1426	1652	2491	1683
224116	1819	2360	1010	3090	3904	1054	2287	2047	1489	2861	1260
224115	1348	8600	5640	7894	11096	6101	2533	9490	7334	7070	7898
224112	2769	3304	4740	4966	6770	1973	4575	2224	2371	6440	2546
224111	9619	10031	12087	10643	4662	11198	6013	11871	11767	12144	8885
224110	10902	11931	11296	12333	20729	10245	13488	12865	9458	12704	9278
224109	4348	5599	4963	7678	10857	4617	6424	6407	4925	8285	4528
224107	2798	5465	3869	7414	11094	6787	8436	11506	3938	9000	6990
224106	5580	5819	3933	8142	9251	5092	6662	4125	5966	8284	4233
224105	477	492	545	611	704	610	515	682	644	886	507
224103	6276	7596	6815	9850	16892	5876	8929	6589	1384	11263	6069
224102	765	1037	861	2015	2234	770	1259	1011	1103	6838	981
224100	11937	13592	13102	15179	23607	11535	15151	14628	12823	17000	11528
224099	288	371	403	411	454	363	427	323	412	547	273

224097	5015	6320	6318	9443	11508	5198	6278	6729	4983	7746	4955
224096	9587	15458	11216	19606	2694	10140	14068	12811	10955	14824	9871
224095	13939	16271	12061	16217	17385	11686	18659	14955	12220	17793	10569
224093	9204	3539	2640	13345	6119	3179	3783	11553	3501	4787	3766
224092	10354	11131	16286	18202	24558	8250	14964	12734	11728	18785	7920
224087	363	609	335	248	787	341	331	516	637	414	345
224086	1801	2176	2122	3071	3052	2098	2279	2431	1827	3758	1784
224083	20033	24006	30207	35509	63696	25097	35416	35465	23239	46085	20485
224079	7181	8468	5239	10225	12804	4034	6637	8814	7850	8869	4488
224076	580706	438890	615578	588578	318767	585913	590711	594437	640684	601912	551923
224075	366324	449085	360978	482346	814272	280296	473292	463187	245316	445127	250331
223946	206	244	267	389	531	207	341	561	506	412	183
223940	222	163	297	391	526	183	272	563	416	448	92
223771	528	578	516	722	887	561	604	752	640	802	537
223766	358	422	279	460	661	312	451	776	385	533	295
223753	291	439	314	547	1175	304	558	469	291	664	313
223745	264	466	277	406	821	483	514	413	332	777	409
223738	349	493	300	422	981	668	902	859	608	1850	444
223737	320	525	490	1006	2055	453	706	616	531	1168	457
223659	315	1686	818	1940	2791	1565	2076	1781	1547	4901	1399
223624	1343	1917	1437	2079	3319	1395	2145	1970	1923	3055	1641
223564	533	1089	457	1217	2972	792	1268	1494	700	1369	1079
223555	404	997	681	940	1225	643	731	788	920	1032	555
223542	1363	1936	1493	2558	3529	859	2130	1681	1347	3408	1308
223535	802	999	613	1011	1153	1177	1061	845	1069	925	718
223522	754	868	608	574	1527	669	1046	905	752	1093	661
223516	1039	1050	1632	2164	3491	1000	1469	1702	767	2623	596
223491	1621	2003	1872	839	1065	1450	2428	2414	464	2603	501
223452	2588	1112	892	3922	2741	1049	873	3692	2682	1397	2231

223421	473	610	429	711	1013	435	631	554	786	679	468
223419	364	365	487	439	610	409	648	562	654	494	573
223418	403	449	502	484	472	430	300	595	482	542	337
223415	1209	1081	1004	1328	2302	915	1289	1243	706	678	904
223412	791	594	672	848	1589	658	813	950	752	1028	620
223405	529	795	490	616	1706	454	759	680	325	968	684
223398	534	717	415	576	1247	485	747	634	603	787	621
223397	456	570	560	959	1009	498	560	645	392	522	391
223394	468	660	537	758	1094	500	729	623	554	762	450
223393	512	689	1080	1005	1375	777	749	995	366	991	594
223373	603	534	732	449	614	565	473	654	653	755	558
223367	513	763	307	702	1072	581	793	815	470	925	562
223363	846	513	642	739	735	638	703	772	738	749	583
223359	640	1004	898	904	1163	652	742	747	627	1412	561
223339	615	834	937	1159	2351	913	1059	1079	764	1389	763
223337	743	1270	777	1453	2919	817	1323	1446	938	1491	1073
223335	637	919	645	1158	2082	681	1168	930	688	2389	765
223334	898	1068	875	1247	1766	797	1269	1099	882	1349	792
223330	901	1374	655	1379	1957	730	1666	1074	841	1612	962
223327	705	1566	964	1996	4746	867	2122	1090	677	2127	1155
223317	715	1273	1167	1896	3888	976	2388	1810	927	2353	1331
223316	987	1345	1033	1452	2192	861	1503	1212	1065	1545	1007
223315	642	1069	740	1086	1502	838	1240	1240	857	679	817
223310	981	1257	1444	1521	2110	886	1344	1194	1105	1464	997
223306	1084	1348	875	1373	1729	835	1501	1244	1079	1490	974
223302	1044	1002	1067	1514	1727	1028	1431	1044	1774	1639	998
223299	1073	1348	1056	1551	2123	892	1422	1339	1171	1679	1099
223298	988	1296	817	1456	2512	797	1468	1217	1057	2486	1033
223295	602	1204	859	1642	4213	1151	1606	1950	800	2035	1173

223293	988	1159	929	1286	1953	876	1374	1267	1042	1805	1021
223292	716	867	1598	1150	1773	953	1104	1404	649	1862	541
223290	1084	1536	854	1287	2766	951	1688	1886	1475	1191	1536
223289	2268	2671	2034	3903	3508	1997	2831	4144	2302	3187	1939
223284	1339	1169	2033	1990	1953	1656	1674	1569	1232	2693	809
223280	1052	676	929	1526	3141	1032	1444	1087	1556	2465	1017
223277	884	1117	1146	1240	2098	945	1129	1233	919	1396	888
223274	781	1196	1424	2068	3244	1144	1421	1683	1110	2047	1021
223272	745	1286	1360	2269	2772	1284	1624	547	622	2102	841
223271	2495	2601	2363	3439	4027	2381	3144	3119	2521	3148	2406
223268	1912	2419	1254	4537	8106	1464	3274	3057	2443	3473	1852
223266	1340	1920	1328	2392	4727	1360	3337	2522	1487	2097	1874
223263	448	913	1394	1745	2549	1451	1727	1586	917	2020	1031
223259	1306	1425	850	1717	1451	960	1839	1621	765	2670	1030
223253	993	1353	1379	1704	2283	1184	1592	1256	1199	1837	1131
223252	1551	1628	1805	1940	2398	1436	1807	1974	1605	2371	1526
223251	1288	1728	1302	2433	3544	1145	2052	1615	1294	3557	1172
223250	1585	1786	1474	2044	4327	1160	2147	1881	1332	3959	1251
223249	1909	2480	1772	2607	3798	1607	2574	2306	1968	2813	1812
223248	2566	2465	2983	4428	6249	1538	3188	2822	3187	8314	1688
223244	1126	1357	879	1144	1545	1214	1700	1730	1248	1227	1127
223243	957	2004	1152	2147	5999	1600	2922	2521	1328	3117	2039
223241	1292	1659	1255	1838	2757	1158	1864	2316	1804	1689	1286
223237	901	1237	1567	2481	2269	1462	1564	1654	1103	2759	1119
223234	1071	1803	1387	1489	2429	1404	1982	1521	1399	3361	1339
223228	1550	3456	2075	5300	8657	2197	5802	4440	2641	6004	4191
223227	1510	1667	1656	2222	2310	1603	2025	2012	2013	2173	1646
223226	1136	2033	1388	2401	3722	1213	1991	1886	2336	3429	1315
223223	2176	1693	1666	1947	1360	1727	2048	1136	2078	1612	1336

223222	1887	1636	2420	2080	1819	2113	2212	2303	2330	2116	1801
223216	1621	2028	1496	1597	2418	1403	2267	1825	1943	1652	1878
223214	1002	1311	2107	2090	2812	1899	1976	2209	1322	2262	1332
223211	1715	2654	1395	3217	4505	1393	3292	2568	1755	3339	1831
223208	2194	2975	1617	2768	5320	1737	2891	2036	1844	3438	2029
223204	2645	2401	1801	2478	3197	1967	1937	2242	2104	2518	1708
223203	1013	1503	2276	1995	3535	1490	3049	1714	1160	2448	1630
223202	1726	2073	1786	2474	3129	1597	2355	2070	1778	4477	1629
223201	1170	1900	1403	1978	3960	2118	1639	2739	1587	1195	2000
223200	1566	2118	2638	3293	4613	2036	2280	3412	2051	5217	1952
223199	1702	2852	2374	6238	8326	1837	5419	2956	3237	11133	1843
223196	1991	2823	3050	3966	2533	1856	2508	2827	2449	4082	1684
223195	1825	2350	1853	2744	3967	1749	2572	2407	1960	2987	1775
223194	5175	3757	4945	7638	4524	4618	5043	636	2913	7180	3242
223192	4132	4669	3423	5118	5362	3123	3886	3948	4694	3168	3500
223187	1253	1604	1741	1151	1810	1877	1234	2929	1980	1747	2128
223186	2485	3452	2072	3526	5406	2180	3148	3019	2304	2940	2357
223185	2165	2912	2191	3100	4400	1677	3153	2580	1962	3877	2072
223184	1796	2347	3507	3414	4589	3423	3392	4054	2109	3922	2401
223177	2048	3261	2330	4118	9502	1909	3741	3191	1920	4741	2597
223175	2496	3770	2259	4477	6235	2445	4017	3768	2104	4660	3367
223173	1232	1672	945	1328	2396	2236	2216	2278	1729	1816	1395
223172	882	2113	1502	4501	5502	2322	3169	3355	1160	4629	877
223171	3511	3764	2235	4703	7309	2832	4970	4677	3411	4534	3281
223169	2241	3128	2127	3478	5176	1925	3351	2756	1707	3870	2025
223167	2930	3933	2668	4292	7093	2531	4518	3224	3025	6061	2949
223165	2059	2838	2724	4508	4821	2320	4028	3891	2307	3862	2219
223164	2434	4470	3814	4163	6850	2281	4829	3795	2965	3453	2673
223160	2640	3694	3563	3810	5219	3656	3517	3892	3348	3835	3335

223159	2005	2411	3825	3191	4526	3200	2580	3989	2846	4595	2502
223158	3566	4315	3987	5490	8497	3545	5740	4747	4018	6216	3492
223156	2050	2433	3099	2966	4544	2305	2531	2671	2125	3353	1907
223155	2685	1194	10136	4156	2868	2997	2656	1933	2510	4603	1872
223154	2873	5270	2469	2973	9180	2003	3888	3306	2016	5648	2627
223153	1390	3469	1859	1477	2330	2485	1851	2857	1530	1100	2532
223152	3667	3072	3806	4876	3084	2575	3652	4106	3189	5158	1502
223150	3101	2543	3168	3276	3482	2647	3267	3265	3265	3678	3068
223149	4193	5336	5030	6831	9702	4215	6120	5778	4324	6872	4145
223148	4257	4944	4200	5996	8068	3838	5470	5184	4478	6255	4205
223146	2026	2730	3720	3289	4670	3518	3110	4063	2048	3437	2670
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223135	5322	4725	5385	4814	7499	3578	5313	8484	6911	8083	4501
223134	3180	3380	4137	4341	5219	3471	3885	3927	3944	6003	3287
223132	2719	3980	2971	4164	7822	3370	4346	3800	4184	4593	4798
223129	3419	4307	3042	5863	7744	4384	5183	3793	2314	578	6355
223126	3429	4021	4297	6620	8276	4299	6826	6534	5030	7320	2588
223125	4930	7256	5837	5240	9977	5151	5125	4139	5200	4795	3510
223122	3222	3090	6064	7252	10908	4701	6625	6519	4551	12965	3040
223120	11946	11776	5424	18179	46246	5348	17920	8436	6145	17671	6553
223117	5133	6151	5012	7144	9983	4501	6969	6596	5406	7757	5252
223116	4104	5279	4968	3399	5489	5492	3390	8218	4835	2779	5867
223113	5955	6471	6999	7398	9695	5583	12434	9290	7551	14096	6821
223109	6129	13043	7266	11916	21880	9514	13181	12945	8054	13065	12174
223108	17467	19971	16333	22694	30768	15061	21319	20308	18313	23211	16734
223107	6492	8013	6296	8826	13153	5765	8642	8139	6765	9756	6394
223106	8030	10251	6948	11046	18198	6407	10885	10125	7639	11904	7887
223100	9376	11566	13149	21577	25052	8725	15792	11626	9294	5248	7466
223099	7795	10749	4953	11682	12085	7501	7636	7681	9992	8699	10004

223098	7567	8989	10720	14969	17906	8212	11342	10953	7220	15964	5436
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223096	7609	9045	9803	16149	18727	9509	13702	12868	8150	24634	7039
223095	7101	9719	12240	14219	20143	10693	12755	13474	8764	16928	8645
223093	6616	9118	11837	12761	16742	12327	11671	15162	7954	14367	8945
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223087	24783	30155	27978	37405	44046	24404	33432	31915	27276	53763	23929
223085	25784	32437	32789	45853	53763	29228	39073	37897	24871	46555	22106
223084	13648	21472	16415	25859	53007	14291	23930	22703	12101	28759	16834
223083	15346	22885	17603	27253	56036	16117	25751	24277	14167	30976	18604
223080	54280	68996	63500	80010	157150	57846	79662	95200	58366	128431	53192
223078	57751	56181	58860	69046	70440	51191	63443	60091	56786	76784	43848
223077	29251	44347	42533	56948	91762	40581	47798	47646	35425	63304	34536
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223071	880457	1065570	800570	1136594	1609124	722345	1169353	1053319	900722	1367959	890919
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222267	553	662	622	14035	5476	604	7535	733	662	12316	679
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217691	16805	18201	17943	19661	26858	18709	20309	21479	17771	22732	17605
217651	2917	4318	4233	4971	7581	3257	4297	628	2925	5543	3384
216193	2505	3404	2523	3895	5593	2321	3562	3319	2839	4080	2646
215896	5013	6557	5622	6840	10011	5553	6852	7529	5346	7347	6139
215857	43402	51218	43836	58914	83297	39380	56056	54091	44454	65273	41729
215855	580	571	549	613	1006	565	574	619	578	824	444
215853	20720	34590	24016	50073	103878	28646	48054	49443	26364	52958	37157
215800	1905	2114	1231	2428	2541	1697	1770	2157	1408	1960	1308

215769	1290	1750	1030	2023	3347	1218	1958	1907	1365	2742	1467
215691	2718	7597	2961	8926	18863	5992	9355	5069	5438	3708	5006
215683	259	209	302	780	494	254	377	239	232	448	177
215676	4032	6394	5457	9395	11296	3316	8563	6110	3130	9121	5094
215671	344	730	793	1868	5475	1715	1410	2108	1054	2106	1220
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215634	1520	3240	1544	4426	16304	2310	5414	6704	2069	5974	4215
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211195	2276	2505	2599	3106	3560	2127	2968	2660	2747	3394	2187
210999	7634	7224	7954	8594	10624	6361	7730	7935	8545	6997	4987
210996	2715	3491	3346	4325	4729	2445	3691	2667	2582	3188	2608
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210885	2163	3102	1901	3052	5347	1930	3053	3022	2232	2915	2444
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210841	1439	3881	3948	2512	4614	3525	4520	5168	3104	2660	1960
210712	1131	1169	584	868	1962	945	1650	1602	1160	1724	1100
210697	2348	2548	2263	3049	4029	1945	2750	3071	2668	3927	2299
210403	770	829	755	1056	1194	754	910	1008	837	1076	749
210321	2017	1591	2699	2085	2195	2342	1948	2802	2630	2265	1952
210316	2014	1702	2778	1987	1908	2212	1992	2630	2434	2264	1927
210297	5963	9645	9714	12268	15345	8297	11058	10915	8594	12959	989
210272	383	373	473	575	587	468	398	466	398	10396	419
210262	544	482	822	615	6526	321	515	3252	562	458	723
209690	455	486	574	579	822	430	534	653	460	677	371

208761	8424	7945	4762	7173	10809	6118	6850	6964	6717	7049	7665
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203809	281	634	472	841	1172	974	533	973	506	557	545
192876	9235	11276	9730	9551	3603	7482	10694	9463	9296	8572	9495
190007	1036	1180	552	1601	1757	291	1451	1130	500	5379	1091
187055	263	655	574	787	1030	282	870	575	620	1022	549
187054	1607	2082	2963	4701	6928	2388	2777	3027	2215	5004	1925
185032	4834	5094	4660	6131	6818	4623	6051	6113	5288	5349	5165
183433	37430	31095	47854	39704	33113	44605	37721	49313	47544	40925	36093
183315	1797916	1373278	2018735	1544056	1795290	1830862	1587356	2030004	1841094	1439132	1624168
183273	325	877	949	1013	1362	723	969	982	925	916	627
183148	4489	5668	4507	6330	12096	5457	6129	5638	4753	10528	5817
183117	745	2074	1165	3491	5922	722	1730	1507	1613	4095	1849
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170974	10355	13253	10782	15281	23363	10128	14964	14366	10978	16240	11037
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170512	693	861	415	940	1301	643	1056	996	861	1075	666
170508	2028	2574	2077	2779	3769	1806	2845	2528	1977	2920	1966
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167348	3255	3763	2574	7128	6669	2373	5029	342	3507	204239	2458
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161376	507	288	307	874	881	360	366	632	306	763	337
146957	683	566	1050	839	2070	637	621	924	813	749	733
146655	2003	2342	2136	2639	3416	2013	2387	2570	2815	2624	2225
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133626	1199	1462	1435	1737	2378	1242	1444	1454	1264	2005	1169
133612	613	695	998	929	1423	1044	1100	793	643	1049	575
133590	4780	6007	4670	6624	6433	3301	6794	5950	4939	9181	3711
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130801	4148	6141	4963	7565	8397	6053	6916	5889	5898	8002	5364

130800	4919	6055	6292	7365	4965	5473	7771	6206	4184	6468	5609
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130781	2286	2564	3667	7055	8370	1384	4748	2277	3712	20290	1663
130746	4742	7598	8276	7067	11377	7003	8872	5455	4444	7675	5273
130741	1657	1588	1935	2795	4358	1341	2214	2243	1092	3204	1435
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130640	12582	16082	14643	21143	29620	11919	19782	18040	14665	23419	13284
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130531	6506	8533	4969	9588	12457	5283	10160	7828	6296	10130	6194
130530	7579	8967	10331	11971	18029	9475	12232	12767	6751	14858	7841
130528	4225	7288	3271	9398	9932	7267	7469	8721	5820	10583	6785
130527	53241	55053	44571	107222	110329	37874	70333	53291	49522	111336	37972
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128034	3754	4750	6195	7745	9000	4184	6099	5812	4667	11018	3705
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125897	5685	7216	4926	9549	12025	6011	7941	7868	4425	10101	6235
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94582	383	279	256	2938	1192	240	1450	263	243	2543	175
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64926	119	108	561	99	345	482	529	494	378	128	398
64751	762	915	1089	1147	933	993	632	1090	899	736	360
64557	2991	4147	3364	5120	9372	3146	4827	4705	4675	7947	3546
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47358	524	729	752	867	930	628	609	690	732	627	562
46377	939	887	1574	1385	1256	1049	959	486	1490	1141	817
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4937	303	136	637	228	250	599	430	481	356	371	244
4929	29862	59280	35447	57329	153017	32941	73804	68433	32736	84039	42750
4837	3734	3245	4056	6182	4507	4925	4283	6055	4529	5732	4960
4793	37616	48333	45973	65650	85047	39738	43797	53483	39408	55495	29656
4746	2550	2484	2259	2511	2366	1964	2446	2642	2133	2876	1176
4744	7928	10524	10657	11935	25142	10128	12332	16413	5023	12659	11287
4723	2341	2543	2269	3719	4818	2921	3098	3627	2385	4323	3053
4721	2556	2618	2348	3735	4466	3192	3323	3907	2439	4416	3275
4550	819	1043	1580	1402	1512	2371	1489	1475	1203	1610	1112
4546	5943	9771	7449	11416	16586	7010	10921	6358	7975	24090	7628
4219	48651	54345	56309	79155	134308	49624	67292	65221	40611	112708	37537
3781	1501104	1833172	1713935	2010978	1249268	1563960	1969463	1640010	1559544	1989447	1330957
3618	1546	1908	2816	2596	6506	2671	1917	2837	2759	2956	1841

3228	1309	1859	1748	2373	2079	1109	2358	1755	1674	2070	936
3207	12473	13731	12055	5255	21093	10444	15902	4843	12789	19283	12109
3188	17838	18482	12728	23771	37729	14975	21863	25486	16353	16075	16310
3044	6087	8076	11299	17341	23134	7259	14849	10677	12119	28658	7146
2900	27777	32728	29766	72878	56126	31569	25381	42212	32169	47834	24147
2806	13028	16944	14417	15030	22684	14704	15290	17035	11272	20045	16933
2749	6722	11520	6755	14715	34278	10591	17574	18990	9469	16944	14494
2673	4120	4895	3247	5398	12618	3640	6087	5933	5423	7898	4865
2667	3510	4711	3432	5330	8943	3186	5025	6160	4486	7323	3451
2665	1922	3821	3590	7088	9216	4024	5702	1971	1258	6487	2420
2662	2351	3145	4040	8169	9823	2176	8182	3607	4259	17125	2541
2659	1353	2654	3291	4230	3591	2516	1959	1763	909	7431	1273
2647	48741	47591	54227	95270	124780	33654	63757	56839	48365	134490	33998
2561	23908	31848	36921	41554	59211	26133	36697	43174	26770	43035	31111
2547	18052	22094	20796	25347	33693	17142	23636	24419	18544	29655	16819
2543	9129	16161	12546	24165	48981	16839	21645	26432	11934	27758	19171
2466	6213	10849	5115	15727	17262	7482	14133	5443	4432	17747	6534
2439	5578	5726	7255	9727	10087	9989	9031	7064	7543	11233	5016
2403	1295	1482	1625	1344	1594	1406	1511	1445	1811	1521	1271
2392	3008	5789	3597	7114	9310	2749	5671	4544	4724	20628	3145
2242	9128	7239	9004	9322	6872	5268	9547	11241	14158	7867	6686
2233	11769	10974	9439	16222	19365	9552	16051	14932	13408	20514	8314
2208	1977	3344	4832	4151	4520	1823	3247	3055	2704	5061	2355
2193	1014	1243	1431	1277	2068	1393	1368	1318	1086	2223	962
2189	870	978	1314	1425	2699	692	1155	1246	640	1833	909
2061	3148	2684	3948	2318	2816	3594	2471	3054	2896	3508	2487
2044	13225	17004	13960	21792	36032	10398	18832	17546	12885	26147	14132
2037	1158	1242	2670	964	1028	1000	640	2711	1033	7494	756
2031	298464	376744	276457	405204	621963	258335	401429	369599	297764	438139	292271

2018	2629	2962	3201	3872	5667	2667	6760	3580	2503	4646	2689
2017	4826	8409	10037	9700	13557	7742	6170	11188	5100	10511	7177
1996	46372	50827	47609	61900	72726	42453	59402	59860	50844	65185	46932
1981	117007	130425	106399	144887	187910	105073	142900	143206	119646	160220	114684
1970	62831	56450	59533	81646	105102	53419	80319	78684	63348	98792	59744
1941	8914	8349	9235	8832	9387	10216	9002	12132	10761	9853	8568
1925	2581	2196	4551	3100	3381	3640	2686	4132	2258	3769	1844
1912	1843	1981	3236	2597	2778	2897	2186	3378	2826	2464	2241
1875	4619	4875	4598	5424	11428	4789	5805	7093	5653	10951	4733
1872	4215	4357	5314	5003	6359	3424	4068	6374	5413	5881	3279
1846	3234	3746	3956	4574	6470	3436	3528	4136	3211	5548	2921
1840	6957	5458	6730	11857	13768	3587	9119	10443	9564	61809	5648
1833	21310	26691	23907	33382	47945	21173	30913	30001	23548	36449	22407
1832	1561	1779	1547	1755	2407	1496	1665	1902	1638	1787	1600
1831	33739	39305	33851	49138	74139	30902	42796	43773	33154	52595	31974
1830	27699	50567	37118	65807	113300	40473	60622	56146	32866	73848	42530
1829	48231	59702	60600	124525	124260	48303	77471	62437	57721	140995	44576
1805	1051	1013	1112	1381	2157	1105	1348	1310	1048	1446	1294
1801	9318	10845	9514	7720	18249	8890	11612	8971	10394	8518	9475
1799	13340	16157	13348	17438	6681	10321	16206	17691	12506	18540	12751
1781	29335	43330	35436	41459	65168	37843	30322	40226	22847	54369	39003
1767	32425	56964	37415	34158	52608	42827	37936	43143	19616	51100	38907
1751	9933	2451	2176	3805	22544	1787	16353	2198	11371	5103	1634
1750	35375	48846	35447	70876	125152	39364	62069	56870	40485	84039	51927
1746	18451	37075	20125	61221	87823	25967	51601	19437	13826	57647	21867
1740	8870	11514	10777	14056	23268	10220	7921	17557	9367	32582	7584
1737	49822	71223	78972	93337	135918	67849	84042	76386	45109	88986	59473
1735	22690	41363	24048	62448	98591	28524	54996	21264	15580	60081	24390
1732	40682	51850	37306	46487	65949	44175	45567	54881	29282	41471	42228

1725	2161	2612	3108	4091	3431	2537	3042	3392	3513	3353	2026
1721	69181	116829	94588	170494	281188	91894	140800	96622	57887	211034	80849
1717	71869	123780	91355	169562	274671	83226	135354	76773	45547	183762	77261
1713	83214	153274	94173	213332	403563	112856	203190	157031	84275	226915	125079
1711	1800	2293	1381	2697	7205	1262	2647	2287	2029	3526	1132
1708	61586	124629	58655	219270	311835	83740	188680	61555	40328	189894	68555
1705	313015	404211	402475	527386	770462	362411	464817	467439	354934	585874	337683
1702	37248	76981	35387	132486	176243	51024	111973	31099	22132	118705	40396
1700	1494	1357	3109	1618	2341	2771	2813	2261	2149	2303	1656
1684	747	766	711	963	1083	677	936	907	1027	1106	561
1661	9242	11890	6891	10701	17523	7275	12075	15296	9786	9999	11223
1380	28075	15156	95230	50206	85719	47678	22689	123293	29250	54716	20746
892	6160	4567	6985	4093	6850	5895	8717	5882	2601	4307	4253
782	437	449	351	4916	1978	418	1604	430	437	3028	275
657	14964	14513	21447	17878	16747	14536	15280	9416	19607	16467	12207
592	13819	18828	17865	34996	49810	16957	19449	22478	15610	28764	15452
453	1387609	1119738	2413604	1716479	3028695	1545270	1185508	2991971	1469951	2167429	941002
307	59761	55963	78678	80617	109436	54347	64065	70905	58740	117148	53285
299	13111	9741	56451	19177	11395	15484	11664	18503	16159	18857	12220
168	452	426	559	714	618	1017	676	979	894	456	1335
137	55242	46284	68845	48279	47894	59909	47840	63544	63001	61374	52492
134	4555	2675	14888	8024	4934	13031	7214	11586	6034	5882	9008
110	8382	8885	8975	9274	9510	9514	9324	12585	9272	8211	6852
91	3358	3739	542	3457	3970	3115	3098	1384	2784	3851	5453
47	6326	4618	8027	6742	4923	5546	7016	5526	7828	5499	4308
39	5679	5345	7563	6816	6561	6556	6553	8258	6814	7326	5238

APPENDIX C

Metabolite	*C. perfringens* JGS 4104											
	Anaerobic jar						Anaerobic chamber					
xylulose NIST	9944	18084	14142	8537	13211	12837	10148	9746	12253	15356	13955	11930
xylose	554	800	577	503	601	548	665	617	587	634	689	678
xylitol	1000	2279	1492	1222	1153	1442	1239	883	1237	1270	1423	1346
valine	5500341	3049851	3678104	4175784	4642714	4470871	3815216	4807895	3438789	3279873	2393073	3261095
uridine-5'-monophosphate	2636	6441	5097	5184	1728	5885	2841	1563	4691	5049	5571	6711
uridine	308	904	698	321	251	584	491	263	556	588	1032	577
urea	10906	1055	19929	13476	11059	21070	18478	10032	15724	21416	17265	21001
uracil	8437	19756	15849	11488	10019	12428	13768	10378	24690	18745	32713	18157
UDP-N-acetylglucosamine	496	840	1235	894	606	849	615	517	876	682	151	760
tyrosol	2484	6698	4084	2991	2969	3479	3434	2678	3440	4595	3880	3926
tyrosine	1543055	2546413	2581578	1818126	1755053	2189355	2269133	1638920	2160802	2602203	2700741	2442132
tryptophol	2098	5783	4746	2747	2625	3170	3199	2341	3074	4396	3703	4392
tryptophan	1820147	4152419	2831795	2191960	2020580	2475530	2399407	1742260	2236049	2984268	2686023	2646208
trehalose-6-phosphate	269	219	239	349	276	273	215	341	280	276	235	201
thymine	465	1325	785	1009	477	614	604	503	802	1007	910	800
threose	1559	2919	2056	932	1151	1494	1576	1503	1140	1683	1243	1575
threonine	670404	1356081	1261447	994997	936280	1086438	1071644	758528	1135999	1080215	1203562	1165613
threonic acid	774	2041	1106	882	840	1054	1001	709	1064	944	1120	1103
threitol	780	775	1365	1205	1256	1116	1026	934	1200	586	962	739
sucrose	15	919	635	674	621	632	444	352	372	528	374	524
succinic acid	22029	65470	36725	25359	26478	34696	30291	23161	32606	31398	34026	36968
stearic acid	223255	199180	243132	206331	236878	205417	189850	185932	222464	220348	284164	237762
spermidine	13831	59062	27363	26127	19196	24254	27846	14853	27527	31274	28870	30821
sophorose	507	959	765	739	928	800	575	756	764	731	874	807

serotonin	2076	7266	5515	4176	3910	3730	4053	3518	3801	4286	4098	4096
serine	370456	1128758	574705	423449	398566	497066	492523	327769	500351	499318	494614	516654
salicylaldehyde	2172	3946	2205	2419	1894	1766	2699	2533	2669	2577	2957	2107
saccharopine	1490	3729	2311	1964	1588	2350	1971	1260	1713	2144	2157	2252
ribose	19688	33995	25807	20575	24293	20893	22256	18566	35555	28050	50415	26576
ribonic acid	5351	16003	7044	5072	6158	6255	7627	5628	5900	6040	6608	6707
ribitol	2250	9230	2636	4031	3913	4886	4989	2390	4760	4275	5877	5617
raffinose	1097	832	1095	846	631	923	635	428	674	946	782	945
pyruvic acid	387903	316901	345445	332223	474726	323913	282766	226724	241923	299595	198891	263260
pyrophosphate	41914	167049	111129	64710	48057	99604	75349	46932	129259	63205	135919	111926
putrescine	34109	92552	56037	42351	41212	50568	48880	35199	47070	47647	55861	55187
pseudo uridine	8855	26054	16487	10919	10974	14319	10671	9928	15614	15416	22623	15934
propane-1,3-diol NIST	64664	83920	69051	67142	58908	74466	75551	54717	62346	20830	76041	74378
proline	3680682	5324973	5024169	3480539	3517894	3722945	3804227	3709613	4287682	3257783	4493862	4189502
pipecolinic acid	5222	20896	12173	7431	7255	7311	6299	7099	8650	4418	7217	9600
phosphoethanolamine	25717	25726	25389	30379	20625	26944	16344	16702	20391	17689	19200	23088
phosphoenolpyruvate	2189	6121	4731	3769	2637	2645	4162	2934	7581	1338	8339	5195
phosphate	608711	348589	495182	641132	636877	117628	504370	485699	544915	506016	511538	470909
phenylalanine	2411380	3095941	2739210	2950824	2871104	2783370	2950179	2714517	2815367	1708701	2556828	1669432
pelargonic acid	16872	11547	12700	18154	19635	9257	11167	13856	14578	11992	16712	10567
pantothenic acid	966	1342	821	1097	878	1052	1031	464	1074	926	712	803
palmitic acid	26936	25780	29950	27293	30810	25257	26438	24128	27812	29310	34151	29201
oxoproline	4783686	6525965	3451024	5802722	5571886	4807641	4496228	5247463	4209759	4821629	4131193	6353479
oxalic acid	666	2374	1178	1181	1607	2013	479	1214	1765	7870	1479	1602
orotic acid	2363	4784	2192	2464	2462	2326	1961	1635	1681	2157	1670	2572
ornithine	4094865	1325538	4728066	4483984	4536736	4545989	4269584	4044538	4541454	4583227	4495286	4573008
O-acetylserine	4617	13575	8410	6468	5936	7962	7353	5664	8069	6800	10094	8751
norvaline	4125	2946	3027	5086	4021	2680	1993	3998	2936	2364	1859	1761
nicotinic acid	55553	146963	89883	65451	62612	78287	74863	54295	83410	87629	96537	87174

nicotinamide	847	1750	1257	1030	546	1045	1207	1107	1225	994	1940	987
n-epsilon-trimethyllysine	862	1491	984	926	941	1218	1125	1067	1023	975	1207	933
N-carbamylglutamate	2881	7607	5055	3636	3783	4721	2623	2466	2901	4771	2754	4592
N-carbamoylaspartate	3464	6174	7172	4070	4312	5301	5985	3755	6527	5175	7950	6231
N-acetylornithine	1758	2503	1777	1529	1560	1284	1292	1413	788	1786	1068	2305
n-acetyl-d-hexosamine	5800	14643	11485	8108	7351	10150	9433	6948	9462	8216	8978	10274
N-acetylaspartic acid	1946	6783	4490	3358	3073	3910	3838	3024	4864	2690	6380	4896
myristic acid	1807	1805	1899	1780	2255	1694	1822	1640	1977	2132	2600	2023
myo-inositol	22913	60735	35545	27649	26006	31496	31533	22515	28862	30901	33392	33513
methionine sulfoxide	787488	2265407	1322571	981339	925479	1148836	1154672	792975	1073979	1112498	1233839	1288411
methionine	351876	1337131	744188	442801	446725	546081	582866	440171	677248	785009	843031	662015
mannose	43723	91328	64205	50986	49617	55710	52121	45545	48793	58893	53576	64609
maltotriose	297	196	186	252	303	224	213	385	251	167	196	232
maltitol	596	680	543	563	710	512	541	621	556	556	620	515
malic acid	2810	8218	4853	3467	3308	4377	4074	3020	4277	3790	4544	4518
maleimide	41239	75725	58671	53146	71264	54627	66006	35145	45102	67835	51784	46452
lyxitol	3305	7802	3834	4248	3650	5149	4660	3510	5054	4678	4022	4035
lysine	2772156	2771260	1274554	2789081	2666269	2464532	3328722	3444144	3484421	2993501	2215847	3031480
levoglucosan	1926	6411	3714	3174	2304	2934	2669	2054	2967	2978	4273	3044
leucine	6016527	1724883	3125768	3258278	3399906	3854032	3190801	5640676	2993337	3509469	2881971	2942122
lauric acid	13351	22923	14137	17593	11519	12310	12932	4807	12642	18489	12466	13403
lactic acid	1489228	642783	791678	1231334	1341839	677126	1210596	1169738	1019925	1115808	900136	1114227
lactamide	2474	3034	2542	2587	2272	1745	2249	2078	2587	2032	2242	2369
ketohexose	1664	1671	2052	1287	1312	1443	1542	1387	1573	1555	1725	1841
keto-hexonic acid	465	572	434	450	443	455	441	448	524	484	425	467
isorhamnose	4336	12612	6891	3488	3301	4428	4440	3113	5720	5980	6512	6093
isoleucine	4017875	3053484	4354367	4707320	4514386	4822771	4793150	4319092	4942667	5342962	5252736	3451055
isocitric acid	1281	3685	2041	1532	1145	1844	1832	1322	1961	1794	1682	1663

hydroxylamine	155000	72625	93551	127962	117451	118644	83918	115450	117286	131249	125030	110502
hydroxycarbamate NIST	19836	16609	24189	26868	18438	26968	18881	17861	23396	18177	24993	19097
homoserine	4678	15931	8725	5974	5604	7146	7483	5175	7720	7249	8969	8301
histidine	310520	190366	222188	295986	290836	238059	229094	319760	248329	228908	106371	211098
hexose	4252	12526	8598	4999	5363	7496	6818	5878	5102	7873	5789	7604
hexitol	588	2143	1072	679	782	979	1434	771	925	1090	1791	1078
guanosine	1096	1250	1167	1032	990	925	1360	775	4242	1725	8380	1807
guanine	1369	2495	1640	1276	1136	1237	1743	1122	4802	2190	7081	1925
guanidinosuccinate	870	2090	1203	1198	1162	1054	1176	776	1103	1221	1416	1039
glycyl-proline	4849	9705	8661	10067	9239	6520	7902	6798	6657	5368	7270	12559
glycyl tyrosine	4231	11531	8412	5270	5576	7892	5521	3641	4700	6458	5902	6553
glycolic acid	6308	8021	6071	6032	5705	6624	6885	6580	8098	6956	10546	6801
glycine	350372	792378	577105	390435	428308	477366	492452	373982	498283	453964	596084	587592
glycerol-alpha-phosphate	4207	10254	5533	5095	3632	5753	3819	2395	6362	5383	5780	6285
glyceric acid	4298	12245	6980	5213	4750	6416	6316	4396	7204	5815	7590	7489
glutaric acid	381	461	306	453	489	143	449	214	268	267	267	292
glutamine	788	3859	1782	1266	1083	1206	1457	1182	2738	1354	2910	1774
glutamic acid	10056	35419	23547	22388	14356	19754	17405	15397	31641	20278	39273	22277
glucose-6-phosphate	1224	2964	11937	7598	1288	1573	8407	7496	14728	2515	19925	12130
glucose-1-phosphate	2253	7079	4328	3191	2987	4625	3309	2916	5193	4393	6357	5097
glucose	76061	161732	28939	83137	85848	104845	59505	72773	62006	97027	39617	112071
gluconic acid lactone	1373	2431	1940	1224	909	1390	1254	1034	2038	1407	1779	1559
gluconic acid	5781	21313	10696	7013	6829	9216	9476	6126	8397	9874	9719	10264
galactose-6-phosphate	660	1318	818	691	631	539	728	387	640	928	1096	613
galactinol	6611	10896	7890	7398	5873	8778	6237	6279	5796	8246	7354	8844
fumaric acid	24363	22696	22457	24992	25593	22148	22100	18989	20344	23291	22580	23443
fructose-1-phosphate	1167	2365	1662	1773	1018	1178	1498	1352	1127	1315	2051	2093
ethanolamine	63627	93337	59159	71911	60505	30125	59905	63223	59702	57949	61060	60562

ethanol phosphate NIST	1072	3738	2444	1466	1147	2213	1746	1086	2441	1685	2712	2278
erythrose	4522	9778	6655	5630	5773	5123	5616	4368	5595	4457	6266	4854
erythritol	1718	5010	3029	2156	2059	2616	2519	1978	2625	2731	3086	2786
dihydroxyacetone	3322	3473	3774	4030	4421	3237	2512	2696	3366	3159	3408	2952
deoxypentitol	933	3362	1510	1164	1234	1532	1515	1099	1425	1533	1737	1836
cytidine-5-monophosphate	38670	53414	30216	29212	33956	48042	35294	20953	24068	45807	36555	51897
cystine	30506	89548	40928	32180	33446	35032	39526	25272	26523	29422	31688	33539
cysteine	54623	118442	67397	64210	69237	64255	74784	48669	53304	94107	62978	70677
cystathionine nist	5451	15551	9300	6959	6100	8358	7751	5291	7057	8201	8341	9231
cyanoalanine	756	2032	1273	1057	1251	426	1049	1162	1337	857	1772	558
conduritol-beta-epoxide	1104	2974	1726	1331	1294	1532	1472	1123	2035	1476	1684	1603
citrulline	33861	78722	51194	40800	37550	38967	48516	38919	49271	37935	52475	42722
citric acid	46460	149473	78215	55349	53046	68432	69086	47045	65886	64475	73544	75370
cis-gondoic acid	457	348	462	675	413	442	312	406	403	412	436	387
cholesterol	466	627	636	757	844	620	608	427	529	363	614	679
cellobiose	4926	8048	5070	5178	3701	5879	4207	2629	3579	4917	2841	5431
caprylic acid	9964	8009	5524	8942	7326	6396	6114	8861	5812	4828	9981	6074
capric acid	642	984	893	840	755	497	952	970	969	1074	1079	929
butyrolactam NIST	110910	247785	167830	125653	121104	146269	140194	110458	144251	136168	176071	155624
biphenyl	851	2448	1074	952	687	883	1398	860	1058	1156	972	807
beta-alanine	741	937	867	881	384	834	535	394	967	892	1079	979
benzoic acid	18682	31722	18683	23515	23542	23800	23721	16702	24028	23691	22378	21111
behenic acid	16093	17432	14892	16183	14050	7788	12396	10459	11309	14360	8100	14217
aspartic acid	258574	1049143	668128	544488	386050	534545	487740	502499	1053217	539621	1263568	814202
asparagine	19291	89226	42139	27360	28030	32516	35967	23623	36041	35190	40324	34814
arachidic acid	78411	50245	77638	104865	77464	74948	59269	55553	68233	71033	96404	67491
aminomalonate	12909	44910	33357	21667	15066	25286	27359	16626	24572	17056	31046	25278
alpha-ketoglutarate	590	946	593	642	537	579	393	421	457	538	394	526

alpha-aminoadipic acid	650	4008	1770	1062	665	1077	1053	654	1215	1167	1704	2182
alanine-alanine	67518	186107	113563	71734	86239	89196	105162	87631	103371	88940	151833	91414
alanine	7059411	4990889	7307337	6870731	7027803	7142675	7023029	6935862	7054265	7043750	7137818	7112086
adipic acid	433	692	505	670	1064	811	618	710	766	318	739	705
adenosine-5-monophosphate	3840	3306	3223	4374	2690	3489	1875	2138	2031	3287	2742	3251
adenosine	3893	5166	5531	4815	3872	3882	6455	3666	19108	10779	34269	8539
adenine	6010	15143	9210	7086	6198	6830	10049	8157	24222	15082	34810	11947
aconitic acid	1310	2762	1637	1552	1545	1274	1509	1274	1113	1220	1603	1185
5-methoxytryptamine	3399	20392	15091	12970	6810	10442	11037	6645	9078	10968	9549	9461
5'-deoxy-5'-methylthioadenosine	398	592	396	466	333	357	459	494	412	607	461	414
4-aminobutyric acid	2019911	1422653	2571555	2227588	2337659	1967738	2122502	1737184	1600388	2082247	1975837	1899118
4-aminobenzoicacid	831	2345	1223	995	779	1258	928	468	542	1215	580	1474
3-phosphoglycerate	22141	80954	40009	31555	21297	28109	26842	18873	49860	30042	54206	36939
3-phenyllactic acid	3765	11722	6707	4796	4575	5873	5568	4195	5792	4420	6851	6433
3-hydroxypropionic acid	13203	20901	17146	15576	13429	16624	15530	10411	15307	15771	15366	15979
3-hydroxybutyric acid	6629	8024	7712	6413	7917	7914	9064	6542	7504	8805	11285	8681
3-hydroxy-3-methylglutaric acid	407	1683	626	395	781	541	511	248	610	991	1016	554
3-aminoisobutyric acid	1928	261	147	2967	51	106	171	121	206	118	266	3381
3'-adenylic acid	370	299	270	155	350	323	262	427	298	344	361	255
3,6-anhydro-D-galactose	41037	79440	59458	46351	51167	53674	43617	39463	48240	51085	53429	49764
2-picolinic acid	604	982	591	920	621	412	624	699	707	652	641	619
2-methylglyceric acid NIST	34540	88014	52797	39761	38261	47958	44513	34250	47076	46098	51266	51411
2-hydroxyhexanoic acid	29721	79842	39651	33871	34778	39252	38053	30204	42075	49130	41801	40157
2-hydroxyglutaric acid	22687	70636	40642	30608	26175	36226	35589	28033	40263	35707	52654	43235
2-hydroxybutanoic acid	98519	166469	131426	83874	94426	109856	143390	140468	144053	147496	202463	119737

2-deoxytetronic acid	6054	13107	7584	7723	6928	9227	6539	5042	6994	4048	7450	9491
2-deoxyerythritol	1712	7506	4448	3338	4132	4069	4170	1991	2867	3229	3173	4103
2-aminobutyric acid	41832	90359	63098	46814	45123	54191	56188	52338	67785	64113	80483	64371
2,6-diaminopimelic acid	2853	9036	5765	4964	2935	4280	5293	4088	8452	6484	11091	6593
2,5-dihydroxypyrazine NIST	826	2129	1185	971	1552	1047	1096	1192	1095	1240	1276	1258
1-monostearin	242	1256	1064	1268	575	730	805	897	742	941	606	1098
227437	689	806	588	669	1456	697	513	520	630	1077	711	853
227427	572	1568	931	794	734	769	816	501	813	874	965	623
227291	576	634	747	634	619	701	654	535	561	617	411	676
227280	703	1551	430	675	312	669	719	518	625	1310	786	754
227272	646	809	854	746	708	749	761	562	402	806	788	856
227260	366	1348	543	748	647	828	824	734	993	997	1229	1039
227255	334	1923	783	762	743	822	539	314	459	1008	844	731
227232	773	277	1204	1022	832	1064	1127	613	764	968	1276	1214
227229	682	1334	1574	1506	1168	1815	1019	1068	797	1425	1530	1037
227223	491	1548	976	705	418	1034	691	458	686	691	1047	549
227216	1208	2445	1694	903	1178	1158	1009	1103	884	1167	1725	1586
227209	1628	2456	1402	1085	1590	686	991	1573	943	940	1184	774
227200	1800	2617	1722	1775	1591	1802	2104	1451	2120	2793	1989	1627
227176	2317	6638	3923	3528	4289	3252	3562	2379	4253	4372	4479	3641
227159	8482	21644	14459	9214	9619	11383	8219	6469	9930	12567	7215	11296
227158	3926	18391	6619	5791	5705	8058	7657	4091	6654	7700	8145	9407
227094	459	386	578	615	388	529	482	428	509	231	572	552
226710	1310	1207	1376	1416	1194	955	1182	944	1026	1069	1423	1044
226556	412	1527	1108	537	960	707	978	811	492	1063	615	1243
226546	748	549	595	723	740	563	898	1011	965	1151	1061	988
226436	3141	8992	4918	3394	3739	3960	4110	894	3650	4667	4434	4755
226431	3638	2756	3438	1154	2373	2170	3209	3171	3005	2532	2967	2268

226008	656	899	837	599	339	601	917	868	644	764	1228	790
225844	601	2290	1158	716	787	1058	972	945	1030	1218	1251	1078
225834	756	3173	1460	1151	1072	1426	1192	856	1397	1508	1202	1428
225831	742	1827	838	683	858	766	758	608	797	1139	654	984
225797	1337	2328	1544	1695	1417	1385	1317	1005	1576	1538	1588	1498
225783	1261	1695	1718	1387	1629	1424	1053	1415	1360	1588	1475	1275
225773	2837	3218	4392	2848	2714	3488	3713	2639	4069	5072	4596	3996
225765	2627	5512	3706	2580	2635	3208	2627	3084	2221	3361	3647	2954
225748	6193	18596	9357	11772	3911	12583	5438	6505	13153	10949	19148	23923
225741	5987	13442	12526	11306	7422	9985	9618	7433	13725	12447	14398	9534
225526	368	648	389	350	360	179	381	348	395	380	501	470
225514	528	875	261	475	389	217	450	395	369	494	239	234
225320	1415	1309	1004	1164	1169	1791	1192	679	774	1658	1504	1939
225305	2091	2155	1920	2413	2868	2521	2112	2049	2224	2618	3440	2206
225303	1243	1702	1530	1265	1151	1122	948	1022	1204	1428	1083	1267
225279	1533	4028	2379	2594	2232	2229	2075	2289	2114	2406	2308	2801
225276	1636	3229	2622	1645	2004	1614	1535	1679	1786	2363	2304	1388
225272	3081	4910	4742	3345	2662	3189	3024	2183	3768	3712	3513	4220
225233	859	460	564	873	482	239	245	702	526	1035	540	720
225226	196	392	296	308	525	156	579	535	200	373	538	563
225211	1075	1036	1059	792	1070	1232	1022	924	1143	1174	1025	915
225203	387	674	496	397	443	286	237	420	587	565	795	622
225193	762	835	542	790	759	502	728	693	511	767	572	522
225192	983	1346	868	652	944	1025	835	1000	856	726	887	731
225185	302	683	400	323	540	730	390	542	384	529	795	508
225184	507	1497	750	769	753	783	664	520	501	999	406	787
225170	1306	958	1153	1435	932	1469	1017	889	904	1735	975	1327
225159	1150	1582	527	1360	1197	1305	1234	1306	1498	1729	1664	1536
225156	480	967	662	492	3864	265	870	532	518	572	881	883

225149	249	950	469	698	616	690	319	599	639	718	716	692
225148	718	1668	1050	1036	844	1073	830	730	928	1330	1033	1045
225146	1090	1965	1222	1115	1155	1279	1330	1074	1187	1158	1423	1651
225143	1899	4033	2883	2623	2103	2576	1731	1448	1923	2663	2208	2753
225134	998	742	626	870	925	863	803	782	838	629	969	734
225130	660	1075	1087	787	854	843	840	823	520	695	960	923
225117	699	2683	1246	898	857	979	1242	678	1050	1227	1005	1325
225114	2168	2916	2854	1814	1271	2245	3032	1296	3713	2434	3212	1291
225112	1256	2058	1557	1378	1369	1199	1385	1303	1838	1223	979	1486
225103	2995	11441	6072	4261	4137	4591	5104	3962	5867	5067	6774	5224
225100	819	865	711	572	814	1234	510	738	831	1071	1037	834
225096	1274	1229	769	769	1122	1038	1245	787	1006	1576	1276	1389
225091	718	2355	1259	761	1102	1021	989	1610	1243	1580	1562	1191
225090	446	3001	1321	1215	653	870	1037	394	771	1603	1016	1097
225088	1583	2569	1604	1452	1430	1686	828	830	1038	2036	1312	1897
225081	1388	2789	1991	1707	1403	2084	1272	1339	1261	1887	1356	1604
225074	2052	2926	2712	2421	1382	2991	1649	1766	1336	2696	1704	2893
225070	1023	3443	1085	1371	734	1773	1618	971	1246	1981	1530	2187
225067	1644	4008	2734	1980	1427	2534	997	1296	2091	2781	2446	3161
225064	1973	3709	2748	1579	2108	1974	2297	1836	1665	2445	2058	2816
225063	2137	2091	1484	1754	1614	2192	1155	1222	1573	1993	1097	2125
225048	1757	5111	2998	2316	1800	2711	2487	1650	2290	3532	2839	3061
225039	1499	4257	2759	1831	1789	2475	2403	1269	1741	3147	1614	2525
225035	6659	15484	8650	8584	6941	9874	5995	4789	5484	9686	6129	10167
225028	1591	1455	902	859	919	1443	1153	786	1101	1506	1487	1695
225020	4125	11945	5619	5086	4021	7106	5880	3998	6599	1951	7710	6708
225017	980	2745	1194	782	752	1531	1020	880	1271	1117	1455	916
225013	2872	12412	5928	3890	2690	6191	5967	2219	6196	4282	8000	6617
225012	6538	58287	21784	9840	18790	16545	18834	8969	16196	17828	19884	13484

225010	7197	17986	13065	8572	8913	10636	10975	7651	10901	13729	12606	12460
225006	8579	18114	6317	10406	10207	9781	12347	8786	12342	12526	13648	14083
224998	629395	1675429	1056319	789167	637237	880007	873765	681958	832474	1055064	1057524	895367
224866	494	594	288	663	439	261	214	377	457	284	691	227
224759	503	404	415	366	534	569	267	698	437	220	518	449
224462	596	1477	1461	2088	792	1269	898	793	1006	603	758	1123
224435	1868	2389	2067	1878	1538	2267	1659	1031	1415	2969	1695	1679
224427	356	6976	3337	1130	949	1351	1228	1444	4405	576	3161	862
224423	1115	3368	1867	1405	1215	1806	1673	1206	1427	2286	1925	964
224377	16263	60332	28283	15433	24034	15796	66447	12943	15404	26756	17001	22744
224374	230	622	551	403	260	431	415	211	266	380	327	578
224359	684	1079	901	673	586	854	397	624	497	573	519	465
224322	613	1782	1346	986	677	913	936	778	1375	616	1590	1011
224312	664	1196	689	488	610	628	448	346	606	623	606	563
224308	305	1382	879	613	619	656	556	407	835	683	809	904
224299	462	1346	903	754	616	733	1045	477	833	918	870	915
224298	860	1155	667	583	456	546	599	435	560	736	646	669
224287	503	1625	906	826	782	744	1176	520	751	869	979	720
224286	427	1328	1101	697	559	747	980	446	689	723	787	784
224273	1135	2340	1381	2055	599	1100	501	1014	840	1191	701	1458
224272	922	1194	1156	1111	938	885	780	742	770	766	1139	815
224271	845	2714	1403	1317	854	1297	1140	779	1246	1054	1607	1815
224265	1282	2498	1665	1520	1420	1623	2302	1234	1405	868	1523	2479
224261	862	1891	1003	1103	1027	1025	793	1001	740	889	621	1074
224260	2867	1041	1286	2407	2072	1551	1157	1207	1234	1661	1232	1621
224259	2056	689	1217	1879	1707	1348	1077	1387	1001	1651	1029	1159
224253	485	1089	484	745	634	826	458	347	485	787	378	449
224250	1764	2432	1275	1016	921	2126	1283	1396	1080	1514	1577	1613
224248	1100	821	922	1083	1200	1113	699	786	847	1532	1012	947

224220	1032	3394	1265	1239	1165	1343	1079	1022	989	1083	1226	1151
224218	439	2260	1157	933	453	1087	868	402	967	1162	811	989
224216	647	2534	1294	709	744	848	752	720	984	1076	1050	799
224215	1132	1456	1056	1027	645	931	873	778	599	1361	895	1081
224214	699	2400	1544	1276	781	1762	1017	767	1278	1124	1414	2216
224211	2186	1427	1611	2169	1457	1669	1164	911	1171	2036	1256	1569
224205	306	2080	804	892	462	1034	752	897	885	1229	1133	1229
224201	2389	4475	2317	3101	2865	2084	3070	2370	2831	2811	2673	2278
224199	759	2382	1330	672	481	1132	1912	1004	1009	1472	466	1361
224198	1055	1515	1496	1152	1445	1320	1007	1163	1272	1295	1392	1130
224193	6289	971	1911	4833	3267	3356	1642	2037	2670	2666	2212	2063
224191	1675	4107	2724	1625	1989	2475	2394	1876	2348	2570	2406	2395
224190	3936	4591	4430	4135	3678	670	2690	2048	3026	2969	1639	1874
224183	1094	3254	2194	1837	1760	1971	1784	1258	1660	1551	1819	1922
224181	3244	2199	2123	2944	3018	2743	912	1179	769	1508	434	1951
224176	1552	4496	3895	2564	1961	2636	2203	2076	3845	1926	3856	2894
224170	540	1351	1347	1170	701	1273	821	1145	1491	1927	1473	1200
224167	1260	2483	2139	1639	1209	1175	1388	1204	2347	1958	2255	1807
224166	1995	684	1039	2040	1788	1509	1064	1236	907	1830	329	1195
224157	1147	3312	2423	2407	1124	2404	1717	946	2135	2734	2208	2736
224156	1491	5469	2971	1689	1514	1660	2339	1679	2855	1998	3439	1720
224155	1291	2239	1551	1426	1933	633	3074	907	890	1950	1269	1217
224147	7955	12826	9120	11055	5661	13661	6303	4320	3316	3565	2529	14481
224143	2041	4069	2561	2381	2084	2901	2387	1393	2234	3796	2421	3271
224142	931	6999	5980	2879	1152	3081	1748	1293	4170	2650	3535	2780
224140	1800	4556	2963	2336	2095	3026	2901	1692	1826	3064	2778	3231
224136	2573	4194	3373	1638	2440	1644	2312	1026	2109	4014	2971	2292
224131	1044	5695	3546	2041	1477	2893	2426	1456	1750	4069	2356	2979
224126	6705	14536	8240	4153	7622	7993	5882	4519	5700	6519	6904	9325

224124	2498	11178	4521	4018	2171	6584	3968	2296	2745	4207	2092	6061
224123	2144	26327	9960	3982	1939	9655	5124	1803	4099	5034	3485	8203
224119	5836	19196	10766	7504	5363	7577	6570	4729	7959	4214	8278	8874
224116	2382	9621	5197	3304	2484	4735	5038	2448	5585	4826	7844	6151
224115	3339	11181	6459	4526	3791	5216	6325	813	5006	5791	6680	6442
224112	1477	3337	1462	2293	2040	3044	2356	1077	560	4404	1356	4195
224111	12411	4556	10946	13650	11333	10866	10010	12033	11361	10684	11501	10359
224110	6265	14308	8977	6841	7487	8639	6520	5726	6487	7244	8447	6550
224109	2599	6598	3835	3357	2913	3810	3330	2433	3271	4496	4049	4466
224107	3786	6340	5584	6159	5541	5316	4305	2425	4186	7473	4878	4268
224106	2959	9572	5480	3592	2921	4667	4301	3009	3821	6358	5162	5244
224105	3238	2695	2881	5706	4011	3622	3251	2333	4383	6385	4656	6244
224103	3883	11241	6844	5107	4606	6280	4739	3680	5193	6403	2572	5443
224102	4109	12528	6518	3652	3840	6997	4352	3427	4825	6487	6183	5128
224100	8369	20605	10899	9993	9211	10949	9733	8385	9536	12517	10893	11294
224099	1472	5815	5212	3890	2377	3980	3197	1739	3860	6100	4924	5491
224097	4646	9937	5916	4934	5116	6532	5686	3573	4798	6620	6192	7178
224096	6247	13464	10274	8186	7104	9322	8328	6109	8228	6775	9541	9106
224095	7990	15758	13208	10303	8745	11038	9064	7560	9668	8506	10804	10961
224093	2739	13004	8703	6729	3363	7401	5459	3058	7924	9346	8604	9907
224092	6759	19233	11791	8288	6985	12525	11318	7569	11740	14448	16542	13462
224087	9210	35980	20365	11929	12586	13185	19359	11412	18946	14176	30770	14234
224086	9107	9869	8624	8604	9791	11984	5962	6215	6741	13200	6805	8929
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224075	164013	520377	268435	198821	177684	258640	183054	129366	190842	227394	214655	268549
223946	490	272	198	212	510	196	199	437	395	521	378	529
223940	227	376	216	102	369	247	152	400	211	122	230	545

223771	636	1148	808	645	686	722	483	697	851	965	604	771
223766	485	522	295	515	299	217	245	152	331	273	312	322
223753	498	619	617	660	610	384	417	473	602	508	595	784
223745	454	271	366	507	418	392	228	329	363	250	355	404
223738	490	709	287	407	395	274	206	351	475	624	369	364
223737	471	1131	644	313	392	639	511	494	352	482	378	584
223659	1208	4171	2714	2074	2131	2740	2662	1559	2442	2788	1225	2505
223624	771	2198	1761	886	846	1647	1063	1131	1056	1070	1547	1561
223564	636	909	741	615	1000	548	547	749	653	582	1090	375
223555	678	1003	534	813	745	550	375	241	623	750	343	499
223542	980	2248	1521	1654	2082	2735	1207	1647	1023	2157	2111	3176
223535	852	1432	557	416	435	168	672	535	426	490	513	545
223522	750	1200	608	809	421	597	530	443	496	561	676	619
223516	1250	2110	1015	980	646	1348	938	974	833	1535	1454	1086
223491	1435	3170	2077	400	1985	1964	586	1126	673	1927	1061	1983
223452	1435	4736	2686	1918	1623	2364	2022	1989	2033	2373	2401	2495
223421	533	718	643	595	576	578	487	498	219	537	372	471
223419	958	946	625	982	548	676	957	783	912	718	662	783
223418	668	808	781	337	602	634	820	549	972	621	1523	796
223415	963	1388	1093	1416	624	1158	640	990	1097	952	799	822
223412	475	688	579	1194	941	1002	804	706	460	634	551	919
223405	530	1169	401	331	338	583	551	284	280	808	410	428
223398	230	953	358	294	388	328	504	602	535	476	709	367
223397	496	1825	837	651	524	863	818	521	1108	1068	797	1051
223394	597	1215	712	613	287	712	356	557	368	525	492	774
223393	1016	1433	1320	1178	745	1456	875	627	1158	1226	1509	1455
223373	491	871	457	391	285	551	438	406	375	517	457	672
223367	625	1013	560	726	206	514	428	261	400	508	523	470
223363	684	855	822	388	366	483	469	366	340	381	498	570

223359	735	851	833	746	819	709	717	609	733	680	443	701
223339	542	778	629	1148	503	609	828	354	420	711	874	641
223337	466	1646	1089	793	721	771	874	525	699	908	899	723
223335	792	1108	704	319	467	658	600	325	821	814	552	799
223334	873	1580	1335	1113	1030	729	730	736	668	1089	1251	1261
223330	387	1460	703	743	213	348	391	263	504	388	525	376
223327	941	2863	1244	974	933	979	1464	591	766	985	825	1319
223317	748	2220	1750	1779	1625	1491	1460	588	797	1815	1571	1424
223316	519	1553	872	595	959	540	766	612	764	774	827	879
223315	459	248	350	350	406	307	292	70	251	341	356	348
223310	550	1828	976	1036	680	816	733	860	795	1336	901	932
223306	464	1380	739	786	505	461	409	427	572	602	561	730
223302	796	1167	908	713	656	753	627	613	665	498	803	262
223299	528	1762	965	703	653	872	869	582	776	979	966	878
223298	418	1731	728	419	494	623	974	409	576	725	768	719
223295	518	1621	1170	776	733	1022	790	457	910	1089	1019	873
223293	830	1937	1144	627	768	1055	1033	837	1047	995	1150	1097
223292	1337	1315	1011	911	881	1080	780	664	996	964	1039	1066
223290	148	704	90	262	302	238	449	693	299	604	511	362
223289	1075	3124	2036	2050	2451	1353	1699	1720	1984	2530	2959	2757
223284	1344	1162	1048	1248	1222	1353	828	660	1251	1104	1188	1176
223280	505	1603	955	1165	705	911	824	509	771	1096	895	954
223277	773	1608	952	806	863	1053	940	598	1116	1191	990	1273
223274	718	1189	718	611	392	1073	830	730	568	842	1033	693
223272	1700	2001	1353	2156	1362	1985	942	429	716	2261	884	2676
223271	3625	8779	6804	4196	4139	4872	4150	3352	4521	4485	4979	4532
223268	1193	4299	1639	1495	1555	1765	1911	1958	1429	2144	1833	2017
223266	1303	2329	2111	1795	1769	1447	1231	1373	2113	2181	1483	1776
223263	622	2123	1510	1918	712	1606	1534	538	1094	1189	1459	1630

223259	450	2338	1184	869	1158	1113	883	550	897	1261	1065	1284
223253	702	1117	860	780	767	938	662	472	605	734	675	826
223252	1465	2349	1968	1666	1171	1856	1578	1044	1487	2516	1569	1477
223251	661	2381	1728	781	720	983	925	594	1326	1081	1029	1669
223250	1221	3153	1675	1354	1227	1386	1633	1046	1361	2843	1547	1879
223249	1147	4656	1931	1394	1308	2613	1722	1127	1565	1493	1762	2784
223248	767	1609	786	656	1221	832	1233	1019	745	1279	649	801
223244	778	1837	952	1145	886	650	949	730	597	1043	841	751
223243	728	2429	1840	1364	1048	1766	1373	698	1005	1767	1264	1377
223241	1097	2419	1176	822	805	1076	1080	675	1038	1103	1156	1140
223237	880	1371	1104	1042	813	1210	797	1046	826	1302	1014	1168
223234	1263	2392	1234	1030	1258	1759	1093	1048	900	1498	1354	1435
223228	2640	5294	4263	4018	2486	4039	3235	1941	2833	3854	2535	3343
223227	1019	1968	2105	1248	1267	1396	1250	1195	1476	1047	1592	1323
223226	1021	2870	1174	998	1245	1666	1142	1284	1203	1526	1322	1628
223223	1089	1922	2109	1481	1198	1738	1402	1775	1997	1451	1700	1842
223222	450	486	383	414	320	466	1848	2150	2283	1253	2313	1864
223216	465	2299	957	383	673	680	1074	561	662	1005	787	812
223214	1156	1082	780	908	598	1311	685	698	826	1096	779	1000
223211	629	3508	2689	2138	968	1676	1378	1162	1824	1782	1453	1449
223208	1459	6061	1661	1184	1082	1638	1900	1063	1457	1661	1576	1726
223204	773	5850	3251	1106	2439	1237	2871	1314	3943	2035	4102	2308
223203	805	4001	1160	1093	1609	1058	1458	602	863	2181	1642	1298
223202	1576	2640	1736	1340	1283	1599	1480	1031	1353	1864	1588	1779
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223196	1143	4476	2110	1763	1283	3716	1737	1099	2645	2400	2573	2817
223195	1139	3241	1799	1993	1154	1676	2018	645	1458	1705	1656	1814

223194	5142	3656	1764	1351	4180	1517	1712	3537	1256	1516	1829	803
223192	5039	7368	7248	6717	6067	5178	4010	5604	6848	2931	7010	4419
223187	1213	1257	1240	1319	1460	1266	1010	1126	1051	1930	1174	1277
223186	164	1538	621	985	741	415	1195	337	644	495	939	357
223185	802	3778	2325	1283	781	1000	1307	791	1431	1177	1322	903
223184	2457	2023	2200	2714	1896	2657	1570	1254	1902	2785	2035	2343
223177	1181	4765	3032	1877	1984	2232	2247	868	1256	3299	2673	2687
223175	1750	4939	3720	2835	1050	2402	1940	1307	2952	3244	3371	3178
223173	1100	2402	1246	1027	833	1300	1157	1060	1942	876	1677	1121
223172	1135	1171	2040	1690	622	2051	1087	572	1166	2753	1415	2479
223171	3092	8154	3955	3323	3137	4216	4309	2735	2941	4794	3824	4220
223169	1011	3966	2147	1458	1437	1788	1559	904	1630	2209	1954	1850
223167	1306	4636	2455	2204	1565	2796	2188	1339	2027	2412	2377	3538
223165	1396	3192	2141	1716	1372	1974	1741	1112	1539	2671	1939	2127
223164	3254	5824	4268	3756	3184	4295	3661	2739	3866	4117	4389	4159
223160	3439	4260	4069	3514	3737	3421	3993	2773	3179	4214	4495	4203
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223158	2626	6067	4691	3781	3666	3540	4133	3818	3626	4249	4621	4527
223156	1576	2856	1724	1435	1583	2204	1491	1168	1448	2804	1919	2440
223155	6594	2649	6850	6123	6580	7052	5694	3530	2141	7599	4218	5003
223154	1165	6003	2069	1417	1118	1846	2038	1086	1563	3945	1846	2633
223153	365	1089	693	489	389	646	305	540	468	428	508	701
223152	1699	2985	2995	2545	2038	2797	2030	1713	2216	3190	1282	2519
223150	2857	2499	3196	3277	3582	3044	3250	2150	1724	3919	2925	3593
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223134	2430	5382	3771	3101	2554	3610	3065	2393	2945	4380	3465	3952
223132	1392	6419	4142	1911	1620	2596	3111	2212	2400	2026	2607	2907
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223126	4408	12006	6191	5284	4051	7297	7507	4305	6632	9329	7500	9373
223125	3023	4676	3220	3239	2633	3833	3105	1595	2242	3915	2375	4738
223122	2936	10610	6050	5539	3278	4611	3749	3230	3982	3736	5195	5674
223120	533	21677	492	13674	1125	1108	4359	9159	16579	8797	16744	2271
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223116	557	1235	729	596	682	796	650	587	636	849	600	793
223113	3145	6419	6432	3835	3691	3830	4283	3530	4350	5590	5224	7356
223109	4434	12599	8931	5796	5589	8031	7882	4416	6490	9092	7448	7324
223108	9330	25657	15709	11683	11515	13605	12961	10326	12435	12733	14790	15114
223107	3369	10816	5983	4173	4051	5179	5036	3276	3932	5917	5644	5634
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223095	7316	9226	8106	8400	6313	9196	5916	3873	6313	8610	6610	8388
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219394	737	674	723	743	695	721	677	719	890	795	781	430
219374	3729	11742	6683	4778	3847	5229	4984	3197	5473	7597	6213	5849
217691	10938	18937	15213	13553	11650	14281	12982	12443	13108	13261	18222	14400
217651	1576	2348	1507	1495	1585	1592	1220	839	1054	1570	973	1570
216193	1416	3415	2487	1753	1659	1270	1609	1684	2130	1927	2308	1620
215896	3273	10263	4230	2882	3703	3498	4809	3404	3359	4932	4026	4987
215857	27737	74532	43971	33628	31706	39662	37494	26775	35440	52394	40891	42619
215855	528	1003	595	543	297	570	549	476	501	508	546	567
215853	20814	47633	32212	32370	28141	30062	28105	17833	26467	36305	27757	25776
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210403	636	1148	808	645	686	722	624	697	851	965	856	771
210321	2940	1619	2080	2689	2860	2346	2085	2459	2264	2534	2606	1993
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210272	1051	2173	625	1104	964	1380	674	443	898	763	645	951
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209690	414	513	223	377	357	230	345	376	251	454	465	438
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203809	426	290	269	396	363	369	276	302	289	317	267	277
192876	10815	1974	6333	9023	10847	8699	7318	9842	7521	7355	8782	6068
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187055	583	703	464	507	513	603	503	479	356	502	459	245
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183433	53761	29470	40886	53577	49973	42718	38415	45973	45090	44369	45649	38176
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183148	3804	8510	5854	3351	3948	5146	4953	2725	4789	5309	4157	5657

183117	1241	4197	1958	1761	1391	2129	1615	1122	1703	2029	2282	2525
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171042	1565	4562	2263	1932	2400	3043	1990	1295	1711	3272	2114	2381
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88052	1197	770	1036	1277	1190	880	1028	1019	1028	1222	1668	907

85198	6888	8757	6630	8773	8800	6743	6435	5820	6231	5557	7698	6511
85104	35698	87253	58816	44158	47295	57202	54595	37911	58918	63034	54852	62277
84721	8853	5705	7120	9243	8548	5543	7607	8921	8483	8005	8415	7579
84116	2995	11441	4817	4261	4137	4591	5104	3962	5867	5067	6774	5224
84086	2698	12158	6035	3499	3245	3987	4233	2779	4844	6797	5836	4551
68529	1310	707	760	1221	844	1330	872	775	694	1170	1074	595
68153	1246	2531	1214	1083	1250	1587	1410	939	1190	1671	1427	1722
67178	9005	2034	3647	8543	5622	4803	3792	3641	4240	6085	4337	4267
67160	7169	1325	2796	6245	4495	4142	2514	2519	3511	4218	2504	2867
66663	1792	1481	1582	1477	1393	1538	1279	867	1369	2149	1353	1494
66608	1509	481	815	1390	1268	997	950	856	944	1385	1115	982
66455	1161	1387	1171	1223	1129	800	976	793	1001	1170	1159	1158
66394	3087	8579	5916	6339	4651	4680	4935	2956	4614	6395	5132	4305
66378	840	1373	962	976	878	910	762	800	909	1018	1009	982
66305	2843	7020	3625	4043	3428	3509	4497	2558	4847	3984	5291	4115
66291	21960	66279	32673	31195	24529	29812	29280	20875	25647	27363	29341	30217
65034	196	385	587	342	161	322	426	442	549	219	145	552
64926	395	167	476	491	403	360	349	343	336	107	128	336
64751	879	432	383	790	743	369	780	580	671	947	762	920
64557	2569	5348	3111	3186	2854	2862	2511	1639	2479	3575	3919	2961
64546	5920	15382	9929	7393	6137	3123	5186	5489	7058	7906	5747	8921
62409	862	2067	1456	1103	1168	1586	1703	1094	1859	1926	2247	1743
62327	2680	8439	5445	3757	3143	5389	5252	2912	5006	5574	6125	5078
62250	46222	75725	65208	57506	71264	62240	66006	35145	49522	77182	59655	51642
53742	5087	3580	3775	4786	4760	3985	3665	4409	3196	4783	4707	3951
48608	1048	1901	2866	1762	1149	2830	1104	769	1146	1532	1652	2598
47358	437	576	938	358	493	516	583	583	571	980	967	468
46377	1775	1455	937	1454	1692	482	1127	1259	1069	764	1600	956
46362	1296	1713	1371	1371	1282	1285	1311	1286	1317	1311	1401	1227

46224	9964	8009	6849	8942	9601	6396	7324	8861	7611	5747	8502	6074
44509	979	1558	1094	1087	1101	1108	921	876	1247	1134	1255	1168
43100	5285	14468	9050	6448	6297	8925	6602	4630	6041	8139	8747	12915
42529	347	333	537	839	351	569	417	439	507	316	466	267
42007	845	8479	4840	3019	2804	6438	4050	2518	4181	5431	3659	4962
41989	468	995	915	897	514	685	528	768	636	864	732	987
41836	709	763	659	459	671	409	663	586	697	675	815	791
34183	1742	3330	1925	1973	1333	1383	1976	1418	2050	2669	2303	2064
34104	713	1392	785	829	840	865	650	451	614	279	383	485
33990	3055	11159	8202	4093	3349	6846	7888	4049	6670	3191	9452	6203
33386	2943	10858	6048	3504	3497	5260	5649	3250	4370	5438	4931	5829
33282	18386	59363	35246	26325	22023	31874	23711	19831	30683	31231	33704	33167
32593	24483	22306	27147	24951	23822	29326	17672	15385	23395	24082	48346	24580
31664	3250	6141	3775	2991	2759	3454	3555	2545	3385	2932	4227	3735
31621	27595	59770	41817	34586	29823	39894	33956	23839	30599	47499	38330	40323
31556	2496	4001	2576	2865	2686	2998	620	912	898	2379	534	2255
31408	7337	9811	11131	8320	7881	5938	9652	8086	7720	10908	10794	11308
31224	18176	16033	16753	17793	23011	11532	20099	15149	10627	11387	9928	11229
23635	5087	2776	3035	3575	3628	3100	2943	3296	4176	3918	3756	2854
22863	45036	51957	57553	51135	48478	67561	40850	40041	51344	64603	73141	42676
22444	1442	1619	2086	1713	1455	1043	1897	1484	2265	1708	2631	1175
21802	103360	112559	141353	103241	93165	142027	141874	131486	141023	153335	242670	184224
21683	1218	2237	1819	1317	1234	1619	1403	1052	1727	1394	2017	1815
21502	845	1062	836	683	582	724	588	859	518	806	711	740
20297	1601	5335	3358	1567	2258	2806	3456	1747	1059	3477	3166	3757
18520	2868	9685	4910	4714	3849	3570	4054	2897	4302	3551	4475	3776
18489	749	1793	767	918	479	652	691	893	1024	1007	755	748
18488	9117	44387	22180	14541	7355	21830	14223	9124	19699	14484	22565	20712
18266	219	519	351	311	176	349	233	193	255	354	291	269

18242	873	2784	1352	1042	942	1314	1393	528	1619	1373	1664	1479
18225	1161	1309	1142	1255	1008	1088	3040	4516	4847	4377	4079	3648
18177	1105	3107	2030	1113	1939	2228	1797	1790	1934	1980	2287	2028
18169	1498	3510	2892	2081	1717	2526	2280	1681	2516	2068	2970	3033
17822	4575	9335	6976	5769	6752	6269	6356	4306	4652	6804	5885	7360
17664	19081	11709	11384	15115	18515	2332	13484	17153	11275	4580	15653	9153
17536	17428	31081	25800	22331	19008	24664	19586	15240	19572	18289	22694	22712
17331	1380	3804	2185	1724	1558	1989	2033	1480	2174	2371	2208	2485
17252	3705	11432	5886	4821	4906	4101	2346	2577	5465	2342	5969	1794
17233	775	4044	1801	1354	1111	2285	1907	1484	2517	3405	2732	2884
17076	40714	101923	38177	39635	41031	37052	35512	41340	33006	37811	39099	36706
17068	1644	477	592	2132	697	557	619	1679	1039	1946	1025	514
17066	2562	5719	3858	691	418	690	3180	484	3055	819	3601	627
16980	643	1029	766	324	302	796	758	487	257	679	824	766
16947	1140	6781	2717	2188	1531	2094	1928	1963	4141	2408	4953	1351
16817	14070	23483	19516	17188	14170	17925	13718	12763	16776	19706	18420	17704
16792	18077	71623	31111	22477	19790	22027	27677	17359	24250	38766	23593	33224
16788	11368	21721	18164	14713	12629	18225	13650	10328	13805	14668	16875	15010
16675	897	1081	1153	1000	902	546	786	546	415	627	742	799
14796	30605	116517	81139	51133	37114	60334	68341	38252	57903	44337	76102	56671
14698	4107	10858	6048	4510	4549	4207	4422	4243	5484	6779	6348	4576
14694	2073	5731	3434	1627	2440	1873	2831	1204	2181	3190	2010	3491
13188	5298	5196	4938	5005	6632	4116	2840	4326	4351	4807	6107	3934
11841	457	838	337	534	631	579	687	691	500	457	374	459
8270	11503	24900	17874	14224	13234	16497	14482	11247	13737	11906	16346	15056
7490	8047	36271	15562	9384	9284	11593	14553	8928	12221	14035	13600	13145
5990	3478	9440	5285	4332	4204	5081	4601	3692	5116	5227	5309	4888
5471	3018	1658	2158	3648	2677	2439	1742	1851	2403	2245	2887	2059
5346	7229	11951	10322	9926	5490	9843	9276	8979	10242	9815	11139	9237

5290	13288	22037	11144	12925	15779	11039	17960	10900	10991	17541	10769	15811
5121	6372	13820	9522	8569	7037	8924	8132	6240	9023	10996	10041	9170
5085	5832	9818	9178	7973	8717	7489	11083	7166	6300	9232	8469	7991
4937	497	288	381	444	320	467	341	453	444	295	464	456
4929	23081	58092	38048	31767	29674	34256	33080	21119	30321	38965	31198	34763
4837	4610	5723	6942	4725	8778	6174	4292	6329	6611	7321	7249	4878
4793	27084	59392	40841	32258	28156	40995	32329	22196	29395	30673	34300	40534
4746	1459	2876	2530	2283	1421	2532	1154	1531	3017	1491	2314	1591
4744	4529	24950	12951	7242	7521	10937	10677	4513	8402	9649	13660	12407
4723	4369	6415	5695	5537	5428	4466	4085	4232	4692	4980	4710	4372
4721	4902	6549	6257	5950	6077	5009	4290	4649	5196	5187	5065	4764
4550	2006	1444	997	1679	974	1272	1050	1211	1560	932	1368	1113
4546	2513	25381	15924	4049	2810	5319	4696	2303	4037	13414	18857	5484
4219	33920	80416	65603	44737	31957	56249	35119	28777	42677	50110	48036	51920
3781	2134	1275	1275	2459	1844	3040	2030	986	976	2949	2563	2952
3618	1384	4123	1801	1974	1979	2634	2299	1732	2519	3180	2995	3103
3228	2253	1459	1278	2021	2235	1834	589	3906	1697	1339	1962	848
3207	7948	18943	11545	9234	7126	11045	9975	8134	10259	12267	11564	11180
3188	20310	50572	26018	22032	20533	26457	28299	19583	23113	28745	23213	27667
3044	9527	10246	10811	12227	9020	12164	7133	4478	7522	13803	5431	9811
2900	12375	35597	37537	37709	15097	37828	18557	28153	37874	31862	40236	39754
2806	15276	35459	22537	17730	17390	21100	21343	15377	19549	21127	24278	22489
2749	5693	14291	11126	9899	10396	8926	9551	4958	7873	12099	9231	7557
2673	3066	6978	3667	3124	4499	3945	4134	2241	3354	4985	4604	3967
2667	2421	7296	4400	3382	2276	4140	2863	2271	2726	5295	3428	4118
2665	5696	5764	4140	6031	4431	6137	2516	2268	1640	6569	1303	5599
2662	1841	2679	2244	2086	1513	2949	1503	993	1692	3048	1167	2151
2659	3211	4225	1818	2734	1873	3003	1799	1813	1234	4727	1463	4136
2647	27788	63185	30770	34266	23104	38053	24198	19089	25759	37516	26943	29761

2561	8845	46683	30867	20005	21121	21068	11613	11249	11532	39256	28165	14743
2547	9770	27114	17682	13749	11323	15260	13566	9560	13388	17800	15546	17194
2543	9671	18655	16613	15645	11958	13343	12399	6855	11315	16165	12594	10872
2466	5853	16910	9345	9239	7440	8832	6257	5698	4582	9300	3763	7815
2439	4614	6884	5650	5851	5757	6033	5996	4398	5173	6163	6734	6056
2403	777	1555	1223	918	922	1138	1208	1092	1347	1156	1402	1099
2392	7880	20006	11869	9389	8987	10512	9377	6815	9369	11163	10118	11548
2242	8844	21023	22302	12983	9258	18275	19244	14985	22768	16529	31725	16225
2233	12040	16885	12916	14218	13369	10735	12456	9988	11534	12595	12525	16575
2208	1321	2848	1901	1276	1285	1521	1546	1186	1950	1421	1277	1602
2193	880	2240	1253	1041	947	1202	1093	820	1099	1047	1301	1158
2189	1032	2142	1053	715	559	1072	578	490	980	1147	1084	1218
2061	4448	2328	3311	4497	3826	3553	3343	4069	3085	3967	3381	3115
2044	4877	29985	10959	9464	7285	10669	8333	5386	10153	10743	10180	11811
2037	356	292	348	467	382	499	241	314	611	376	912	405
2031	157836	518319	269694	180675	181470	233870	230389	160299	215387	219482	254580	258158
2018	5669	9680	7751	7022	7149	7334	10404	4935	11802	7953	15994	11468
2017	10837	17802	12379	10845	3623	14295	9349	3115	8716	14044	10721	15187
1996	30481	54208	39912	35032	33605	37819	34624	30242	37163	24541	39566	40600
1981	62881	153606	96826	74605	69376	90803	86041	66371	87823	83755	97806	96006
1970	38452	104771	56630	41630	42588	49061	46408	37478	47271	52526	54790	52358
1941	10421	8269	8841	10557	9326	8222	8216	9765	9711	8762	10156	7436
1925	4184	2647	3052	3379	3346	4177	2159	2581	3737	3519	3987	3341
1912	2640	1999	2527	3124	2418	2255	1936	2024	2112	2763	3036	2519
1875	5211	5278	5883	2014	3518	4685	5039	3931	2782	5298	6303	5315
1872	3621	4745	3707	4010	3824	3866	3410	3737	4168	3638	4291	3630
1846	5512	8252	4727	5314	5019	5655	7154	4357	6611	6355	11622	6760
1840	3339	5741	4450	2649	2752	4101	3575	1982	3940	5085	6202	4959
1833	13078	36521	22445	17879	14459	20367	17966	11236	16773	28126	20535	21494

1832	1529	2315	1388	1605	1434	1396	1324	1717	1437	1503	2076	1766
1831	18691	56825	30232	24192	21032	28762	26902	18288	25497	27078	29509	28676
1830	28045	70274	41187	37167	29421	43075	31677	21584	27030	46292	33907	40139
1829	38776	87654	48787	42859	36576	49822	39338	26115	33933	48743	39980	46754
1805	1327	2285	1537	1707	1817	1236	1681	1339	2873	1535	2798	2654
1801	6871	16001	11452	8653	8040	11173	11391	7662	11405	12687	14445	13845
1799	812	22038	12755	5147	7735	11606	11233	8385	10894	11054	12284	10928
1781	50513	179449	87287	69628	58213	76684	67376	46553	61968	81048	60002	80735
1767	55958	131833	97966	81398	63868	88440	56457	45712	64635	91470	58187	81212
1751	14012	23028	30498	33440	21029	22132	15222	21067	29931	16569	29326	20877
1750	27599	68744	46126	39272	35382	41750	40278	25901	37116	49962	39220	43462
1746	32817	68845	36737	40717	33656	43733	24456	22098	15834	35054	13371	38224
1740	24309	61414	38157	30224	26173	36928	31137	19874	28629	33414	32850	36918
1737	76562	123266	83695	78275	67940	93861	68744	46305	59533	102789	71478	102231
1735	31474	67748	35100	39324	32841	43352	25671	20944	18092	43161	16380	40142
1732	46939	120660	71461	57140	51829	65752	60602	43274	60944	80379	66931	70057
1725	2438	3232	3486	2852	3423	3251	2614	2473	2801	3766	3766	3435
1721	92454	153060	95198	91587	80963	120877	68047	51944	55982	97842	56288	112322
1717	97190	204758	107511	99081	90144	129218	82862	59036	82036	102078	77833	132400
1713	105033	241442	137735	141793	121116	145699	106263	72646	73898	161790	89521	133955
1711	465	1510	743	438	403	575	717	521	700	444	768	602
1708	129655	242777	123821	153501	128916	152624	81418	78607	51274	120098	43421	135886
1705	217973	489692	327575	277840	227155	312397	280360	168347	235542	452976	290382	336079
1702	78792	131780	66867	91861	78673	91926	46187	46074	28688	71173	25603	79831
1700	2148	2055	2568	2330	1543	2102	1735	1995	2120	1972	2320	1856
1684	653	2231	636	1320	1385	1723	2103	876	2256	1570	1234	1872
1661	8891	33757	21467	11802	11188	13166	20329	11832	23132	22950	39796	21857
1380	44450	78406	42755	40921	37106	75768	86966	22104	51166	200345	169951	55544
892	6439	2335	5402	5318	1902	4618	1957	4848	3922	5980	5276	2808

782	11053	35475	16345	13409	10827	19784	10127	7435	7655	12869	4699	20688
657	17948	12375	11940	14210	18515	2659	14558	15566	12049	5198	16515	9887
592	12429	58923	29792	17767	14275	28337	20128	13078	26325	22519	36645	29381
453	1196846	2123450	1150513	1140088	1107641	1649435	1886191	874912	1320786	3855238	2472692	1462077
307	59032	65806	28966	49162	46718	58951	43204	33819	35390	54617	42598	62950
299	26324	14579	23431	27960	18971	30290	17689	16785	17584	25861	18767	26644
168	633	549	774	1301	615	451	599	902	758	741	938	602
137	75676	38099	56786	69888	57692	50593	51246	53937	59565	63758	69305	53149
134	8473	3744	5143	20659	9132	6496	5394	4368	4824	16170	9077	34759
110	6530	9832	8945	7657	5229	7727	3892	5804	7907	7905	8547	7015
91	3943	3364	2950	6300	3146	1548	2969	6671	5940	2967	4815	2864
47	6105	4222	5309	7024	6394	7711	5577	6584	6460	6022	6061	7034
39	8128	6513	6453	7789	7209	5912	5422	6997	6706	5495	6580	5535

APPENDIX D

Metabolite	**Control (0 kGy)**						**EBCP (10 kGy)**					
2,4-Dimethylbenzaldehyde	564556	703108	655127	553817	577802	679654	540834	771987	719297	807193	873449	1169325
2-Hydroxycinnamic acid	2.9E+07	3E+07	5.1E+07	2.2E+07	2.2E+07	5E+07	2.3E+07	4E+07	3.3E+07	1.2E+07	4.2E+07	3E+07
3-(2-Hydroxyethyl)indole	93276	98285	90591	98045	102086	91119	93102	83932	83835	84176	74813	84492
3,5-di-tert-Butyl-4-hydroxybenzaldehyde	1166109	988966	808401	834882	1020843	910086	1026043	995567	996185	1103372	1069242	1033776
4-Aminobenzoic acid	434329	452802	404164	460801	422165	435872	427481	427715	426239	449147	87226	377968
4-Ethoxy ethylbenzoate	5570598	5469929	5478509	4442178	5205855	5783850	5464531	4877309	4725207	5049744	6180869	5504692
4-Indolecarbaldehyde	200323	164503	172100	161473	171538	206954	184437	166628	171846	165968	168351	159431
5'-S-Methyl-5'-thioadenosine	101442	200482	123863	209935	140000	124854	147666	98453	144705	181259	136166	198494
6-Methylquinoline	815311	842681	819777	852278	790797	834234	817359	857587	799821	761957	877613	750205
7-Hydroxycoumarine	123192	115497	115734	115193	122270	118561	112800	113355	117747	108089	118393	123443
Acetylcholine	2107436	1965357	1935145	1961047	1946908	2102696	1780227	1880067	1871190	1815716	1786951	1783762
Acetyl-L-carnitine	1624556	1523738	1589272	1689828	1538902	1611847	1816250	1678717	1494182	1556267	1580870	1503749
Adenine	1749756	1775757	2041340	2040491	1855371	1903715	1891578	1916726	2149688	2216227	1998329	1972804
Adenosine	298488	294615	384752	299906	319403	242378	429711	446303	421708	413288	562715	417586
Asparagine	6283850	6196271	5986203	5732515	5853886	5870927	5606969	5781034	5605452	5306436	6029511	5861425
Betaine	3.4E+07	3.4E+07	3.9E+07	7.3E+07	5.7E+07	2.5E+07	2.1E+07	4.5E+07	4E+07	4.6E+07	2.6E+07	3.8E+07
Bis(2-ethylhexyl) phthalate	50148	89872	50718	70398	105711	87835	158494	403972	439317	1117983	1135148	569895
Bis(4-ethylbenzylidene)sorbitol	3347753	1701112	871771	1138992	2779240	1972792	2043594	2265036	2040639	2377204	2392734	2863701

Bis(methylbenzylidene)sorbitol	3203856	4062462	2975223	2752570	3772926	4107630	3542210	5890783	3685117	4653970	7205437	7669420
Carnosine	1596280	1999771	2034418	1253033	1630342	1427727	1426382	1533388	1362423	1747881	1070233	1397574
Choline	3.9E+07	4.4E+07	3.9E+07	3.9E+07	4.5E+07	4E+07	4.4E+07	3.7E+07	3.8E+07	3.9E+07	4E+07	4.3E+07
Creatine	5.5E+07	5.4E+07	5.4E+07	5.4E+07	5.4E+07	5.4E+07	5.5E+07	5.6E+07	5.2E+07	5.4E+07	5.4E+07	5.2E+07
Creatinine	5.5E+07	5.3E+07	5.4E+07	5.3E+07	5.1E+07	5.3E+07	4.8E+07	5.2E+07	4.9E+07	5.2E+07	5.4E+07	4.1E+07
Cyclo(leucylprolyl)	840024	776156	759762	917385	836341	896755	905412	750329	800405	902348	805373	725647
Cyclo(phenylalanyl-prolyl)	289206	281120	253009	292902	321693	318841	308083	269679	265329	266020	277185	269046
Cyromazine	1448124	1641030	1301767	921876	1147059	1030621	1314586	1452595	1337320	1542611	1535987	1628229
D-(-)-Aspartic acid	2929254	2860820	3089429	2874027	2494460	2595246	3537058	2730800	3073122	3409119	2625737	2617855
D-(+)-Pipecolinic acid	1.8E+07	2E+07	1.9E+07	2.2E+07	2.2E+07	2E+07	2.2E+07	2.2E+07	1.8E+07	2.2E+07	2.1E+07	2E+07
D-(+)-Proline	7.7E+07	8.7E+07	8.4E+07	8E+07	7.8E+07	8E+07	8.3E+07	8.9E+07	8.5E+07	9.4E+07	8.7E+07	8.6E+07
D-(+)-Pyroglutamic Acid	4.1E+07	6.9E+07	4.9E+07	4.1E+07	1E+08	6.9E+07	8.8E+07	7.3E+07	1.2E+08	3.6E+07	3.8E+07	5.3E+07
D-Cysteine	3649023	3156765	4511270	3908049	3827108	3878373	1682346	2049336	2119527	2178900	2292439	1765685
Decanamide	236472	305148	340815	178177	178420	280555	428188	357489	259038	193297	261871	223404
Decarbamoyloxysaxitoxin	1.3E+07	9842410	8024366	8192769	1.1E+07	9635079	1.1E+07	9406373	9985192	9210697	1E+07	9152907
Diethanolamine	568745	697762	800844	781053	958203	1068228	958506	521069	662720	540289	703490	789075
Diisobutylphthalate	1.1E+07	7322562	6875512	1E+07	1.5E+07	1.1E+07	8062178	9001166	1.3E+07	1.4E+07	1.2E+07	1.6E+07
DL-Carnitine	3981317	4485646	4368894	6229593	4371188	6112772	8561962	5167491	3297291	5453565	4075883	3920332
DL-Homoserine	3E+07	3.1E+07	3.1E+07	3.1E+07	3E+07	3E+07	2.9E+07	3E+07	2.9E+07	2.9E+07	3.1E+07	3E+07
DL-Lysine	1.5E+07	1.5E+07	1.6E+07	1.7E+07	1.8E+07	1.6E+07	1.9E+07	1.7E+07	725566	1.7E+07	1.6E+07	1.5E+07
DL-Serine	1190942	1291828	1654342	1236272	1380513	1742244	869484	975147	1085494	1107060	1467441	1335098
DL-Tryptophan	2E+08	1.9E+08	1.8E+08	2E+08	2.1E+08	2E+08	1.9E+08	1.9E+08	1.9E+08	1.9E+08	1.9E+08	1.7E+08

Docosahexaenoic acid ethyl ester	93186	107644	94672	113374	120012	111897	118853	91084	159480	114789	122006	179588
Dodecyltrimethylammonium	128118	108651	71493	70707	117589	74065	101030	110155	97452	80796	89940	63446
Ethyl paraben	854996	793933	760663	752457	973531	848491	875158	716095	859609	850767	901230	867230
Glycyl-L-leucine	194373	240839	179387	209788	188395	189621	222194	199418	167755	179827	179323	218783
Guanine	1144564	1152660	1092118	1148628	1057964	1087481	1138351	1171105	1230203	1294403	1157877	1120129
Hexanoylcarnitine	110764	122546	119922	101137	117615	120820	144289	101212	132376	129984	150873	120150
Histamine	1.2E+08	1.2E+08	1.3E+08	1.3E+08	1.4E+08	1.4E+08	1.4E+08	1.2E+08	1.2E+08	1.3E+08	1.1E+08	1.2E+08
Hypoxanthine	2.3E+07	2.6E+07	2.7E+07	2.6E+07	3E+07	2.7E+07	2.4E+07	3.2E+07	3.1E+07	3.1E+07	3.1E+07	3E+07
Indole	2348628	2285754	2173946	2278423	2411199	2415823	2075333	2147364	2234819	2207733	1835295	2009760
L(-)-Carnitine	1793333	1769291	2609841	2271371	1839601	1527444	2414574	1862343	2310125	1916595	1540680	2453405
L-(-)-Methionine	4.2E+07	4.6E+07	6739756	3.8E+07	1932069	3.9E+07	3.8E+07	1374911	4.2E+07	4213811	3.9E+07	4E+07
L(-)-Pipecolinic acid	557688	235370	142915	81910	394478	781251	678431	432677	554986	138016	507672	672241
L-(+)-Citrulline	3.7E+07	3.7E+07	3.6E+07	3.5E+07	3.5E+07	3.7E+07	3.9E+07	4E+07	3.4E+07	3.5E+07	3.6E+07	3.5E+07
Leucylproline	3938503	5068020	3057487	2900341	4272240	3441310	5006789	4914529	3661149	3890468	2815511	4117812
L-Glutamic acid	1142171	1313096	1345485	1556193	1227256	1204207	1205582	1258983	1148337	1550050	1212789	1443301
L-Histidine	1208023	1166977	1110080	1102784	1121453	1089518	962772	1074749	967198	936886	839347	959747
L-Methionine sulfoxide	3961359	4395679	3895675	4120417	4538692	4353907	6212924	6192881	5713844	5817363	5977076	5642944
L-Phenylalanine	4.4E+08	4.2E+08	4.5E+08	4.1E+08	4.2E+08	4.3E+08	4.5E+08	4.4E+08	4.1E+08	4.6E+08	4.5E+08	4.2E+08
L-Tyrosine	3.2E+07	3.1E+07	5.2E+07	2.4E+07	2.4E+07	5.2E+07	2.4E+07	4.2E+07	3.5E+07	1.3E+07	4.4E+07	3.1E+07
L-Valine	2.4E+08	2.3E+08	2.3E+08	2.4E+08	2.3E+08	2.3E+08	2.4E+08	2.5E+08	2.4E+08	2.5E+08	2.5E+08	2.3E+08
Muramic acid	6448853	6628289	6933400	7350474	6685560	6865880	6034347	6989923	6234179	6622838	6779597	6566775
N-Acetyl-L-cysteine	991144	1128517	885055	1045467	1220022	801454	1381680	1082723	731013	964347	1177516	1089636

N-Benzylformamide	105780	81438	3692694	2479845	1183797	1333266	2341488	2134234	1758791	2601042	3135209	1657931
Neosaxitoxin	522525	711697	1012114	767064	765157	691183	1044515	674671	802706	1109477	490060	654170
Nicotinic acid	5175617	5200222	5599410	5608004	5743570	5725418	5149368	5273331	5308866	4547289	5540546	5422402
Olomoucine	814577	863554	564205	879543	889573	858090	1021761	1132514	1153607	503301	541907	919831
PEG n5	1.7E+07	1.5E+07	1.6E+07	1.2E+07	1.4E+07	1.5E+07	1.5E+07	1.6E+07	1.5E+07	1.5E+07	1.2E+07	1.3E+07
PEG n6	2.1E+07	2.2E+07	1.5E+07	1.9E+07	1.7E+07	1.8E+07	2E+07	1.7E+07	2.2E+07	1.9E+07	2E+07	1.9E+07
Phenethylamine	68441	87150	77018	66202	70383	83846	293747	155447	130137	173176	146614	160505
Pilocarpine	4135960	4326344	4179513	4267123	4209315	4448452	4143854	4083480	4091860	3819967	3912481	3865007
Prolinamide	1.5E+07	1.6E+07	1.6E+07	1.8E+07	1.8E+07	1.6E+07	1.9E+07	1.8E+07	1.6E+07	1.7E+07	1.7E+07	1.6E+07
Prolylleucine	1314786	4221637	2964957	4039442	4132561	3655836	2547872	5854337	3463780	4226125	2076594	4641019
Spermidine	336697	403463	390136	254000	300241	323937	513120	317768	367544	457869	298817	324036
Taurine	1065521	1047146	987041	1187297	1040718	897695	1078305	1040545	1120751	1037811	1133830	1020570
Thymine	709966	493037	669411	727380	657211	711447	710747	749636	713411	716383	676353	679974
trans-3-Indoleacrylic acid	2E+08	1.9E+08	1.8E+08	2E+08	2.1E+08	1.9E+08	1.9E+08	1.9E+08	1.9E+08	1.9E+08	1.9E+08	1.7E+08
Tributyl phosphate	1111717	978201	1040289	1399550	1650844	1477220	724787	1188583	1323689	1572066	984483	1547235
Triethanolamine	219547	177853	188894	184862	209126	188827	671337	233089	173674	169236	175175	190138
Triisopropanolamine	861813	818360	844389	825336	839580	891517	857402	740109	999328	793364	936105	1048396
Valylproline	563316	601734	618539	560520	486776	819336	523843	475675	574518	843497	540215	311531
?-Aspartylphenylalanine	784491	749132	739048	692058	863359	797501	658490	793919	722142	651333	758548	697888