MOLECULAR RESPONSES OF NON-O157 SHIGA TOXIN PRODUCING *E.COLI* WHEN EXPOSED TO ACID STRESS AND ELECTRON BEAM IRRADIATION IN STRAWBERRY MATRIX

A Dissertation

by

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ABSTRACT

Non-O157 Shiga toxin producing *E.coli* (STEC) serogroups are responsible for a growing number of food-related illnesses around the world. These serogroups experience dramatic pH fluctuations either by organic acids introduced during food processing or by inorganic acids in the stomach, which induce acid resistance in the pathogens. The main non-O157 STEC serogroups were analyzed for their acid sensitivity by exposing them to acid buffer, inorganic acid buffer and strawberry puree for 24h and room temperature. The results show that bacterial inactivation depends on the nature of the acid and the strain ($P<0.01$). Each of the serogroups exhibits different levels of resistance to acid stress with O103 as the most resistant strain and O26 and O111 as the weakest of all to acid stress ($P<0.01$). The pattern of microbial inactivation of the acids is inorganic acid> strawberry > organic acid. An untargeted metabolomics analysis identified that peptidoglycan, nitrogen, and unsaturated fatty acid biosynthesis are activated in *E.coli* O26 when exposed to inorganic acid buffer, to protect the structural integrity of the cells. D-Glutamine/D-glutamate metabolism was activated in both strawberry puree and inorganic acid exposed cells to possibly maintain the homeostasis of the cellular pH. Application of 1kGy of eBeam results in a 4-log inactivation of a cocktail of non-O157 STEC serogroups in strawberry puree and a significant (>99.99%) reduction in public health risks. A lethal dose of 3 kGy of eBeam activated metabolic pathways related to DNA repair, virulence and glutathione metabolism in an attempt to repair the lethal

damage. Transcriptomic analysis results indicate that when *E.coli* O26 cells are maintained for 24 hours in phosphate buffered saline (PBS) buffer 88% (5358 genes) of its 6089 genes were up-regulated. However, in the cells are stored at room temperature in a strawberry matrix, only 71 genes (1.1%) were up-regulated. When *E.coli* O26 cells were exposed to 3 kGy eBeam dose and stored in PBS buffer and strawberry matrix, 5379 and 2250 genes were upregulated respectively. Though the cells are inactivated after exposure to lethal doses of eBeam radiation, the metabolomic and transcriptomic analysis indicate that they are still metabolically active.

DEDICATION

To whom I owe the air I breathe in

Maman & Baba

And *Shadi*, my other half

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NOMENCLATURE

eBeam	Electron Beam Irradiation
E.coli	*Escherichia coli*
kGy	kilogray
STEC	Shiga toxin producing *Escherichia coli*
CFU	Colony forming unit
DE	Differentially expressed

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CHAPTER I

INTRODUCTION

Globalization has affected our lives like no other time in the history and the world of food is no exception. Fresh fruits and vegetables indigenous to different parts of the world are available in the local supermarkets all year round irrespective of the season. Although globalization in the food industry has diversified the shopping basket, it has also generated a globalization of different microorganisms that might affect public health through dissemination of pathogens and epidemics around the globe. Many of common pathogenic bacteria in fresh produce happen to be in Enterobacteriaceae family and in the genus, Escherichia (De Roever, 1998). The prevalence of recent food borne outbreaks has increased the concerns about the safety of the fresh produce. On the other hand, the FDA's dietary guidelines encourage the public to increase their fresh produce consumption in order to get more of the health benefits associated with their consumption (USDA, 2015).

Relevance of Research

There is an extensive amount of literature on the incidence, virulence mechanisms and resistance of *E.coli* O157:H7 to different "pathogen hurdles" that have been adopted by the food processing industries (Delbeke et al., 2015; Han et al., 2004b, Knudsen et al., 2001, Nguyen et al., 2014, Yu et al., 2001; Keshun et al., 2001). The inactivation of *E.coli* O157:H11 cells with interventions such as heat (Cheville et al.,

1996), chemical agents (Yu et al., 2001; Keshun et al. 2001), freezing (Knudsen et al., 2001), organic acids (Cheville et al., 1996; Conner & Kotrola, 1995), high pressure processing (HPP) (Linton et al., 1999) , pulsed electric fields (PFE) (Evrendilek et al., 1999), irradiation (Clavero et al., 1994), etc. is well documented. However, the non-O157 *E.coli* serogroups are relatively less studied and therefore, fewer commercial scale interventions applicable to the food industry are available (Shayanfar et al., 2016; Gould et al., 2013). The recent advances in pathogen detection methods have enabled scientists to identify and characterize these strains with greater accuracy. Consequently, more food borne outbreaks associated with non-O157 strains are being reported. The USDA-FSIS (Food Safety and Inspection Service) has categorized the most common non O157 strains the "Big Six". The Big Six strains include O111, O103, O45, O145, O26, and O121 (FSIS, 2012). Recently few outbreaks associated with two of the Big Six namely *E.coli* O26 and *E.coli* O121 in the Chipotle grill restaurant chain and General Mills' Gold Medal™ wheat flour have also been reported (CDC 2015; CDC 2016) Similarly, fresh produce can get contaminated with different strains of *E.coli* . As mentioned earlier there is only limited data on the resistance of Big Six to different interventions available. Therefore, understanding their virulence mechanisms, sensitivity to different treatments, growth kinetics, and quantification of risks associated with them will enable the food industry to adopt more effective hurdles to prevent outbreaks. However, in order to survive in today's competitive market; product quality should also be taken into consideration (Grunert, 2005). Considering the expanded food distribution channels, extending the shelf life of fresh produce is an advantage to increase sales opportunities.

In recent years there has been a movement toward "green technologies" that are both chemical free and do not endanger the environment (Selfa et al., 2008; Cardello, 2003; Sukant et al., 1991). A number of technologies have been introduced to address both microbiological safety and sensory quality. However, none of these technologies on its own is capable of addressing safety, quality and sustainability at the same time (Pillai & Shayanfar, 2015).

One of the most effective non-thermal technologies is electron beam technology (eBeam) that targets the microorganism's nucleic acid. Ionizing radiation inactivates microorganisms by directly causing breaks in DNA strands or indirectly by the generation of radio lytic byproducts that interact with DNA causing DNA breakages (Pillai & Shayanfar, 2015). Electron beam (eBeam) irradiation is an FDA approved non-thermal ionizing radiation based food processing technology (FDA, 2015). An extensive amount of literature indicates that eBeam at low dose of 1 kGy is applicable to increase the shelf-life and inactivate microbial pathogens on fresh produce such strawberries (Shayanfar et al., 2016; Smith, 2015).

One of the naturally existing hurdles in some fruits and vegetables is their intrinsic low pH (3.3 – 3.5) that limits the growth of these pathogens within them (Delbeke et al., 2015; Knudsen et al., 2001). There is competition for nutrients or antagonism from the fruits' phytochemicals (Liao & Fett, 2001). Survival of *E.coli* O157 in fresh and frozen berries has been described in literature (Delbeke et al., 2015; Han et al., 2004b, Knudsen et al., 2001, Nguyen et al., 2014, Yu et al., 2001); however, no study on the survival of non-O157 *E.coli* serogroups in fresh produce has been reported.

The initial population of *E.coli* O157:H7 cells decreased between 2.5-3.9 log in strawberries when stored at 4°C, 7°C, 15°C and 20°C (Delbeke et al., 2015; Nguyen et al., 2014). Other studies suggest *E.coli* O157:H7 survival but not growth on the surface of berries (Keshun et al., 2001; Knudsen et al., 2001). Despite the reduced survival of pathogens in low pH fruits, outbreaks linked to high acidic food items such as apple cider, strawberries, blueberries, etc. continue to occur. Thus, the microbiological safety of high acidic foods is now becoming an increasing concern (Knudsen et al., 2001; Dingman 2000; Asplund & Nurmi, 1991). When *E.coli* cells are exposed to acid, stress inducible proteins are triggered that endow the cells with the capability to survive acidic conditions (Bearson et al., 1996; Lu et al., 2013; Large et al., 2005; Leyer et al., 1995; Abdul-Raouf et al., 1993; Zhao et al., 1993). When microorganisms are grown at pH values higher or lower than that of the cytoplasm (pH 7.6), their protective responses are induced to maintain internal pH homeostasis and to promote cell survival for later exposure to more extreme pH conditions (Castanie-Cornet et al., 2001; Ma et al., 2002; Small et al., 1994). *E.coli* cells are capable of surviving extremely low pH conditions (1.5 -2.5) for hours in the GI tract (Foster, 2000). Different studies indicate that acid tolerance is an important component of virulence for *E.coli* cells (Brown et al., 1997; Leyer et al., 1995). The pH difference across the cell membrane can provide energy in the form of proton potential that supports motility, ATP syntheses and catabolite transport but at the same time increases the uptake of acids that dissipate the proton potential (Russel et al., 1998). A significant number of catabolic enzymes and catabolite transporters are regulated by pH (Foster, 2000). Decarboxylase enzymes such as lysine

and arginine decarboxylases catabolize amino acids and generate alkaline amines as by product that help the cell against external acidification (Argaman et al., 2001).

Rationale

Despite all the attempts to elucidate the molecular and physiological changes associated with acid resistance (Foster, 2000; Lin et al., 1995), the information about different factors affecting acid resistance is limited. It is hypothesized that organic and inorganic acids affect virulence in pathogenic *E.coli* in different ways. Similarly, irradiation of *E.coli* cells with eBeam can also induce some other stress induced resistance mechanisms in the cells. To the best of our knowledge, there is no information on how eBeam processing itself or jointly with low pH can affect *E.coli* specific virulence genes such as Shiga toxin producing genes. It is also not clear what metabolic pathways are triggered by either of low pH or eBeam processing interventions. The main metabolic pathway reported to be triggered in stressed bacterial cells is the Sigma factor (RpoS), which is the central regulator for a variety of stress conditions (Weber et al., 2005; Small et al., 1994). There is also not much information on the key metabolites triggered by either acid stress or ionizing radiation, since it is hypothesized that metabolites induce resistance in bacterial cells. Information on global gene expression and metabolic pathways in Shiga toxin producing non O157 *E.coli* serogroups are lacking. Bacterial DNA is known to be cleaved during eBeam irradiation (Nikjoo et al., 2001). However, there are questions about the ability of the DNA to repair itself (Von Sonntag, 2006). Exposure of the damaged cells by eBeam to low pH might

synergistically contribute to cell inactivation Therefore, there was a need to understand to what extent the final inactivation of non O157 Shiga toxin producing *E.coli* (STEC) in fruits of low pH (in this case, strawberries) is achieved by the "introduced hurdles" (eBeam) or the intrinsic conditions (low pH) that are unfavorable for growth.. Such detailed information will enable us understand how virulence genes in STEC are affected by different interventions and how such food borne pathogens can be effectively controlled in low pH fruits through synergistic food processing technologies.

Major Objectives

This study attempted to identify the molecular responses of *E.coli* O26 in terms of identifying the virulence genes that are differentially expressed and the primary metabolites that are produced within these *E.coli* O26:H11 cells when exposed to low pH (pH 3.6) and lethal eBeam dose (3 kGy).

Specific Objectives

The specific objectives of the current study were:

1. Quantifying the reduction of non-O157 Shiga toxin producing *E.coli* (STEC) in strawberries when exposed to 1 kGy eBeam dose.
2. Comparing the survival of different strains of Big Six in low pH (3.6) matrices such as of organic acid, inorganic acid, and strawberry puree.

3. Studying the transcriptomic response of *E.coli* O26:H11 cells when exposed to 3 kGy eBeam dose and incubated for 24 h in phosphate buffer as compared to strawberry matrix (puree)

4. Identifying the metabolic pathways occurring in *E.coli* O26 after exposure to inorganic acid (pH 3.6) buffer and strawberry puree (pH 3.6) for 24 hours

5. Identifying the metabolic pathways occurring n *E.coli* O26 when exposed to 3 kGy eBeam dose and incubated for room temperature for 24 hours

Relevance to Food Safety

This dissertation research is relevant to food safety as it relates to strawberries and STEC. The study **objective # 1** dealt with quantification of the reduced risks of non-O157 STEC in strawberries when exposed to 1 kGy eBeam dose. This dose is only permitted for phytosanitary reasons in fresh produce; however, there is a body of literature from our lab that supports microbial inactivation of eBeam treated fresh produce. Fresh produce items are mildly processed and in some cases like fresh strawberries are not even washed. Therefore, introducing a non-thermal intervention that not only maintains the quality but also ensures food safety is of great value. On the other hand, using a measure such as QMRA to facilitate communicating the microbial risk reduction in food is beneficial to promote food safety.

Objective # 2 focused on the microbial inactivation effect(s) of naturally occurring organic acids in low pH fruits (i.e. strawberries having a pH value of 3.6). These organic acids present in low pH fruits and vegetables contribute to microbial

inactivation to some degree. To determine the extent to which organic acids contribute to the overall inactivation when strawberries are eBeam processed, an acid sensitivity study was performed to mimic strawberry's acidic content.

Objectives # 3 focused on transcriptomic responses of E.coli O26:H11 (the most commonly occurring non-O157 STEC in foods) to eBeam when present in strawberry puree. Identification of the transcriptomic responses of E.coli O26:H11 as a result of both lethal dose of eBeam and acidic matrix (e.g. strawberry puree) can elucidate the pathogen's gene expression even when they are not culturable. Understanding such responses supports the process of decision making about the safety of various eBeam treated foods of different pH values.

Objectives #4 and **#5** focused on metabolomic responses of *E.coli* O26:H11 when present in different acidic matrices and when exposed to lethal dose of eBeam. The metabolites and the pathways activated in this study objective would determine the destiny of *E.coli* O26:H11 in terms of metabolic activity and the mechanisms these cells would adopt in order to resist the imposed stressors. Therefore, identification of these metabolites and their role in pathogenicity is important in supporting the safety aspect of low pH eBeam treated foods.

All the inoculation studies were performed using fresh strawberry puree. The rationale for using strawberry puree rather than whole strawberries is as follows. The different sizes and geometrical shapes of whole strawberries would make it impossible to keep the conditions in these inoculation studies constant and therefore the results would not be reproducible. On the other hand, in order to ensure the uniformity of the

eBeam dose a physical status of strawberries that can be controlled uniform is of great value. It was critical that inoculated pathogen experienced the acidic conditions. Therefore, performing the studies in a puree rather than the surface was the most logical approach. Therefore, the results of the current study can be extrapolated to various conditions in acidic fruits.

CHAPTER II

LITERATURE REVIEW

Overview

Strawberries are popular because of the relatively high content of phytochemicals, which are beneficial to human health. Among these phytochemicals, anthocyanin and ellagitannins are the major antioxidant compounds (Giampieri et al., 2012). Strawberries have at least a 2X to 11X greater antioxidant capacity than apples, peaches, pears, grapes, tomatoes, oranges, or kiwifruit (Wang et al., 1996). The hypothesized health benefits related to strawberry consumption include their role in the prevention of inflammation, antioxidant capacity, cardiovascular disease (CVD), certain cancers, s; type 2 diabetes, obesity, and neurodegeneration (Huntley, 2009; Seeram, 2008).

Strawberries are, however, perishable with extremely short postharvest shelf life (Holzwarth et al., 2012). Strawberries are generally not washed after harvest and so fungal spoilage is inevitable (De Roever, 1998). Since they are not washed, the potential risk of foodborne pathogens being transmitted via fresh strawberries is, therefore, relatively high (Delbeke et al., 2015) (Table 1). According to the CDC between the years 1998 and 2014, 18,211 outbreaks associated with consumption of strawberries contaminated with viruses and pathogenic bacteria such as STEC have been reported (CDC, 2015) (Table 1). In 2012, Germany experienced the largest recorded foodborne illness outbreak it its history from frozen strawberries imported from China causing at

least 11,000 cases of norovirus (Food Safety News, 2014). Likewise, in the US, there were similar outbreak reports from Oregon (Food Safety News, 2011) and Massachusetts (CDC, 2015) associated with *E.coli* O157:H7 and *E.coli* O26:H11 respectively (Table 1). Both *E.coli* O157:H7 and *E.coli* O26:H11 are Shiga toxin producing *Escherichia coli* (STEC) meaning they are pathogenic types of *E.coli* that produce a potent toxin called Shiga toxin (Stx).

Table 1- Food-borne outbreaks associated with consumption of Shiga toxin producing *E.coli* contaminated strawberries.

Year	Month	State	Serotype	Illness	Hospitalizations	Deaths	Food Vehicle	Contaminated Ingredient
2006	July	Massachusetts	O26	5	1	0	strawberries; blueberries	
2011	July	Oregon	O157:H7	15	7	2	strawberries	
2011	July	Minnesota	O157:H7	6	1	0	fruit	strawberries; watermelon

Adopted from CDC, 2015.

Strawberries can get contaminated with a variety of microbial pathogens (protozoan, bacterial, viral and fungal) from soil, irrigation water, insects, wild and domestic animals, equipment and human handling (Delbeke et al., 2015) (Table 3). Infection rates of non-O157 STEC were reported to be as high as that of O157 STEC. Non-O157 STEC were responsible for a number of deaths in Germany when they entered the food supply chain via contaminated sprouts (Mora et al., 2011, Gould et al., 2013; FSIS, 2012; Werber et al., 2002). These pathogenic serogroups have become an

important cause of food borne illnesses associated with consumption of fresh produces and meat products (Hsu et al., 2014).

New technologies to extend the shelf life (e.g. modified atmosphere packaging, irradiation, etc.) coupled with international trade agreements have facilitated imports of fresh produce to fill shortfalls where domestic supplies were unable to meet consumer demands and willingness to pay (Clemens, 2004). Since a large portion of fresh produce is consumed raw, the number of foodborne outbreaks associated with these products has increased correspondingly (Buck et al., 2003; Lynch et al., 2009; Olaimat & Holley, 2012). Since fresh produce cannot withstand any thermal pathogen inactivation methods, there is a need for robust non-thermal intervention technologies to ensure microbiological safety in fresh produce.

Shiga Toxin Producing *Escherichia coli* (STEC)

Shiga toxin producing *Escherichia coli* (STEC) has emerged as one of the major causes of food-borne infections since the early 1980s. The main serogroup of STEC that is extensively studied and documented is *E.coli* O157:H7 (Perelle et al., 2007); however, there are other serogroups of STEC that even though they have not been investigated as much, the number of food borne outbreaks associated with them has increased over the past decade (Hsu et al., 2014; Shayanfar et al., 2016). The main non-O157 STEC are the six serogroups of O26, O121, O103, O111, O145 and O45 that are often referred to as the “Big Six” STEC (USDA-FSIS, 2010). These are considered as adulterants in beef (Smith et al., 2014). Among all the non-O157 STEC, *E.coli* O26 is considered to be the

most prevalent stain (Table 3). Between 2000 and 2010, about 2006 cases of non-O157 STEC infections were reported by FoodNet in the United States, indicating the infection incidence of non-O157 STEC has increased from 0.12 to 0.95 per 100,000 populations during 2000 and 2010 (Gould et al., 2013). In year 2011, STEC O157 serogroups caused 40.3% illnesses, whereas the non-O157 STEC serogroups resulted in 59.7% of the cases (Scallan et al., 2011). These outbreak data confirms that the non-O157 STEC are responsible for a bigger portion of the total STEC cases in the USA. The majority of the cases were related to food and food items (Table 2 and Table 3). According to the same reference, the majority of the reported cases were food-borne. The STEC infections result in diarrhea and hemorrhagic colitis and could eventually lead to cause hemolytic uraemic syndrome (HUS) (Perelle et al., 2007). HUS is characterized by acute kidney failure but the central nervous systems, pancreas, lungs and even heart might be also affected (Smith et al., 2014). The main source of STEC in foods has been identified to be the rumens faeces (Locking et al., 2001).

Table 2 - The incidence of the infection associated with the non O157 STEC between the years 2000 and 2010

Isolate	**Incidence (%)**
O26	26
O103	22
O111	19
O121	6
O45	5
O145	4

Adopted from Gould et al., 2013.

Table 3 – Foodborne disease outbreaks associated with consumption of strawberries.

Year	State	Species	Location of Preparation	Illnesses	Hospitalizations	Deaths
2014	Minnesota	*Norovirus Genogroup II*	Restaurant - Sit-down dining; Caterer (food prepared off-site from where served)	8	1	0
2014	Ohio	*Cryptosporidium unknown*	Private home/residence	6	1	0
2013	Colorado	*Hepatitis A*	Private home/residence	2	1	0
2012	Michigan		Private home/residence; Grocery store	6	0	0
2011	Oregon	*E.coli O157:H7*	Other (describe in remarks)	15	7	2
2007	Georgia	*Norovirus Genogroup I*	Private home/residence	10	0	0
2007	Florida	*Hepatitis A*	Restaurant - other or unknown type	3	2	0
2007	Illinois		Caterer (food prepared off-site from where served)	13	0	0
2006	Massachusetts	*E.coli O26*	Other (describe in remarks)	5	1	0
2005	Washington	*Norovirus Genogroup I*	Private home/residence; Caterer (food prepared off-site from where served); Other (describe in remarks)	20	0	0

Table 3 Continued.

Year	State	Species	Location of Preparation	Illnesses	Hospitalizations	Deaths
2005	Georgia	*Norovirus Genogroup I*	Other (describe in remarks)	40	0	0
2005	Massachusetts		Caterer (food prepared off-site from where served)	98	0	0
2004	Colorado	*Norovirus Genogroup I*	Long-term care/nursing home/assisted living facility	62	5	0
2003	California	*Salmonella enterica*	Other (describe in remarks)	13	2	
2002	Minnesota	*Norovirus Genogroup I*	Restaurant - other or unknown type	15	0	0
2002	Washington DC		Grocery store	11	0	0
2000	Pennsylvania		Private home/residence; Grocery store; School/college/university; Other (describe in remarks)	14	0	0
2000	Massachusetts	*Hepatitis A*		8		
2000	Florida		Private home/residence	10		
2000	California	*Norovirus Genogroup I*	Restaurant - other or unknown type	100		
1999	Minnesota	*Norovirus Genogroup I*	Office/indoor workplace	63	0	0

Table 3 Continued.

Year	State	Species	Location of Preparation	Illnesses	Hospitalizations	Deaths
1999	Florida	*Cyclospora cayatenensis*	Restaurant - other or unknown type; Other (describe in remarks)	94	1	
1999	California	*Shigella sonnei*	Restaurant - other or unknown type	3	1	0
1998	Texas	*Hepatitis A*		29		
1998	New Hampshire		Restaurant - other or unknown type	28		
1998	Iowa	*Norovirus Genogroup I*	Restaurant - other or unknown type	41	0	0

Adopted from CDC, 2015.

Mechanism of virulence in STEC

The pathogenic serogroups of *E.coli*, unlike the generic non-pathogenic serogroups express traits that enable them to resist host defenses, proliferate, and cause diseases. The genes associated with pathogenic STEC colonization and virulence factors are present within specific regions of DNA that are called pathogenicity islands (PAIs). There are various genes in PAIs that contribute to the virulence of pathogenic STEC (Swenson et al., 1996). Ingested STEC cells remain in the lumen of the intestinal tract and adhere and attach to the epithelial cells using specific adhesion factors (A/E lesion). The cell adhesion to epithelial cells is mediated through the outer membrane adhesion protein, Intimin (encoded by *eaeA* gene). Subsequently, distinct morphological structures called pili or Fimbriae are formed (Kaper et al., 2004; Carey et al., 2009). The other virulence factor is flagella that endows mobility and encoded by over 40 genes. Flagellar structural protein is flagellin and is encoded by *fliC* (Carey et al., 2009). Then locus of enterocyte effacement (LEE) and non-LEE effectors are injected into the host cells through type three secretory system (T3SS). LEE and non-LEE effectors can upregulate a specific set of genes to manipulate the host cells through a variety of functions including hemolysis, inhibition of phagocytosis, repression of host lymphocyte response, destruction of microvilli, making lesions on enterocytes, etc. (Kaper et al., 2004). The main metabolite related to virulence in STEC is Shiga toxin that appears as Stx1 and Stx 2, with Stx2 being a more potent toxin that Stx1 (Smith et al., 2014). The STEC serogroups isolated form patients with HUS are mainly positive for the *stx2*, *eaeA* and *hlyA* genes (Monaghan et al., 2011). Shiga toxin is transported to the Golgi

apparatus and endoplasmic reticulum. Shiga toxin can cleave ribosomal RNA and thus inhibit protein synthesis within eukaryotic cells. Inhibition of protein synthesis may result in apoptosis of kidneys endothelial cells and HUS subsequently (Ivarsson et al., 2012; Kaper et al., 2004). Despite certain differences in different serotypes of STEC, their virulence is mainly associated with the presence of pathogenicity islands (PIs) that express genes for motility, attachment to epithelial cells and secretion of Shiga toxin (Perelle et al., 2007).

STEC in fresh produce

In recent years, fresh produce has been identified as the vehicle for transmission of many pathogen outbreaks (De Roever, 1998). STEC serogroups are known to colonize the intestines of cattle and are shed into the environment where it could persist for several months (Bolton et al., 2011). This environmental release could contaminate irrigation water or could directly result in produce contamination. There are still many questions about the transmission of pathogens from their reservoirs to fruits and vegetables. The reason for this level of uncertainty is that despite the common handling factors in all produce items each fruit or vegetable has its own physical characterization, growing, harvesting and post-harvesting practices (De Roever, 1998). Since fresh produce does not undergo any major kills step the probability of harboring STEC on fresh produce is relatively high. Therefore, it is not surprising that there are a number of STEC related foodborne pathogen outbreaks (Table 4).

Table 4- Foodborne outbreaks associated with the non-O157 STEC during 1998 -2016.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2016	May	Multistate	O121	Flour	42	11	0	flour	flour
2015	Dec	Multistate	O26	Chipotle Mexican Grill	55	21	0	Chipotle Mexican grill	
2014	April	New Mexico	O26	Private home/residence	4	2	0		
2014	May	Multistate	O145	Private home/residence	8	3	0	ground beef	
2014	June	Arizona	O26	Restaurant – Sit-down dining	2.	0	0		
2014	June	Multistate	O111	Restaurant – Sit-down dining	16	2	0	cabbage	
2014	June	Minnesota	O111	Restaurant – Sit-down dining	15	4	0	cabbage	cabbage

Table 4 Continued.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2014	Oct	Multistate	O103:H2	Restaurant - other or unknown type	12	0	0		
2014	Oct	Minnesota	O121	Unknown	3	1	0		
2014	Nov	Minnesota	O111	Restaurant - "Fast-food"(drive up service or pay at counter)	3	0	0	salsa	
2014	Nov	Utah	O26	Other (describe in remarks)	4	0			

Table 4 Continued.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2014	May	Multistate	O121	Restaurant - "Fast-food"(drive up service or pay at counter); Restaurant - Sit-down dining	19	5	0	clover sprouts	clover sprouts
2013	Apr	Multistate	O26	Restaurant - "Fast-food"(drive up service or pay at counter)	26	5	0	lettuce	
2013	June	Tennessee	O26	Fair, festival, other temp or mobile services	3	0	0		
2012	Jan	Multistate	O45	Private home/residence	21		0	sandwich, unspecified	

Table 4 Continued.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2012	Apr	Louisiana	O145		5	3	1		
2012	July	Colorado	O111:NM	Private home/residence	2	0	0	raw milk	milk
2012	Oct	Multistate	O145	Restaurant - "Fast-food"(drive up service or pay at counter)	16	6	0	lettuce	
2012	Dec	Multistate	O121	Private home/residence	35	9	0	frozen meal	
2011	May	Maine	O26:NM		2	2	0		
2011	Sep	Kansas	O26	Unknown	4	3	0		

Table 4 Continued.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2011	Sep	Wisconsin	O111; O26		12	0	0		
2011	Dec	Multistate	O26	Restaurant - "Fast-food"(drive up service or pay at counter)	29	7	0	clover sprouts	
2010	Feb	Washington	O26:H11	Other (describe in remarks)	6	0	0	milk, whole milk unpasteurized	
2010	June	Multistate	O26		3			ground beef	
2010	Sep	Idaho	O121:H19	Fair, festival, other temp or mobile services	6	3	0		

Table 4 Continued.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2010	Nov	Multistate	O45		7	3	0	sausage	
2010	Nov	Minnesota	O103:H2; O145:NM	School/college/ university	29	2	0	venison	Venison
2010	Nov	Oklahoma	O157:H7; O84:NM	Prison/jail	21	0	0	multiple foods	
2009	July	Wyoming	O111	Private home/residence	2	0	0		
2008	July	Nebraska	O111	Other (describe in remarks)	34	2		pork, BBQ	
2008	Aug	Oklahoma	O111	Restaurant - other or unknown type; Religious facility	344	71	1		
2007	Mar	Maine	O11		8	0	0		

Table 4 Continued.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2007	July	North Dakota	O111	Private home/residence	23	0	0		
2007	July	Colorado	O121; O26; O84	Prison/jail	135	10	0	American cheese, pasteurized; margarine	cheese
2006	July	Utah	O121	Restaurant - other or unknown type	3			lettuce-based salads unspecified	
2006	July	Massachusetts	O26	Other (describe in remarks)	5	1	0	strawberries; blueberries	
2005	Aug	New York	O45	Prison/jail; Other (describe in remarks)	52	3	0		

Table 4 Continued.

Year	Month	State	Serotype	Location of Preparation	Illnesses	Hospitalizations	Deaths	Food Vehicle	Item
2004	Sep	New York	O111;	Other (describe in remarks)	212	14	0	apple cider, unpasteurized	fruit, un-specifi-ed
2000	July	Washington	O103	Caterer (food prepared off-site from where served); Other (describe in remarks)	18	2	0	punch, unspecified	
1999	June	Texas	O111	Camp	55		0		
1998	Oct	Montana	O121		40				

Adopted from CDC, 2016.

Survival of STEC in low pH matrices

STEC cells experience different acidic environment along their journey from the cattle rumen to the land, low pH crop in the land and finally ingestion in human GI tract with a pH value about 2.low pH food. Each of these environments can induce acid stress responses in STEC and subsequently provide them with acid resistance trait.

Low pH (3.3-3.5) in fresh produce is known to be inhibitory to pathogen multiplication (Delbeke et al., 2015; Knudsen et al., 2001; De Roever, 1998). Strawberries are highly acidic (juice pH 3.6) and contain citric acid (0.73 to 1.58 g/100 ml) and malic acid (0.22 to 0.69 g/100 ml) (Kallio et al., 2000). If any bacteria happen to occur in a low pH fruit such as strawberries, the acidic environment can induce bacterial injury, inactivation, and growth inhibition (Han et al., 2004b). Low pH (less than 4.0) is presumed to be a barrier against the growth of *E.coli* O157 (De Rover, 1998); however, food borne outbreaks associated with consumption of strawberries contaminated with STEC are reported (Table 3). Thus, the microbiological safety of high acid foods is now becoming an increasing concern (Asplund & Nurmi, 1991; Dingman 2000).

Survival of *E.coli* O157 in strawberries(fresh and frozen)and strawberry juice has been documented (Delbeke et al., 2015; Han et al., 2004b, Knudsen et al., 2001, Nguyen et al., 2014, Yu et al., 2001). However, there is no published information on the survival of non-O157 STEC in fresh produce and specifically in strawberries. The initial population of *E.coli* O157:H7 decreased about 2.5-3.9 log, when strawberries were stored at different temperatures such as 4°C, 7°C, 15°C and 20°C, (Delbeke et al., 2015). This reduction trend continued for all the storage temperatures to almost

undetectable amounts. However, the samples incubated at 22°C could not be used for analysis due to growth of mold and rot. The survival of *E.coli* O157:H7 in strawberry juice (pH 3.6) at two different temperatures of 4°C and 37 °C was evaluated (Han et al., 2004b). Three days of storage at 4°C did not change the initial population of *E.coli* O157:H7 in strawberry juice; however, using plating on selective media suggested that almost 2 log of the cells were injured during cold storage. Incubation of *E.coli* O157 cells in strawberry juice at 37°C resulted in their inactivation (Han et al., 2004b). Other studies report that after 24 and 48h incubation at room temperature, no substantial change of *E. coli* 0157:H7 population in strawberries (whole or sliced) was observed. However, when whole strawberries were refrigerated, there was between 1 to 2 log reduction (Knudsen et al., 2001; Yu et al., 2001). The initial population of *E.coli* O157:H7 on bruised strawberries declined by 1.9 log, when berries were stored for 24 hours at 2°C (Nguyen et al., 2014). However, their populations remained stable on undamaged strawberries. *E.coli* O157:H7 can survive but not grow on the surface of fresh strawberries (Keshun et al., 2001; Knudsen et al., 2001). But there is the possibility for the microorganisms to penetrate through the pores on the surface of the strawberries and internalize within the fruit (references needed to support this claim). Despite the poor survival of STEC in low pH fruits, outbreaks linked to high acidic foods such as apple cider, strawberries, blueberries, etc. continue to occur.

Acid stress response in STEC

When *E.coli* cells are exposed to acid, stress inducible proteins are triggered that endow the cells with the capability to survive acidic conditions (Bearson et al., 1996; Lu et al., 2013; Large et al., 2005; Leyer et al., 1995; Abdul-Raouf et al., 1993; Zhao et al., 1993). Growing microorganisms at pH values higher or lower than that of the cytoplasm (pH 7.6), induces protective responses in order to maintain internal pH homeostasis and to promote cell survival for later exposure to more extreme pH conditions (Ma et al., 2002; Castanie-Cornet et al., 1999; Small et al., 1994). *E.coli* cells are capable of surviving extremely low pH conditions (1.5 -2.5) for hours in the HCl acidified environment of the GI tract (Foster, 2000). The numerous reported food-borne outbreaks associated with STEC indicate that STEC cells can survive this hostile environment and maintain their virulence (Table 4). There are previous reports detailing the genomic responses in *E.coli* during acid exposure. The results of genomic responses in *E.coli* during acid exposure indicate that low pH enhances expression of numerous virulence factors (Maurer et al., 2005). The pH difference across the cell membrane can contribute cell energy in the form of proton potential that supports motility, ATP syntheses and catabolite transport but at the same time increases the uptake of acids that dissipate the proton potential (Russel et al., 1998). A significant number of catabolic enzymes and catabolite transporters are regulated by pH (Foster, 2000). Decarboxylase enzymes such as lysine and arginine decarboxylases catabolize amino acids and generate alkaline amines as by product that help the cell against external acidification (Argaman et al.,

2001). Low pH accelerates acid consumption and proton export in *E.coli* K12 cells (Maurer et al., 2005).

There are three main inducible decarboxylase mechanisms associated with acid resistance in *E.coli*, namely acid resistance system 1 (AR1), acid resistance system (AR2), and acid resistance system (AR3), whose activities are medium dependent. AR1 is apparent in cells grown to stationary phase in Lysogeny broth (LB) media to pH 5.5 and is glucose repressed and its mechanism is unclear. AR2 and AR3 have similar clear mechanism and dependent on glutamate and arginine respectively (Foster, 2004).

When cells are exposed to an acidified environment, *gadC* encodes a putative glutamate/ϒ-amino butrate antiporter which is required for the glutamate-dependent acid resistance system in *E.coli*. The glutmate-glutamine cycle is trigged by upregulation of *glsA* that activates L-glutamine anminohydrolase that catalyzes degradation of L-glutamine to L-glutaminc acid and ammonium ion. Furthermore, this enzyme supplies the nitrogen required for the biosynthesis of a variety of metabolic intermediates (Sinsuwan et al., 2012). The amino acids glutamine and glutamic acid are known enhancers of *E.coli* survival in acidic conditions (Lu et al., 2013; Foster, 2000; Lin et al., 1995). The increase in these nitrogen containing amino acids could facilitate the action of glutaminase, which results in release of gaseous ammonia which would ultimately neutralize the increasing proton levels within such cells (Lu et al., 2013).

Despite all the attempts to elucidate the molecular and physiological changes associate with acid resistance (Foster, 2000; Lin et al., 1995), our understanding of this phenomenon is still incomplete and there is still uncertainty about how virulence factors

including Shiga toxin producing in *E.coli* are affected by low pH. The main factor extensively studied in acid stressed bacterial cells is sigma (RpoS) factor as the central regulator for a variety of stress conditions (Weber et al., 2005; Cheville et al., 1996; Waterman &Small, 1996; Small et al., 1994).

Electron Beam Technology

Electron beam (eBeam) irradiation also known as electronic pasteurization is one of the main three principle ionizing radiation techniques that are available to the food industry. The whole working principle is based on speeding up electrons (from commercial electricity) that are generated off a cathode in a vacuum environment. An electron gun consisting of a cathode, grid and anode generates and accelerates the beam. The generated beam of electron is focused using a magnet to control the pattern the beam leaves the gun. Application of high voltage increases the efficiency of the beam power (Clemmons et al., 2015).

Electron beam technology is a "switch-on/switch-off" technology meaning when needed it can be turn on or when not needed, switched off. . This key feature differentiates eBeam technology from radioisotope based, cobalt-60 or other isotope radiation technologies. Ionizing results in DNA and RNA strand breakage in microorganisms and therefore inactivates the growth in both spoilage and pathogenic microorganisms. Unique features associated with eBeam have made it a functional alternative to other non-thermal technologies to address microbiological safety, food quality and what environmental sustainability in food (Table 5).

Table 5- Benefits and challenges associated with application of eBeam technology.

Benefits	**Challenges**
• Non –thermal technology • No loss in nutrients, flavor or appearance of food • Applicable on packaged products (avoids cross contamination) • No chemicals • Low carbon footprint • Low energy requirements • Higher dose rate delivered over time No radioactive material storage on site or waste • Adjustable for varying doses and different applications • Continuous process • Precise doses	• High operating costs if product volume is low • Labeling requirements • Requires education • Widespread confusion about the technology • Limited vendors providing the technology • Lower penetration depth when compared to gamma irradiation for pallets

The accelerator used for eBeam should provide the required electron energy required to penetrate the packaged product. Food irradiation is permitted with eBeam technology until at below 10 MeV. Significantly, lower energy is needed for surface sterilization or treatment. Alternatively, the distance of accelerator's scan horn and the target on the conveyor belt can be adjusted (Brown, 2015).

The unit of measure of irradiation is dose that is measured in Gy (Grays) and kGy (kilo Grays). Different applications of eBeam are defined by the dose of eBeam approved by FDA (Table 6). Electron beam processing of food can be broadly categorized into 3 different dose ranges namely,

1) Low dose treatment (<1 kGy); mainly for phytosanitary, insect disinfestation, delaying the maturity in fruits, preventing germination in tubers (potato, onion, ginger,...)

2) Intermediate dose treatment (1-10 kGy), pasteurization and extension of shelf life of foods

3) High dose treatment (10-44 kGy), commercial sterilization of food.

The original patent on food irradiation technology is over 100 years old. The technology has matured over time as a safe and efficient food processing technology (Farkas et al., 2014). Early food irradiation was centered around γ-rays and commercial irradiation of food dates back to 1960, when it was suggested as a new method to replace canning process (Roberts, 2014). The development of electron beam linear accelerators took place during the 1930 and; thereafter, the technology matured with lower production costs and was introduced as an optimum method of food irradiation (Lung et al., 2015). Considering the source of energy, speed, safety, etc., electron beam accelerators turned to be more successful compared to γ-rays (Clemmons et al., 2015).

An investigation into the published data, questionnaire survey and direct visits on the status of food irradiation in the world in 2005 indicated that the quantity of irradiated foods in the word to be 405,000 ton compromised 46% for spice and dry vegeTable disinfection, 20% for fruit and grain disinfestation, 8% for fish disinfestation, 22% for sprout inhibition of garlic and potato and 4% for other items including health foods, mushroom, honey etc. (Kume et al., 2009) (Table 7).

Table 6– FDA approved applications of ionizing radiation technology for food processing.

Application	**Dose (kGy)**
Control of Trichinella spiralis in pork carcasses	0.3 – 1
Inhibition of fresh food growth and maturation	<1
Microbial disinfection of dry or dehydrated enzyme preparations	<10
Microbial disinfection of dry or dehydrated aromatic vegetables, herbs and spices	<30
Control of food-borne pathogens in fresh poultry products	< 4.5
Control of food-borne pathogens in frozen poultry products	< 7.0
Sterilization of space food	Min 44
Control of food-borne pathogens in fresh meat products	< 4.5
Control of food-borne pathogens in frozen meat products	< 7.0
Control of *Salmonella* in fresh shell eggs	< 3.0
Control of microbial pathogens on seeds for sprouting	< 8.0
Control of *Vibrio* bacteria and other pathogens in fresh or frozen molluscan shellfish	< 5.5
Control of food-borne pathogens and extension of shelf life in fresh iceberg lettuce and fresh spinach	< 0.4
Control of foodborne pathogens and extension of shelf life of chilled, frozen, raw, cooked, partially cooked or dried crustaceans	< 6.0

Adopted from FDA, 2015.

Table 7- The global status of food irradiation in 2005.

Food	**Application**	**Percentage (%)**	**Amount (ton)**
Spices and dry vegetables	Disinfestation	46	186,000
Garlic and potato	Sprout inhibition	22	88,000
Grains and fruits	Disinfestation	20	82,000
Meat and fish	Disinfestation	8	32,000
Others	Mushroom, honey, etc.	4	17,000
Total		**100**	**405,000**

Adopted from Kume et al., 2009.

The sensitivity of microorganisms to eBeam is expressed as D10-value, which is the dose required in order to reduce the initial population of microorganisms by one log (D10 for different microorganisms). Different microorganisms indicate different level of sensitivity with the fungi and spore formers to be the most resistant of all (Table 8). Another factor that facilitates eBeam irradiation is the moisture content of the product as the higher the amount of free water the more effective eBeam can be applied. The reason is generation of radiolytic products in water that can themselves indirectly affect microbial DNA. The results of some research claim that at doses higher than 10 kGy the oxidation of fat content of the food being irradiated should be of concern; however, such high doses are rarely applied in the majority of food groups. The environmental factors including temperature, water activity, pH and chemical composition of food can affect the irradiation efficiency too (Roberts, 2014; Sommers, 2012). Areal density (g/cm^2) is also of importance in order to ensure eBeam penetration into the product. Reconfiguration in the packaging design in order to meet the range of areal density to be able to conduct eBeam processing is helpful.

Table 8- The D-10 value of some microorganisms in different food products when irradiated with eBeam.

Microorganism	**D10 (kGy)**	**Food/Matrix**	**Temperature (℃)**	**Reference**
Campylobacter jejuni	0.08-0.20	Food	RT	Farkas, 2005
Campylobacter jejuni	0.18-0.32	Frozen food	RT	Farkas, 2005
E.coli K-12 MG1655	0.18	Gelatin	RT	Rodriguez et al. 2006
	0.45	Cantaloupe	RT	Rodriguez et al. 2006
E.coli O157:H7 933	0.13	Gelatin	RT	Rodriguez et al. 2006
E.coli O26:H11	0.11	Buffer	RT	Shayanfar et al., 2016
E.coli O111:NM	0.07	Buffer	RT	Shayanfar et al., 2016

Table 8 Continued.

Microorganism				
E.coli O45:H2	0.07	Buffer	RT	Shayanfar et al., 2016
E.coli O103: H2	0.06	Buffer	RT	Shayanfar et al., 2016
E.coli O121: H19	0.14	Buffer	RT	Shayanfar et al., 2016
Listeria monocytognes	0.18	Gelatin	RT	Rodriguez et al. 2006
Listeria monocytognes	0.15	Cantaloupe	RT	Rodriguez et al. 2006
Listeria innocua	0.66	Gelatin	RT	Rodriguez et al. 2006
Salmonella enterica	0.38	Gelatin	RT	Rodriguez et al. 2006
Salmonella Poona	0.13	Gelatin	RT	Rodriguez et al. 2006
Bacillus cereus spores	>3.3	Distilled-water	RT	Valero et al., 2006
Bacillus cereus spores	3.8±0.40	Distilled-water	RT	De Lara et al., 2002
Bacillus cereus spores	0.95±0.01	Ham	RT	Aguirre et al., 2012
Bacillus cereus spores	0.87±0.01	TSA	RT	Aguirre et al., 2012
Bacillus subtilis spores	> 3.3	Distilled-water	RT	Valero et al., 2006
Bacillus subtilis spores	3.6±2.40	Distilled-water	RT	De Lara et al., 2002
Rotavirus	1.29 ± 0.64	Spinach	RT	Espinosa et al., 2012
Rotavirus	1.03 ± 0.05	Lettuce	RT	Espinosa et al., 2012
Shigella spp.	0.22-0.40	Food	RT	Farkas, 2005
Shigella spp.	0.22-0.41	Frozen food	RT	Farkas, 2005
poliovirus	2.35 ± 0.20	Spinach	RT	Espinosa et al., 2012
poliovirus	2.32 ± 0.08	Lettuce	RT	Espinosa et al., 2012
Vibrio spp.	0.02 -0.14	Food	RT	Farkas, 2005
Vibrio spp.	0.04-0.44	Frozen food	RT	Farkas, 2005
Yersinia enterocolitica	0.04-0.21	Food	RT	Farkas, 2005
Yersinia enterocolitica	0.20-0.39	Frozen food	RT	Farkas, 2005
Salmonella spp.	0.048	Sprout		Rjkowski & Thayer, 2000

Application of eBeam in fresh produce

Fresh produce including fresh, unprocessed fruits and vegetables are voluble agricultural products that given the fact that they are presented raw, they might carry pathogenic microorganisms that jeopardize the lives of people. Between 1980 and 2001, per capita consumption of fresh fruits increased by 19 percent (Huang & Huang, 2007). The health benefits of fresh fruits and vegetables are widely known. In the US, at the federal level, there is an ongoing set of promotions to encourage fresh produce consumption for example, the replacement of the USDA food pyramid (MyPyramid) with My Plate in support of dietary recommendations in 2011 to fill half of the daily plate with fruits and vegetables (USDA, 2015). The World Health Organization (WHO) also encourages the daily intake of at least 400 g of fruit and vegetable per day for the prevention of chronic diseases, such as heart disease, cancer, diabetes, and obesity (Callejón et al., 2015). Fresh produce are mainly consumed for the naturally occurring phytochemicals, which are highly susceptible to heat treatments. Therefore, the non-thermal status of eBeam, makes it an exciting technique in order to ensure both safety and quality in fresh produce.

In a study by Grasso et al. (2011) application of 2.3 kGy resulted in more than 4 log reduction in the bioburden population of cabbage. They showed that increasing the dose to 4.0 kGy decreased the population of *E.coli* K-12 by about 7 log. In another study only 0.7 kGy of eBeam could reduce 5 log of the test organism in baby spinach leaves packed under modified atmosphere packaging (MAP) with 100% (Gomes et al., 2011). Application of 7 kGy of eBeam on tomato, cantaloupe and lettuce seeds reduced

Salmonella levels by 3-5 log (Trinetta et al., 2011). Viruses are the major cause of food borne illnesses associated with consumption of fresh produce. Espinosa et al (2012) showed that eBeam at 4 kGy could significantly reduce rotavirus and poliovirus in iceberg lettuce and spinach about 2.5 and 3 respectively.

The majority of the studies on irradiation of fresh produce are performed using γ-irradiation and thus the number of studies performed with eBeam on fresh produce is not as exhaustive as that of gamma irradiation. The first marketed irradiated strawberries were treated with gamma in January 1992 (Marcotte, 1992) and the success encouraged the irradiation of strawberries.

Thomas et al (1986) reported that strawberries may tolerate irradiation up to 2 kGy. Żegota (1988) noticed loss of color in strawberries upon irradiation up to 2.5 -3 kGy and Johnson et al (1965) reported texture softening at 1-4 kGy in strawberries. Yu et al (1995) were the first to irradiate strawberries with eBeam and noted that even at 2kGy no chemical or physical changes occurred in the fruit. As a result of the aforementioned study it took 4 days for the unirradiated fruit to reveal visible mycelia, while in 2kGy eBeam treated strawberries it took 8 days for the mycelia to appear suggesting application of eBeam can extend the shelf life in strawberries.

It is worth mentioning that an acidic environment would favor the disappearance of the aqueous electron according to Equation 1 (Stewart, 2001), which can be valid in irradiation strawberries with the intrinsic pH value of 3.6.

$e^- + H^+ \rightarrow {}^{\cdot}H$

Equation 1- The interaction of positively charged hydrogen in acidic environment with electron.

It is hypothesized that the intrinsic low pH value in strawberries might sensitize the microbial cells and have a synergistic effect along with eBeam in reduction on the initial concentration of the microbial cells. Several studies have indicated that application of doses up to 2.5 kGy on strawberries is the maximum dose that can be applied in order to control the microbial growth without affecting the sensory quality attributes with little visual color change or in the content of anthocyanins extracted from the berries (Thomas, 1988). Horubala (1964 & 1968) suggested there is a relationship between the amount of anthocyanin pigment in the fruit and its susceptibility to color change during irradiation. He also suggested that strawberries are the most resistant berries among all other berries when exposed to 1.5-7.5 kGy and the pigments were regenerated during storage. Strawberries exposed to 2.5 kGy destroyed about 20% of the anthocyanins; however, during 6 days of storage the pigments were regenerated but

at higher doses this possibility was failed as a result of irreversible changes in the changes in anthocyanin molecules (Horubala, 1964; Horubala 1968). Deschreider & Vigneron (1973) did not report any changes in the coloration of anthocyanins detected in strawberries after exposure to doses of 1, 2, and 5 kGy. While does of 1.0-3.5 kGy caused no visual color changes, control samples showed higher content of anthocyanins photometrically with color intensity decreasing in indirect proportion of dose (Lovel & Flick, 1966), meaning the higher the anthocyanin content the more significant the changes in the final color would be. Table 9 summarizes the findings on the application of eBeam for different fresh produce items.

Table 9- Summary of studies that investigated the use of electron beam processing for fresh produce.

Fresh produce	eBeam Dose (kGy)	Summary of results	Reference
Blueberries	0.25, 0.5, 0.75, 1.0	• Texture effect was related to the blueberry variety. Texture in 'Sharpblue' was significantly affected by increase in dose but firmness in 'Climax' berries was not affected. • Flavor and texture were negatively affected with the increase in dose for both what cultivars; however neither the flavor nor texture was rated unacceptable by the sensory panelists. • Weight loss, decay, peel color, total soluble solids and titraTable acidity were unaffected by target doses. • Insect infestations could be controlled by a dose of no higher than 0.75 kGy.	Miller & McDonald, 1995
Blueberries	1.1, 3.2	• Doses higher than 1.1kGy affected the texture. • Color was affected at 3.2kGy • Irradiation reduced the respiration rates of blueberries. • Blueberry quality was unacceptable at 3.2 kGy • Irradiation did not affect the density, pH, water activity, moisture content, acidity and juiciness of the fruits	Moreno et al., 2007
Cantaloupe	0, 1.5, 3.1	• Quality was unaffected up to 1.0 kGy. • Carotene content increased as irradiation dose increased.	Castell-Perez et al., 2004

Table 9 Continued.

Fresh produce	eBeam Dose (kGy)	Summary of results	Reference
Cabbage	0,1.0,2.3, 4.0	• At 2.3 kGy, about 4.0 log reduction in bioburden is noticed. • At 4.0 kGy, about 7 log reduction was noticed in *E.coli* K-12.	Grasso et al., 2011
Grapefruit	1.0, 2.5, 5.0, 10.0	• The acidity decreased with increasing the dose, whereas the total soluble solids increased. • At 1kGy no change of vitamin C was reported. • Lycopene level decreased as eBeam dose increased, while β-carotene level increased. • Naringin increased over the control at 10 kGy.	Girennavar et al., 2008
Iceberg lettuce	0, 1.5, 2.0, 3.0, 4.0, 5.0	At 3.0 kGy the risk associated with consumption of a serving size (~14 g) of lettuce contaminated with 10 PFU/g of poliovirus and rotavirus reduced about 85% and 99% respectively.	Espinosa et al., 2012
Mushrooms	1.0	The fresh color was maintained while the pathogen level decreased.	Yurttas et al., 2014
Mushroom	0.5, 1.0, 3.1, 5.2	Dosage higher than 0.5 kGy reduced total plate counts, yeast and mold and psychrotrophic counts to below detection levels and prevented microbial induced browning. Firmness did not change. Irradiation maintained the color. The polyphenol oxidase was not affected.	Koorapati et al., 2004

Table 9 Continued.

Fresh produce	eBeam Dose (kGy)	Summary of results	Reference
Onion	0.1, 1.0, 2.0, 3.0, 5.0	Electron beam had no significant effect on pH, moisture, ascorbic acid and color. Doses of higher than 3.0 kGy became softer. The sensory scores changed only at doses higher than 3.0 kGy.	Lu et al., 1988
Romaine lettuce	1.0, 1.5, 3.2	• No significant change of color in lettuce. • The firmness decreased as dose increased. • Sensory attributes were less acceptable at high doses.	Han et al., 2004a
Spinach	0.20, 0.50, 0.75, 1.0 , 1.25	• 5 log reduction in *Salmonella* was noticed under 100% O_2 atmosphere at 0.7 kGy	Gomes et al., 2011
Strawberries	0, 1, 2	• Fruit firmness decreased from 7.01 (N) to 5.93 (N) and 5.35 (N) as the eBeam dose from 0 increased to 1 and 2 kGy respectively. • Total pectin and non-extractable pectin were not affected by eBeam. • The oxalate-soluble pectin content and firmness of irradiated strawberries increased.	Yu et al., 1996

Table 9 Continued.

Fresh produce	eBeam Dose (kGy)	Summary of results	Reference
Strawberries	0, 0.5, 1, 2	• The intensity of red color rated by sensory panelists decreased as dosage increased to 2 kGy. • L values were higher for fruits treated with 2 kGy • Panelists rated irradiated fruit lessfirm than nonirradiated fruit stored 1, 2 and 4 days. • Irradiation suppressed fungi growth. • The doses of 1 and 2 kGy extended the shelf life 2 and 4 days respectively.	Yu et al., 1995
Tomato	0, 0.7, 0.95	• Electron beam reduced microbial population of *Salmonella* spp. About 1.8 and 2.2 log using 0.7 and 0.95 kGy respectively.	Schmidt et al., 2006
Watermelon	1.0	• Electron beam had a significant impact on total count and mold and yeast of the watermelon cubes. • Electron beam had no negative effect on firmness or color • The eBeam processed samples were scored higher by the consumer panelist in terms of odor and flavor.	Smith et al., 2016

Despite the fact the eBeam technology is extensively used all around the world, there are some country regulations that limit the type of foods that can be treated with eBeam. In the EU and US there is a specific list of food items that can be irradiated. In the USA, the FDA does not allow the cooked food to be irradiation. Additionally, up to this moment there is no regulation available on irradiation of juice or dairy products (FDA, 2015). However, in countries such as India or Brazil all food items can be treated using this technology. For example in Brazil there is no limit over the doses allowed to be applied in different food items as long as the consumers are willing to purchase and consume them (Niemira & Deschenes, 2005). European Commission has put a draft proposal forward to extend the items on the "positive list" to fresh fruits and vegetables too, which is considered favorable by the EU Scientific Committee for Food (SCF). Due to the complexity of this issue and different pros and cons, a broader debate is ongoing and irradiation of fresh fruits follows the local regulations in different European countries such as Belgium, Czech Republic and UK. However, in the US all fresh produce can be treated to extend shelf life as long as the dose does not exceed 1 kGy (FDA, 2015). The irradiated food items in the US disregarding the dose or application should be labelled with the "radura" symbol (Figure 1). The radura symbol has to be accompanied by the phrases phrases "irradiated for food safety" or "irradiated for extending the shelf-life" depending on the dose employed.

Figure 1- Radura symbol

The eBeam technology is commercially in use in different parts of the world. The system can be also made modular to fit an existing production line. Electron beam technology is a chemical-free, solvent-free technique and therefore can be considered a green technology. However, facilities have to be designed in order to ensure the control of the radiological hazard for the personal and the environment. The safety of food irradiation under 10 kGy has been proven by a Joint Expert Committee ON Food Irradiation consisting of WHO, IAEA and FAO (JECFI, 1981). In addition to meeting the regulations set by food regulatory organizations there are some operating regulations to be put in place at the eBeam processing facilities. Since eBeam equipment is rated as "radiation-producing device" shielding is required in the construction design (Brown, 2015). All the personnel that are exposed to eBeam radiation process should wear dosimeters and should be protected from exposure to eBeam.. Electron beam processing of food is regulated by the FDA in the USA and in Europe Directive 1999/2/EC and Directive 1999/3/EC regulates food irradiation.

Bacterial response to ionizing radiation

Application of eBeam can inactivate microorganisms either directly through damaging the DNA of the microorganisms so that the cell division is impaired or indirectly through interaction of electrons with water molecules and creating hydroxyl radicals (Tahergorabi et al., 2012; Lung et al., 2015) (Figure 2). Therefore, as it was mentioned earlier water activity of the products affect the efficacy of the irradiation process (Farkas et al., 2014; Tahergorabi et al., 2012).

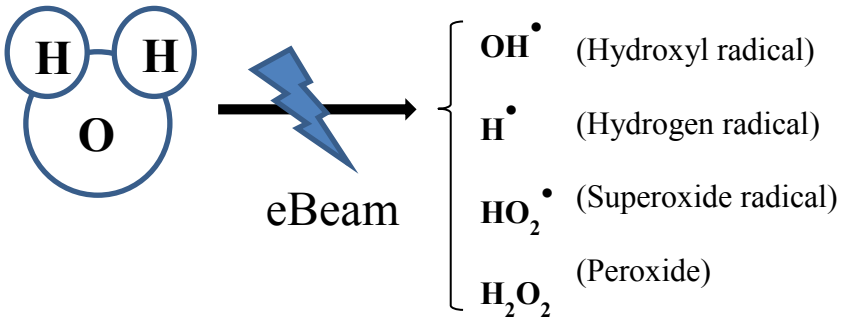

Figure 2- The effect of eBeam on water molecules and creating hydroxyl radicals.

When water is irradiated with eBeam it is hydrolyzed to a number of highly reactive entities (e.g. reactive oxygen species (ROS) are (Stewart, 2001) (Figure 3). Presence or absence of oxygen during irradiation can influence the course of radiolysis toward oxidation or reduction. The hydroxyl radical is a powerful oxidizing agent, while the aqueous electron and hydrogen atom are both reducing agents; therefore the food

undergoes both oxidation and reduction reactions during eBeam irradiation (Stevenson, 1992).

Some species of microorganisms (e.g. *Deinococcus radiodurans*) can survive the common low doses applied for food decontamination and might even resume growth (Patterson et al., 1993). In order to completely inactive the eBeam resistant microorganisms higher doses are required which might not only be permitted by regulatory organizations such as FDA but also can damage the organoleptic properties of the food (Tahergorabi et al., 2012). Therefore, when the food is eBeam treated both pathogen sensitivity to eBeam and quality parameters should be taken into account. Sub-lethal damages can increase the sensitivity of the cells to other environmental stress factors and synergistically inactivate the microorganisms (Szczawinska, 1983). Therefore, considering other hurdles in addition to eBeam to synergistically ensure both safety and quality are helpful.

The *Salmonella* cells in the meat sample surviving irradiation (1-3 kGy) were inactivated during storage of meat at 0-2 °C (Szczawinska, 1984). In another similar study the irradiated *Salmonella* cells with only 1 kGy were sensitized against curing salts (NaNO2 and NaCl) in meat products (Szczawinska, 1985). In case of fresh produce application of modified atmosphere packaging (MAP) in conjunction of low-dose eBeam can be helpful in reducing the numbers of both spoilage and pathogenic microorganisms (Patterson, 1988; Smith et al., 2016).

Most of the microbial inactivation studies are validated based on conventional plating methods; however, it is proven that some microorganism might go into viable but

non cultural (VBNC) status; meaning they do not growth but they are still alive and metabolically active (Oliver, 1993). Bacteria possess mechanisms to response to changes in environmental conditions and adapt their structure and physiology based on multiple genes expression (Quillardet et al., 2003).

Molecular response of bacteria to eBeam

The chromosomal DNA is constantly exposed to damage and repair that induces SOS mechanism upon the DNA damage. The SOS is induced as a result of any sort of stress including irradiation or chemicals disrupting DNA and cell division (Quillardet et al., 2003; Kenyon & Walker, 1980). The SOS response to DNA damage in *E.coli* cells requires the products of genes lexA and recA genes and mutations in SOS genes make cells highly sensitive to any sort of stress including irradiation (Janion, 2008; Quillardet et al., 2003). Ionizing radiation, irrespective of its type (gamma, eBeam or X-ray), damage the DNA sugar-phosphate backbone both directly though formation of DNA double-stranded breaks (DBSs) and indirectly through generation of reactive oxygen species (ROS) byproducts from water molecule radiolysis (Byrne et al., 2014). The DBSs are the most lethal form of DNA damage (Hutchinson, 1985; Liu et al., 2003) and ROS can directly attack DNA, RNA, protein, lipids, etc. (Cabiscol et al., 2000). The oxidative stress in *E.coli* against ROS is mediated by two major transcriptional regulators namely OxyR and SoxRS that each contains at least ten genes. The activation of genes *OxyR* and *SoxRS* increases the cellular resistance to oxidative agents such as hydroxyl peroxide (Cabiscol et al., 2000), which is one of the ROS by-products during eBeam processing (Figure 2).

The analysis of proteome (de Groot et al., 2009; Tanaka et al., 1996) and transcriptome (Tanaka et al., 2004; Liu et al., 2003) changes after ionizing radiation over time has provided helpful information into the bacterial response to ionizing radiation. The role of σs (Sigma factor) as the global regulator of stationary phase gene expression (RpoS) is documented in stressed *E.coli* cells (Weber et al., 2005; Membrillo-Hernández et al., 1997; Cheville et al., 1996). The most resistant microorganism to ionizing radiation is *Deinococcus radioduran* whose genes are extensively studied for its extensive resistance to extreme ionizing radiation and DNA repair (Byrne et al., 2014; de Groot et al., 2009; Tanaka et al., 2004; Liu et al., 2003; White et al., 1999; Tanaka et al., 1996). The genome of other microorganisms such as *E.coli* K12 (Krasin & Hutchinson, 1977; Krisch et al., 1976), Bacillus subtilits (Hariharan & Hutchinson, 1973), and Micrococous radiodurancs (Burrel et al., 1971; Kitayama &Matsuyama, 1968) have been mainly studied for DBS repair.

The first systematic study on damage inducible (din) genes was carried out by subjecting *E.coli* GW1000 cells to UV and random inserting a lac reporter gene into *E.coli* chromosome to identify the promoters that were upregulated as a result of DNA damage (Kenyon & Walker, 1980). In the same manner various studies identified the other din genes involved in the recovery of damaged DNA (Courcelle et al., 2001). There are a number of genes involved in the DNA repair of *E.coli* (Byrne et al., 2014; Kuzminov, 1999) to coordinate the homologous recombination process in the DNA of *E.coli*. During their vigorous exponential growth, *E.coli* cells contain four or five

haploid chromosomes that are integral for repair of DSBs in *E.coli*. Therefore, the cells that have no homologous DNA molecules cannot repair their broken strands. Repairing the BDSs may occur by a recombinational event with another DNA double helix of the same base sequence (Krasin & Hutchinson, 1977; Kuzminov, 1999). Typically only three to four DBSs per completed genome (2·5 × 109 daltons) is repaired through upregulation of recA gene (Krisch et al., 1976). The ionizing radiation resistance phenotype *E.coli* is explained mainly by three DNA metabolism genes of *recA*, *dnaB* and *yfjK* (Byrne et al., 2014). The gene *recA* is required for genetic recombination and regulation of cellular response to DNA damage in *E.coli* (Sargentini & Smith, 1986); while *dnaB* gene functions in the propagation of replication forks in the bacterial chromosome (LeBowitz & McMackens, 1986). The medium in which the *E.coli* cells are irradiated can also increase their ionizing radiation resistance (Sargentini & Smith, 1985). About 46 genes are involved in DNA repair of irradiated *E.coli* cells, from which 21 genes are in involved with DNA metabolism and 7 in cell wall structure and biosynthesis (Byrne et al., 2014). It is worth mentioning that all mentioned studies are based on DNA array method which despite generating valuable data has its own technical difficulties (Fadiel & Naftolin, 2003). Therefore, despite the fact that *E.coli* is the most extensively studied microorganisms the functions of one third of the genes in *E.coli* are still unknown (Byrne et al., 2014). There is a need for modern screening methods such as Next Generation Sequencing (NGS) to discover new genes with particular functions (Hurd & Nelson, 2009).

In all the mentioned studies gamma or X-ray irradiation were used as the source of ionizing radiation. Irradiation of *Salmonella* Typhimurium at lethal doses of eBeam resulted in minimal differential gene expression after 24 hours of storage in PBS buffer at 4°C; however, incubation in growth media (TSB) at 37°C led to unique gene expression. The genes coordinating DNA and membrane repair were mainly upregulated; while the genes regulating citric acid cycle were down-regulated (Hieke, 2015), indicating the cell is allocating its energy to more vital mechanisms such as DNA and membrane repair to survive. The repair of the damaged DNA and other cellular components are detrimental for bacterial cells to survive ionizing radiation (Byrne et al., 2014).The study of the eBeam treated *E.coli* transcriptome facilitates identification of the ionizing resistance induced by eBeam and virulence genes affected. Due to the complexity of bacterial metabolism it seems unlikely that ionizing radiation resistance is mainly supported by DNA repair and amelioration of oxidative damage to proteins (Byrne et al., 2014). Therefore, a broader evaluation of ionizing radiation resistance in bacteria (i.e. *E.coli*) is needed. Furthermore, there is no information on how ionizing radiation can possibly affect virulence genes in any microorganisms and how possibly they can repair themselves.

Quantitative Microbial Risk Assessment (QMRA) in the Context of Ionizing Radiation

Quantitative microbial risk assessment (QMRA) is a pragmatic approach in collecting data on hazards and calculating the possibility of their incidence through

mathematical models and documenting and communicating them. Risk analysis is a valuable tool in microbial food safety management that enables the food industry to make more powerful decisions in terms of accepting or rejecting food, water or other items that may be the source of microbial exposure to the public (Duffy et al., 2006; Haas et al., 1999).This approach is increasingly advocated for use in estimating order of magnitude risks associated with specific scenarios as it is mainly based on probabilistic models (Hamilton et al., 2006). The risk in this context is a combination of chance, hazard, exposure and consequence (QMRAwiki, 2016).

Risk assessment consists of main steps of hazard identification, dose response, exposure assessment, risk characterization and risk management. After identifying the risk (i.e. pathogen) the dose response needs to be identified. Dose response represents the estimate of a response (e.g. infection, illness or death) to a known dose of a pathogen. Dose response is calculated using mathematical functions. Exposure assessment deals with the dose of the pathogen that an individual comes in contact with. The dose response is used to predict the probability of infection. Risk characterization integrates the data on the dose exposure and dose response to estimate the probability of the risk. The final step is managing the risk through different strategies or interventions to reduce the risk effectively and communicate it with public.

An effective QMRA model requires accurate conceptual and quantitative distinction between “variability” and “uncertainty”. Variability in this context refers to the changes introduced from nature over time, space, among samples, or any other sources; whereas uncertainty corresponds with limited information about a parameter

(Schmidt & Emelko, 2011). The main two models that are used for QMRA dose response with regard to such variation in pathogens are the exponential and beta Poisson models. The two mentioned models are similar to each other but the dose response curve in beta-Poisson is more shallow that that of the exponential (QMRAwiki, 2016).

Figure 3- Risk management framework.
Adopted from QMRAwiki, 2016.

The published QMR models include risk assessment of piped water supplies (Howard & Pedley, 2003), enteric virus infection associate with reclaimed–water irrigation of vegetables such as cucumber, lettuce, etc. (Hamilton et al., 2006), *E.coli* O157 in beef (Duffy et al., 2006), *E.coli* O157 and Staphylococcus aureus in cooked meat products (Pérez-Rodríguez et al., 2007), human salmonellosis through consumption of fresh minced pork meat (Bollaerts et al., 2009), etc. The only studies reported on application of QMRA in the context of irradiation are limited to the risks associated human norovirus (NoV) and Hepatitis A (HAV) in oyster (Praveen et al., 2013) and poliovirus in lettuce and spinach (Espinosa et al., 2011).

Application of eBeam in oyster could reduce the risk associated with NoV and HAV by 12% and 16% respectively in a serving size of oyster contaminated with 105 PFU at 5kGy (Praveen et al., 2013). Similar study on lettuce and spinach eBeam treated with 3 kGy resulted in 95% and 99.2% risk reduction associated with consumption of a serving size of lettuce and spinach respectively when contaminated with 10 PFU/g poliovirus (Espinosa et al., 2011). Viruses have high D10 values to eBeam (Table 8) that accounts for relatively low amount of risk reduction when contaminated foods with viruses are eBeam treated. Since the D10 values of STEC strains are lower (Table 8) the amount of risk reduction associated with the application of eBeam in the foods contaminated with STEC is expected to be higher.

CHAPTER III

QUANTIFYING THE REDUCTION IN POTENTIAL INFECTION RISKS FROM NON-O157 SHIGA TOXIN PRODUCING *E.COLI* IN STRAWBERRIES BY LOW DOSE ELECTRON BEAM PROCESSING[1]

Overview

Strawberries are vulnerable to harboring microbial pathogens because they are generally not washed due to their perishable nature. The focus of this study was to quantify the reduction in infection risks associated with non-O157 Shiga toxin producing *E.coli* serotypes contaminated strawberries if the strawberries are exposed to low doses ~ 1 kGy (kiloGray) of electron beam (eBeam) irradiation. A cocktail of six serotypes of non O157 *E.coli* namely, O26:H11, O45:H2, O103:H2, O111:NM, O121:H19, and O145 was employed. The results show that when these serotypes are exposed to 1 kGy eBeam dose, there is approximately 4-log reduction in their numbers when they are present within a strawberry matrix. Quantitative microbial risk assessments suggest that if a typical strawberry serving (150 g) was heavily contaminated (~ 10^5 CFU/serving size), 2 out of 10 susceptible individuals (20%) would get sick (without eBeam treatment). However, if these contaminated strawberries had been treated with 1 kGy of eBeam dose, the infection risks would have be significantly reduced to approximately 4 out of every 100,000 individuals (0.004%). Similarly, even at low levels of contamination (~

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102 CFU/serving), the infection risks would be reduced from 6 out of 10000 susceptible individuals to approximately 4 out of 100 million susceptible individuals.

Introduction

Strawberries are considered healthy and therefore popular because they contain an abundance of antioxidants which have demonstrated anticancer properties (Folmer et al., 2014). The US is among the world's leading producers of strawberries accounting for about 30% of the world's production (FAO STAT, 2015). Approximately 1.3 billion metric tons of strawberries were produced in 2012 with an estimated market value around $2.2 billion. Given their highly perishable nature, strawberries are, however, neither washed nor a validated pathogen kill-step employed to remove or eliminate microbial pathogens that they may harbor. This inability to adequately wash strawberries increases the likelihood of them being linked to foodborne illnesses. Berries such as strawberries have been attributed to disease outbreaks all around the world involving viral, protozoan, and bacterial pathogens (CDC, 2012, Miller et al., 2013; Niu et al., 1992). Infection rates of non-O157 Shiga toxin-producing *E.coli* (non -O157 STEC) strains are reported to be as high as that of O157 STEC strains (Gould et al. 2013). Non O157 *E.coli* strains were responsible for a number of deaths in Germany when they had entered the food supply chain through contaminated sprouts (Mora et al., 2011). The "Big Six" non O157 serotypes namely, O26, O45, O103, O111, O121, and O145 have been identified to be the key disease-causing non O157 strains (FSIS, 2012).

Electron beam (eBeam) irradiation is a FDA approved non-thermal ionizing radiation based food processing technology. We and others have already shown that this technology is applicable to increase the shelf-life and inactivate microbial pathogens on fresh produce such strawberries (Predmore et al., 2015; Espinosa et al., 2012; Sanglay et al., 2011). The use of ionizing radiation technology is stymied in the US because the FDA has approved the use of ionizing radiation for microbial pathogen reduction only for spinach and lettuce. However, the FDA has allowed the use of ionizing radiation such as eBeam technology for extending the shelf life of fresh produce, provided that the delivered dose does not exceed 1 kGy (FDA, 2015). We have previously shown that even at low doses (~ 1 kGy), eBeam processing results in extending the shelf life of strawberries when stored under refrigerated conditions (Smith, 2015). Our goal in this study was to obtain empirical evidence about the reduction of key bacterial pathogens (non-O157 Shiga toxin producing *E.coli* serotypes) on strawberries at a dose already approved by the FDA.

The underlying hypothesis of this study was that the application of low (~ 1 kGy) eBeam doses on strawberries results in a defined reduction of non O157 *E.coli* strains which translates to a reduction of potential infection risks associated with these pathogens. The objectives of this study were three-folds. The first objective was to determine the sensitivity of selected non-O157 *E.coli* strains to high energy (10 MeV) eBeam irradiation. The second objective was to determine the reduction of these pathogens in a strawberry matrix; the third objective was to quantify the reduction in infection risks achievable if contaminated strawberries were processed using eBeam

irradiation at the low dose (1 kGy). We employed laboratory inoculations of a cocktail containing the following Shiga toxin producing *E.coli* strains namely, O26:H11, O45:H2, O103:H2, O111: NM, O121:H19, and O145.

Material and Methods

Bacterial cultures

The bacterial pathogen serotypes O26:H11, O45:H2, O103:H2, O111: NM, O121:H19, and O145, were obtained from the culture collection of the Food and Feed Safety Research Unit (USDA-ARS FFSRU) in College Station, Texas. These cultures were grown on either Trypticase Soy Agar (TSA) plates or TSB liquid media at 37 °C for 24 hours. Prior to each experiment, a loop of each strain was individually transferred to TSB and shake incubated at 37°C overnight. The overnight culture was washed using Phosphate-Buffered Saline (PBS) by three consecutive rounds of centrifugation (3000 × g for 10 min) using a floor-mounted high speed centrifuge. The optical density of the washed cells was measured (using 620 nm absorbance) and standardized to approximately 10^9 CFU/ml. A cocktail of the six non-O157 serotypes were prepared by combining equal aliquots of the six strains. The cell density in the cocktail was adjusted to yield approximately 10^9 CFU/ml.

Sensitivity of non O157 E.coli strains to varying eBeam doses in phosphate buffered saline

The objective was to calculate the D-10 value (dose required to achieve 90% reduction in titers) of the different strains. The serotypes O26:H11, O45:H2, O103:H2, O111: NM, O121:H19 were used in this study. Defined concentrations of the different serotypes were individually exposed to varying eBeam doses to determine the sensitivity of these strains to eBeam irradiation. The surviving bacterial concentrations after eBeam exposure (log CFU/mL) were plotted as a function of the measured eBeam dose (kGy). The inactivation of the bacterial pathogens was assumed to be linear (Hieke & Pillai, 2015). Linear regression analysis was performed and the negative reciprocal of the slope was calculated to be the D-10 value. The Student's t-test was performed to determine whether there was any statistically significant ($p < 0.05$) difference between the D10 values.

Inoculation of strawberry samples

Fresh strawberries were purchased at a local farmer's market to reduce the likelihood that they were treated with antimicrobial sprays or other disinfectants. These samples were refrigerated prior to the experiments. Preliminary studies showed that attempting to inoculate intact strawberries on the surface or internally were not reproducible. Also, inoculating the surfaces of the strawberries would not be realistic since it is possible that pathogens may be internal to the fruits. Thus, to simulate natural contact of the pathogen to the fruit matrix we used a strawberry "puree". The puree was prepared as follows: twenty five grams of strawberries were placed in sterile Whirl-Pak

bags (Whirl-Pak, NASCO, Fort Atkinson, WI) and kneaded by hand to obtain a homogenous puree. One milliliter aliquots of the pathogen cocktail were added to these samples. The bags were sealed and samples were further kneaded to achieve a uniform mixing of the bacterial cells with the fruit matrix. To comply with the university biosafety regulations, all pathogen -spiked samples were placed in heat-sealed double-bagged Whirl Pak® bags (Nasco, New York, NY). These heat-sealed bags were then placed inside a "specimen transport" bags that were rated up to 95 kPa (Thermosafe, Arlington Heights, IL). Only such triple-bagged samples were permitted to be treated at the commercial scale eBeam facility on campus.

Electron beam processing

The eBeam processing was performed at the National Center for Electron Beam Research at Texas A&M University using a 10 MeV, 18 kW, linear accelerator. To verify the actual eBeam dose received by the samples, alanine (L-α-alanine pellet) dosimeters (Harwell Dosimeters, Oxfordshire, UK) were used. The alanine dosimetry system that was employed was traceable to international standards. The dosimeters were measured using the Bruker E-scan spectrometer (Bruker, Billerica, MA) to measure the delivered eBeam dose. The target dose was 1.0 kGy. A number of preliminary trials were performed to ensure that the dose was ≤ 1 kGy. All treatments were conducted in triplicate and repeated three times on separate days.

Enumeration of non O157 strains after eBeam exposure

To estimate the numbers of surviving non-O157 strains, the entire content of the eBeam processed sample bags were mixed with 225 ml of PBS in stomacher bags and mixed for 1 minute in a "stomacher" at the medium setting. The sample was serially diluted in PBS and aliquots were plated on TSA plates as well as *E.coli* specific media namely, modified m-TEC agar (m-TEC Agar) (Difco). We chose m-TEC agar and TSA agar rather than relying on non-O157 selective media to avoid possible errors arising from potentially injured cells (from eBeam exposure) not growing on the selective media. Morever, m-TEC agar is a standard *E.coli* media approved by the US EPA for isolating and enumerating *E.coli* in environmental samples (EPA, 2002). The plates were incubated for 24 hours at 37 °C. The red/magenta colonies characteristic of *E.coli* cells were enumerated and reported as CFU/g.

Quantitative microbial risk assessment

We estimated the infection risks that would arise from exposure to non O157 serotype -contaminated strawberries. The reduction in the titers of the non-O157 cocktail when exposed to eBeam dose (≤ 1 kGy) used in this study was used as the basis for calculating the reduction in risks. We assumed a standard strawberry serving size of 150 g (Ashfield-Watt, P.A. 2004). Reduction in infection risks associated with various levels of non O157 serotype contamination loads (on 150 g serving sizes) with and without eBeam irradiation was estimated. The initial non –O157 pathogen titers (per serving size) were assumed to be 102 CFU, 103 CFU, 104 CFU and 105 CFU. The infection

risks were estimated using the beta-Poisson (modified exponential model) where the defined parameters were N50 = 2.11 X106, α = 0.155 and serving size =150 g (CAMRA, 2015; Dupont et al., 1971). We assumed that all the bacterial pathogens were infectious and that all of the exposed individuals were also susceptible to infection.

Results

Table 10 shows the D-10 values for the five non-O157 *E.coli* strains when exposed to eBeam irradiation in phosphate buffered saline solution. The results show that a dose of approximately 0.068 kGy will achieve at least a 90% reduction in the pathogen titers in PBS. There was no significant difference in the D-10 value among the five strains.

Table 10- D-10 values for the selected non-O157 Shiga toxin producing *E.coli* serotypes in phosphate buffered saline when exposed to 10-MeV eBeam

	Non O157 *E.coli* serotypes				
	O26:H11	**O111:NM**	**O45:H2**	**O103:H2**	**O121:H19**
D10 value (kGy)	0.119^{a}± 0.005	0.074^{a}±0.005	0.071^{a}± 0.006	0.066^{a}± 0.002	0.142^{a}± 0.012

Values are means and standard deviations (SD) of three triplicate experiments. Within a row, means with different letters are significantly different as determined with Student's t test (α = 0.05).

Table 11 shows the reduction of the non-O157 *E.coli* strains in the strawberry puree after exposure to 1 kGy eBeam dose. Over 4-log reduction (average 4.23 log

reduction based on inactivation observed using the two media) of non-O157 serotype cocktail was observed at this low eBeam dose. Table 12 shows the reduction of infection risks if strawberries contaminated with non-O157 *E.coli* serotypesare processed at low (~ 1 kGy) eBeam doses. If the strawberries were contaminated at 100 CFU per serving size, the infection risks without eBeam irradiation would be approximately 6 persons out of every 10,000 susceptible individuals (0.06%).

Table 11- Inactivation of a cocktail of non O157 *E.coli* serotypes (O26:H11, O45:H2, O103:H2, O111: NM, O121:H19, and O145) in a strawberry matrix (puree) and exposed to 1 kGy electron beam (eBeam) dose.

Measured eBeam Dose (kGy)	**TSA (Log CFU/g)**	**m-TEC (Log CFU/g)**
0 kGy	9.34± 0.15	9.48 ± 02
0.99 kGy	5.23± 0.23	5.14± 0.10
Mean reduction	4.23 log	

The values shown are mean ± standard deviation based on n=3 independent trials with each trial having 3 replications.
The samples were plated on either Trypticase Soy Agar (TSA) or m-TEC media.

However, if these same strawberries are treated with ~ 1 kGy eBeam dose, the infection risks are significantly reduced to only approximately 4 persons out of every 100 million individuals. Table 3 also shows that if the strawberries are heavily contaminated (~ 105 CFU/serving size), 2 out of 10 susceptible individuals (20%) would get sick (without eBeam treatment). However, if these heavily contaminated strawberries are treated with 1kGy of eBeam dose, the infection risks would be significantly reduced to approximately 4 out of every 100,000 individuals.

Table 12- Risks of infection associated with non O157 Shiga toxin producing *E.coli* with and without eBeam (1 kGy) processing.

Initial *E.coli* Concentration CFU/serving (before treatment)	**Infection Risks Before Electron Beam Irradiation**	**Final *E.coli* Concentration CFU/serving (after 1.0 kGy treatment*)**	**Infection Risks After Electron Beam Irradiation**
100,000	$2.2x10^{-1}$	5.89	$3.7x10^{--5}$
10,000	$5.2x10^{-2}$	0.589	$3.7x10^{-6}$
1,000	$6.2x10^{-3}$	0.0589	$3.7x10^{-7}$
100	$6.3x10^{-4}$	0.00589	$3.7x10^{-8}$

Assuming *E. coli* exposure; strawberry serving size = 150 grams
*Log-reduction of 4.23 assumed.

Discussion

Strawberries are of high economic value to US agriculture with an estimated value of around $ 2.4 billion. However, this commodity is highly perishable and therefore no traditional pathogen kill step can be employed. Given their growing practices, they are therefore vulnerable to microbial contamination and since no wash step of pathogen kill-step is employed they are prime suspects in a number of foodborne illness outbreaks (Olaimat and Holley, 2012). Non-thermal technologies such as eBeam processing are suiTable for highly perishable commodities such as lettuce and spinach (Espinosa et al., 2012). The FDA has approved the use of eBeam and other ionizing radiation technologies for use with fresh foods such as strawberries (FDA, 2015).

To make this study relevant to the commercial strawberry industry we focused at the FDA approved dose of 1 kGy. We have recently reported that that eBeam at ~ 1 kGy can extend the shelf-life of strawberries without negatively impacting its sensory of consumer acceptability (Smith et al., 2015). The D-10 value of non O157 *E.coli* strains have been reported previously (Li et al., 2015; Kundu et al., 2014). They reported values ranging between 0.090 kGy and 0.127 kGy. In this study we obtained a D-10 value of 0.068 kGy in phosphate buffered solution. This should have translated to greater than 10 log reduction in strawberries. The reason we observed only observing a 4.23 log reduction of these pathogens in the strawberry puree reflects the differences in response of microorganisms to ionizing radiation depending on the surrounding matrix. It is well known that the presence of free water molecules enhances the inactivation kinetics. A number of different hypotheses have been proposed for the reduced inactivation of

microorganisms in matrices that contain non aqueous compounds as compared to pure water or buffer solutions. These include the possible free radical scavenging effect of organic compounds (Shenoy et al., 1975), reduced radiolysis of water molecules (Song et al., 2009) and antioxidant activity of phenolic compounds present in strawberries (Wang & Lin, 2000; Heinonen et al., 1998).

The FDA has regulations in place currently for labeling irradiated foods such as fresh strawberries. Presently, if this technology is used for strawberries, all retail packages (at point of sale) should display the radura symbol with the phrase, "treated by irradiation for food safety" or "treated by irradiation for shelf-life extension" clearly indicated. Even though this study has shown that 1 kGy eBeam dose results in significant collateral reduction of pathogenic non-O157 *E.coli* strains, the labeling cannot state, "treated by irradiation for food safety" since the FDA has permitted the use of 1 kGy for shelf-life extension purposes only. Nevertheless, the extension of shelf life will be accompanied by a defined reduction in public health risks if this technology is adopted.

This study has shown that even at a low eBeam dose such as 1 kGy, at least a 4-log reduction of non O157 Shiga toxin producing *E.coli* serotypes can be achieved in a strawberry matrix. The significance of achieving this level of bacterial pathogen reduction in fresh produce such as strawberries is that it translates to significant reduction in potential infection risks.

CHAPTER IV

QUANTIFYING THE EFFECTS OF VARYING SOURCES OF ACID STRESS ON NON-O157 SHIGA TOXIN PRODUCING *ESCHERICHIA COLI*

Overview

In the United States, non-O157 Shiga toxin-producing *Escherichia coli* (STEC) may account for up to 20%-50% of all STEC infections. With an estimated 37,000 cases annually, the total annual cost for the United States is $51 million. Because of the high incidence rate and the relatively little that is known about non-O157 STEC, it is important to study and understand how these bacteria react to certain stresses. This study examined the effect of acid stress, a method used as one of the main 'kill steps' in many food industries, on non-O157 STEC, specifically, the "Big Six", six of the most common serotypes that have been linked to foodborne outbreaks in the United States: STEC O26, O45, O145, O111, O121, and O103. An initial titer of 10^9 CFU/ml bacteria cells were incubated in various liquid acids: an organic acid buffer, and strawberry puree, all with a pH of approximately 3.6. The samples were then analyzed for the survival of the microorganisms. A pH of 3.6 was used to emulate acid stress routinely occurring in fresh produce such as berries, which have a pH in the range of 3-4. The results show that bacterial inactivation is depending on the nature of the acid and the strain ($P<0.01$). Each of the Big Six indicates different level of resistance to acid stress with O103 as the most resistant strain and O26 and O111 as the weakest of all to acid stress ($P<0.01$). The microbial inactivation of the acids is strawberry > organic acid.

Introduction

Non-O157 Shiga toxin producing *Escherichia coli* are one of the many pathogens that have been cited in these outbreaks. Non-O157 STEC infection rates are as high as O157 STEC rates (Shayanfar et al., 2016; Gould 2013; Kalchayanand et al., 2012). In 2011, the deadliest E. coli outbreak in history occurred in Europe when fresh sprouts contaminated with non-O157 STEC were identified as the source of the outbreak that lead to 4,075 reported cases, with 50 deaths across 16 countries (CDC, 2013). In 2012, the "Big Six" non-O157 STEC serotypes (O26, O111, O121, O103, O145, and O45) were named as the main non-O157 disease causing strains (FSIS 2012) responsible for 71% of non-O157 STEC diseases in the USA (Kalchayanand et al., 2012).

As consumers become more health conscious, their consumption of fresh fruits and vegetables has increased dramatically. Strawberries has long been considered an extremely nutritious food, high in antioxidant phytochemicals that have been linked to reducing the risk of cardiovascular disease as well as having anticancer activity (Hannum, 2004). It is reported due to low pH in fresh produce the growth of pathogens is not supported in fresh produce (Delbeke et al., 2015; Knudsen et al., 2011); however, there are still outbreaks reported associated with the consumption of low pH fruits such as apple cider, blueberries, strawberries, etc. (Asplund & Nurmi, 1991; Dingman 2000; Arnold, Kaspar, 1995; Harrwas et al., 2006). Thus, the microbiological safety of high acid foods is now becoming an increasing concern.

In strawberries, the main acids present are citric, malic, and ascorbic acid, which contribute to the pH of strawberries being between 3.0-3.5 (Castro, 2002; Kallio et al., 2000). Weak organic acids such as these have long been used in the food industry to control microorganisms and render a food safe (Buchanan et al., 2002). Even, upon consumption of the food the potential pathogens present are exposed to HCl as a strong inorganic acid in GI tract (Smith et al. 2014) but there are still food borne outbreaks reported. A considerable number of studies have been conducted in order to investigate the survival of *E.coli* O157:H7 in different acids and at different pH values (Delbeke et al., 2015; Han et al., 2004b; Knudsen et al., 2001; Nguyen et al., 2014; Yu et al., 2001); however, there is no literature available on acid sensitivity of the Big Six STEC.

Various studies have reported the ability of *E.coli* O157:H7 to survive acidic conditions in various foods, in stomach and in vitro suggesting acid tolerance of *E.coli* O157 (Brudzinski & Harrison, 1997; Garren et al., 1997); however, little is known about the acid sensitivity of the non-O157 STEC Big Six. Considering the fact that acid sensitivity in different STEC isolates may have implications for virulence too (Waterman & Small, 1996), it is of interest to have information about the sensitivity of the Big Six in different (organic, inorganic, fruit) acidic conditions. The objective of the current study is to collect empirical information about the acid sensitivity of the Big Six STEC in order to support the efficacy of the hurdle suggested in the food processing to control the virulence associated with these pathogenic strains.

Materials and Methods

Bacterial cultures

Non-O157 E. coli serotypes of O26 (TW 1597), O45 (KSU 2566-58), O103 (KSU 156124), O111 (KSU 7726-1), O121, and O145 were obtained from the USDA-ARS culture collection sourced from cattle faces (USDA-ARS-FFSRU, College Station, Texas). The cultures were grown on Trypticase Soy Agar (TSA) at 37 °C for 24 hours. Before each experiment, a single colony was transferred into a falcon tube containing 10 ml Trypticase soy broth (TSB) (Difco, USA) and shake incubated at 37 °C overnight. The overnight culture was then washed with Phosphate Buffered Saline (PBS) three times via centrifugation at 4000g for 10 minutes each time. After the last washing, the culture was suspended in either PBS (pH 7.4), strawberry puree (pH 3.6), organic acid cocktail (pH 3.6) and incubated at room temperature for 24 hours. The cultures were washed three times in the same method as mentioned before. After the last washing, the bacteria were suspended in 10 ml of PBS. The bacteria were enumerated at 0 hours and 24 hours in order to determine the starting titer and survival after being exposed to varying levels of acid stress for 24 hours. The samples were serially diluted and plated on TSA. The strawberry puree samples were not washed after the 24 hours of incubation. Previous experiments showed no difference between strawberry samples that were washed and those that were diluted without a washing step. All experiments were done in triplicate.

Preparation of strawberry puree

A strawberry puree was used in order to simulate a realistic and homogenous growth environment for the bacteria. Fresh strawberries were purchased from a local farmer's market in College Station, Texas in order to reduce the chance of antimicrobials or other industrial sprays interfering in the experiment. Fresh strawberries were blended in a standard kitchen blender before being centrifuged at 10000 rpm to separate a majority of the seeds. The pH of the strawberry puree was measured using a calibrated pH probe (calibrated with stock solutions of pH 4 and pH 7) (Corning, Corning, NY). The puree was kept at -80 °C in approximately 40 ml aliquots until needed for each experiment at which point it was thawed overnight before use.

Preparation of acid buffers

The organic acid cocktail buffer solution was comprised of citric, malic, and ascorbic acid buffers, combined in a 7:1:0.3 ratio to the final pH of 3.6. This ratio was based off of organic acid concentrations found in literature (Castro 2002). This was done in order to most accurately isolate the effects of only the organic acids in strawberries. The pH of the strawberry puree also was measured to be 3.6. The pH of the prepared acid buffers and strawberry puree was verified before each experiment using pH indicator strips (Sigma-Alrich, Location).

Bacterial viability staining

The instructions in the Live/Dead *BacLight* bacterial staining kit (Invitrogen, Inc.) was followed in order to determine the viability of *E.coli* O26 cells when exposed to different low pH acidic conditions namely; inorganic buffer, organic buffer, strawberry puree. The cells were visualized using a fluorescence microscope (Olympus BX50, Japan) equipped with a digital camera (Olympus Qcolor3, Olympus, USA). In order to quantify the live cells different ratio of live/dead *E.coli* O26 cells were prepared from the control sample. The dead cells were prepared by suspending1 ml of *E.coli* cells (108 CFU/ml) in a 20 mL vial of 70% isopropyl alcohol (ESP Chemicals Inc. USA.) for 2 hours. The live cells were prepared by diluting 1 ml of *E.coli* cells (108 CFU/ml) in a 20 mL vial of sterile 0.85% NaCl. The vial was shaken every 15 minutes and later was mixed with live cells to make different ratios of live/cells from 0-100% live cells. The tubes of different ratios of dead/live cells were stained using the Live/Dead *BacLight* protocol and pipetted in a 96 well glass bottom plate in three technical replications and using a Fluorescence microplate reader (CLARIOstar, BMG Labtech. The plate was stored away from the light before measuring the fluorescence intensity. The first emission (green) was read with the excitation wavelength centered at 488 nm and the fluorescence intensity at the wavelength of 575 nm. The second emission (red) was read with the excitation wavelength centered at 575 nm and the fluoresce intensity at 630 nm. The obtained values from the first emission were divided by the ones from the second emission and reported as Ratio G/R, which was later graphed versus the ratio of live

cells. The regression equation was used to quantify the amount of live cells in each of the treatment.

Transmission electron microscopy (TEM)

One milliliter of *E.coli* O26 cells from different treatment groups was collected and centrifuged at 11,000×g for 10 min. The supernatant was poured off and the pellet was suspended in 1 ml of Trumps fixative buffer (McDowell & Trump, 1996) for three times. Then, the cells were mixed with 2% w/v low-gelling temperature agarose and centrifuged at 11000 X for 10 minutes. Small (1 X 1 mm) cubes of agarose gel containing the bacteria were prepared and fixed in in osmium tetroxide. The samples were dehydrated with 10% steps of ethanol (10%-100%) over the course of 2 days and embedded in Quetol 651-modified Spurr epoxy resin (Ellis, 2015) and polymeruized at 55 °C overnight. The 100 mm sections of the fixed dehydrated samples were stained using a 400-mesh copper grid, rinsed and stained for about 5 minutes in Reynold's lead citrate (Wright, 2000).

TEM images were taken on JEOL 1200 Ex microscope performing at 100 kV equipped with SIA 15C CCD camera (SIE, Duluth GA) at Microscopy & Imaging Center – Texas A&M University. TEM images were adjusted for contrast in ImageJ software (Rasband, 1997).

Results

The goal of this study was to examine the Big Six non-O157:H7 strains' response to being exposed to various forms of acid stress for 24 hours (Figure 4). The analysis of variance (ANOVA) shows that both the matrix and the strain are significantly effective in resistance /sensitivity to acid stress (Table 13). Across the 6 strains, an average log reduction of and 1.45 ± 0.59 log was observed in strawberry puree, while the organic acid cocktail resulted in a 0.45 ± 0.41 CFU/ml log reduction (Table 14). The PBS was used as a negative control in each experiment, with minimal reduction observed after 24 hours (0.29 ± 0.19 CFU/ml).

Table 13– Table of analysis of variance (ANOVA).

Source	N	DF	Sum of Squares	F Ratio	Prob> F
Strain	5	5	8.80999	4.953	0.0007
Matrix	3	3	423.55935	396.9078	<0.0001

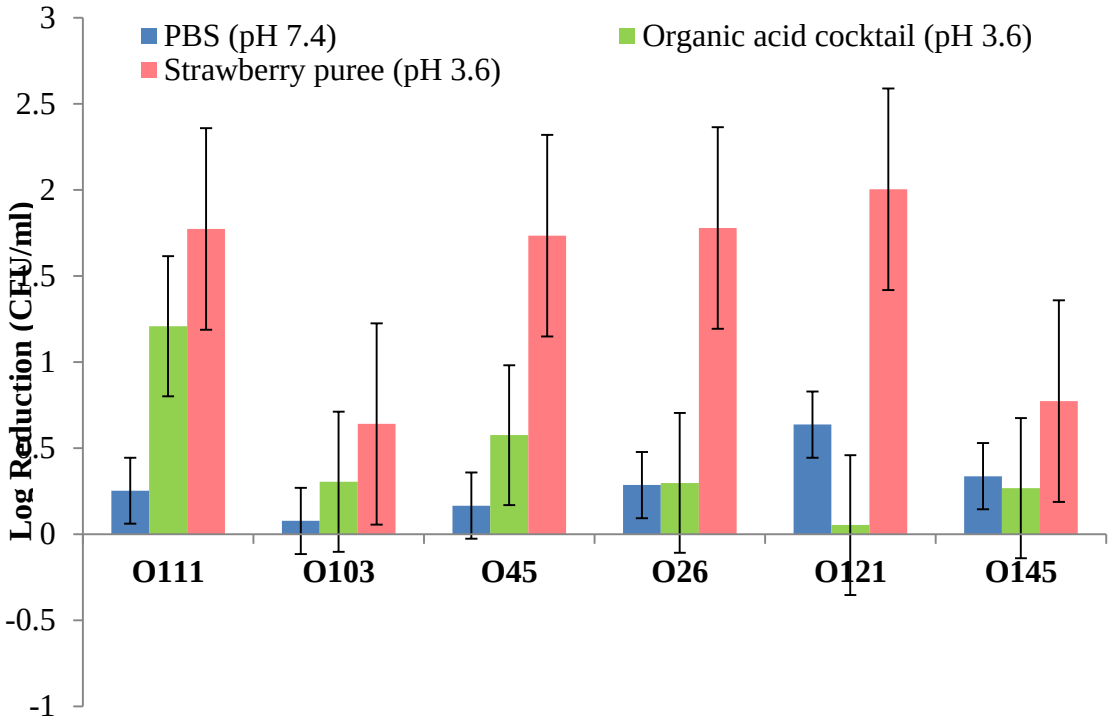

Figure 4- Log reduction of non-O157 STEC serogroups O26, O45, O103, O111, O121, and O145 after 24 hour exposure to PBS (pH 7.5), organic acid mixture (pH 3.6), strawberry puree (pH 3.6) .

Overall, O103 was the most resistant to acid stress, followed by O45, O145, O121, O26, and O111 (least resistant) (Table 14). While O121 was most resistant to the organic acid cocktail, it was least resistant to the acids in the strawberry puree. The results of the fluorescent spectroscopy (Table 15) based on the calibration equation achieved (Figure 6) show that there is no significant difference between the number of live cells in *E.coli* O26 when incubated in strawberry puree (Figure 5B) or organic acid buffer (Figure 5C), which is supported by quantifying the number of live cells in these two treatment groups ($P<0.01$). On the other hand, TEM images do not show any

different in the cell membrane structure or wholesomeness as a result of different acidic treatment; however, in the cells exposed to strawberry puree bright particulates are formed inside the cells (Figure 7C).

Table 14- The log (CFU/ml) reduction of the non-O157 STEC serogroups O26, O45, O103, O111, O121 and O145 after exposure to 24 hours of varying acidic matrices.

	Log CFU/ml Reduction After 24 h						
	O111	**O103**	**O45**	**O26**	**O121**	**O145**	**Average**
PBS (pH 7.4)	0.25	0.08	0.17	0.29	0.64	0.34	$0.29^{c} \pm 0.19$
Organic acid (pH 3.6)	1.21	0.31	0.58	0.30	0.05	0.27	$0.45^{c} \pm 0.41$
Strawberry puree (pH 3.6)	1.77	0.64	1.73	1.78	2.00	0.77	$1.45^{b} \pm 0.59$

The mean values are the average of the surviving colonies (CFU/ml) in three technical and three biological replications in each strain. Different lowercase alphabets (a, b, c) represent significant statistical difference ($P<0.01$).

The mean values are the average of the surviving colonies (%) ± standard deviation. Different lowercase alphabets (a, b, c) represent significant statistical difference ($P<0.01$).

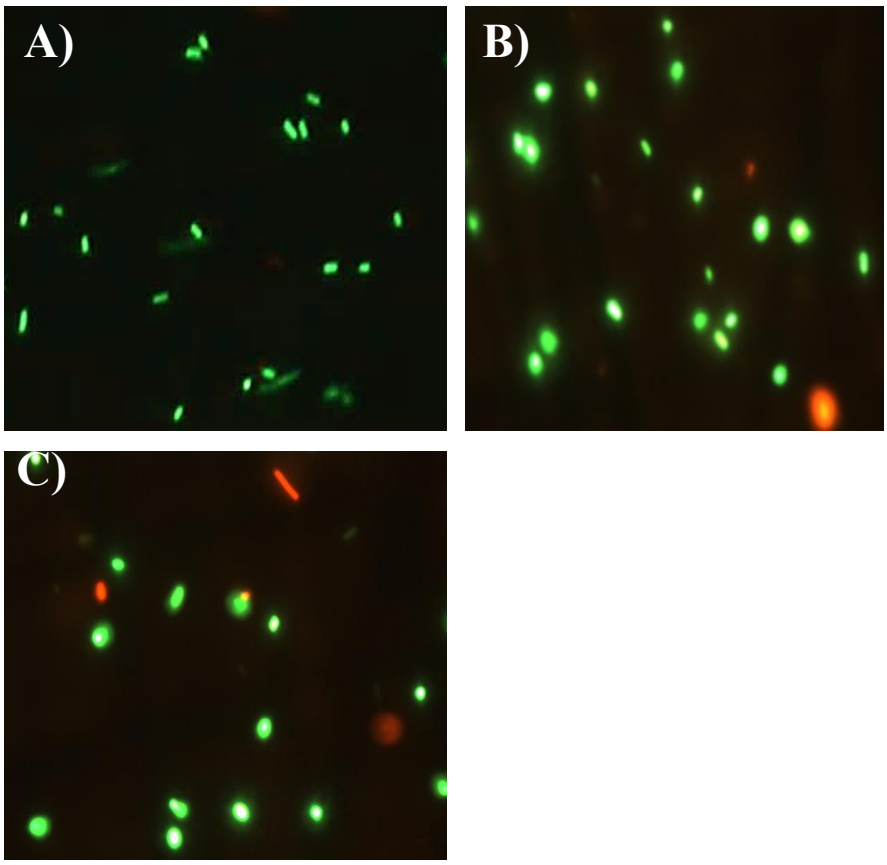

Figure 5 -The fluorescence images of *E.coli* O26 cells after 24 hours of incubation in A) PBS (pH 7.5), B) Strawberry puree (pH 3.6) ; C) Organic buffer (pH 3.6).

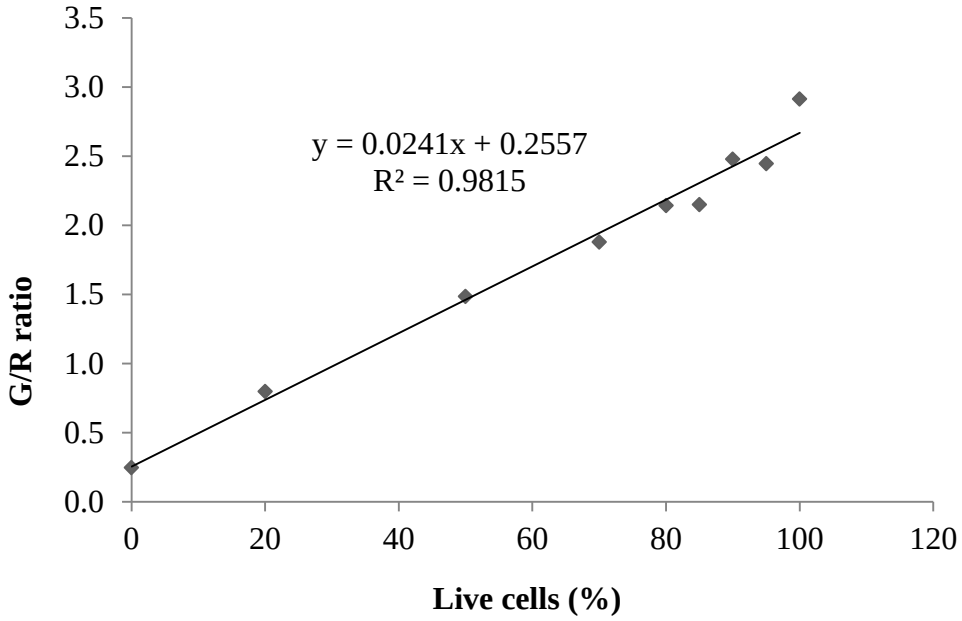

Figure 6- The calibration graph used to quantify the amount of surviving *E.coli* O26 cells.
G/R ration values are the ration of the fluorescence emission reads in green spectrum versus red spectrum. The live cells are the ratio of live *E.coli* O26 cells.

Table 15- The % of live (viable) *E.coli* O26 cells remaining after exposure to 24 hours of varying acidic matrices.

Acidic Matrix	**Live Cells (%)**
PBH (pH 7.4)	100^{a} ±0.07
Organic acid cocktail (pH 3.6)	97.29^{a} ±0.27
Strawberry puree (pH 3.6)	98.18^{a}±0.08

The mean values are the average of the surviving colonies (%) ± standard deviation. Different lowercase alphabets (a, b, c) represent significant statistical difference ($P<0.01$).

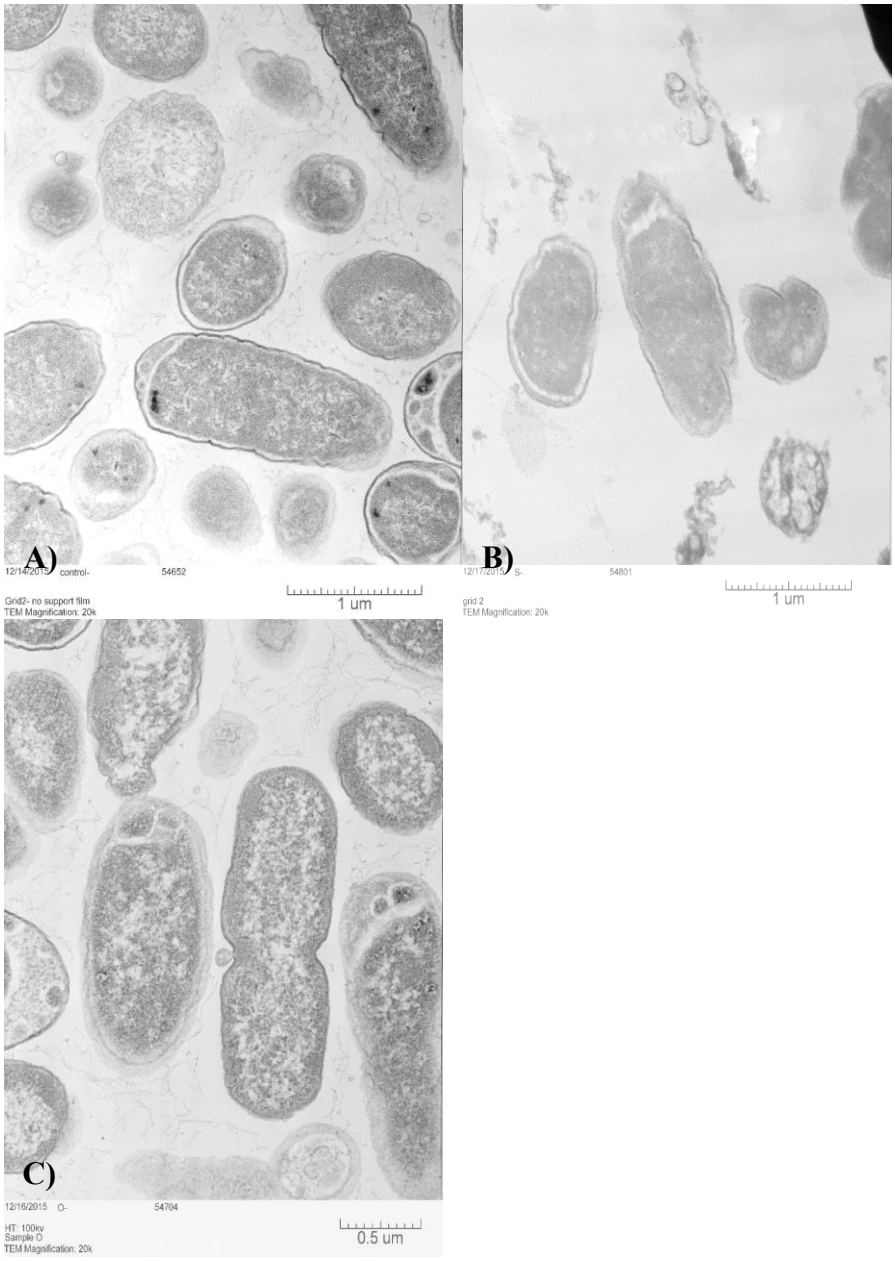

Figure 7- The TEM images of *E.coli* O26 cells after 24 hours of incubation A) PBS (pH 7.5), B) Strawberry puree (pH 3.6), C) Organic acid buffer (pH 3.6).

Discussion

The loss of viability in all STEC strains cells at pH 3.6 disregarding the type of acid used can be linked to lowered internal pH as opposed to external cellular damage (Brudzinski & Larrison, 1998). The H+ ions in the acid can enter the bacterial cell, increase the intracellular acidity and lead to cell inactivation (Smith et al., 2014; Hirshfield et al. 2003). Marinating the internal cellular pH value when exposed to different external pH values is the result of physiologically triggered inducible pH homeostasis system, which requires regulation of a group of proteins in charge of imparting protection effect (Gareen et al., 1997).

Shiga toxin producing *Escherichia coli* (STEC) strains are a group of enteric pathogens with the ability to survive pH 2.5 due to sigma factor (RpoS) regulating genes required for acid resistance (Waterman & Small, 1996). However, despite long exposure (24 h) to relatively low pH value (3.6) acids, there are still surviving *E.coli* cells (Figure 1) that raises concerns about the acid tolerance response (ATR) (Brudzinski & Larrison, 1998) and cast doubts on the significant effect of low pH acids as an effective microbial killing method. It is worth mentioning that all the strains where studied at their stationary phase, which is the most resistant phase in the cell growth cycle (Garren et al., 1998) and stationary phase cells are 1,000-10,000 times more resistant than exponentially growing cells to acid (Waterman & Small, 1996).

Organic acids are used in food processing as preservative and it is believed that it can cause *E.coli* adapt and tolerate pH values that in normal situation would inactive the organism (Brudzinski & Harrison, 1998). Treatment with 4% lactic acid could contribute

to 1.6-3.1 log reduction in the initial concentration of the Big Six (Kalchayanand et al., 2012). In another study Leyer et al (1995) reported a significant acid-adaptive response in *E.coli* O157:H7 in the presence of lactic acid. In a similar study Garren et al. (1998) exposed the *E.coli* O157:H7 and non-O157:H7 strain (ATCC 23716) cells at their stationary phase to lactic acid (pH 4.0) as an organic acid and reported their acquired acid resistance. The results indicate (Table 2) that the microbial inactivation in strawberry puree is significantly ($p<0.01$) higher than that of organic acid. In a similar study incubation of *E.coli* O26:H11 cells in strawberry puree for 24 hours in room temperature resulted in 1 log reduction of the initial concentration of the strain (Shayanfar & Pillai 2016).

Survival of *E.coli* O157 in fresh and frozen strawberries and strawberry juice has been described in literature (Delbeke et al., 2015; Han et al., 2004b, Knudsen et al., 2001, Nguyen et al., 2014, Yu et al., 2001); however, no literature on the survival of non-O157 STEC in fresh produce and specifically strawberries is reported. During storage of strawberries at different temperatures of 4°C, 7°C, 15°C and 20°C, the initial population of *E.coli* O157:H7 decreased about 2.5-3.9 log (Delbeke et al., 2015). This reduction trend continued for all the storage temperatures to almost undetectable. The results of some complementary studies suggest that *E.coli* O157:H7 can survive but not grow on the surface of fresh strawberries (Keshun et al., 2001; Knudsen et al., 2001). Nguyen et al (2014) showed that the initial population of *E.coli* O157:H7 on bruised strawberries declined by 1.9 log when berries were stored for 24 hours at 2°C; however, the microbial populations remained stable on intact samples. In another study by Han et

al. (2004b) the survival of *E.coli* O157:H7 was also reported in strawberry juice (pH 3.6) at two different temperatures of 4°C and 37 °C. Three days of storage at 4°C did not change the initial population of *E.coli* O157:H7 in strawberry juice; however, using selective medium suggested that almost 2 log of the cells were injured during cold storage. Incubation of *E.coli* cells in strawberry juice at 37°C inactivated the bacteria. In other studies conducted by Knudsen et al. (2001) and Yu et al. (2001) it was concluded that after 24 and 48h incubation at room temperature no substantial increase or decrease of *E. coli* 0157:H7 population in whole or sliced strawberries was observed but there was almost 1-2 log reduction reported on the whole strawberries when stored in refrigerator.

Considering the availability of nutrients such as glucose or fructose in strawberries unlike the organic pH buffer, where no nutrients are available needs more investigations. Berries are good source of phenolic compounds including tannins that are known both for their antioxidant properties and antimicrobial activity against pathogenic bacteria (Heinonen, 2007), which can justify the higher inactivation of strawberry puree when compared to acidified organic buffer.

It was previously reported that *E.coli* cells were capable of surviving extremely low pH conditions (1.5 -2.5) for hours in the GI tract (Foster, 2000). There were previous reports detailing the genomic responses in *E.coli* during acid exposure. The results of studies on different pathogenic strains indicate that low pH enhances expression of numerous virulence factors (Maurer et al., 2005). The pH difference across the cell membrane can contribute cell energy in the form of proton potential that

supports motility, ATP syntheses and catabolite transport but at the same time increases the uptake of acids that dissipate the proton potential (Russel et al., 1998). It is known that a significant number of catabolic enzymes and catabolite transporters are regulated by pH (Foster, 2000). Decarboxylase enzymes such as lysine and arginine decarboxylases are known to catabolize amino acids and generate alkaline amines as by product that help the cell against external acidification (Argaman et al., 2001). It is hypothesized the white particles accumulated inside the cells (Figure 7 -D) to be the consequence of concentration of acid stress induced metabolites.

Different strains showed different acid sensitivity and in another study *E.coli* strain was identified as an important variable in the acid survival (Garren et al., 1997; Kaalchayanand et al., 2012). The various level of acid sensitivity in different STEC isolates (Table 13) may have implications for virulence too as it is expected for more acid –resistant STEC to require a lower infective dose than acid-sensitive one for inducing sickness (Waterman & Small, 1996). The other point is that since except for the acid stress induced by strawberries the other acidic media did not provide any nutrients (e.g. glucose) it is hypothesized that *E.coli* isolates could benefit cross-protection effects through carbon starvation and expressed protective proteins (Brudzinski & Harrison, 1998). However, more epidemiological data need to be collected in order to be able to address virulence of STEC with regard to acid sensitivity. Among all the Big Six strains *E.coli* O26 and O111 are the most sensitive to acid stress (Table 14).

Despite all the efforts to elucidate the molecular and physiological changes during acid treatment, our understanding in this area is still incomplete and more studies

are needed to clarify how acid treatment might possibly affect flagellar motility, Shiga toxin producing, virulence and the metabolites formed as result of cell homeostasis to impart cell resistance to acidic condition. The results of our study indicate that pH is not the only factor to take into account if it is intended to use acid treatment as a hurdle to ensure food safety. The organic/inorganic status of the acid is also an effective factor in cell inactivation. Despite losing the ability of the acid treated cells to grow on the media they have not lost their viability; hence, culture methods should not be taken as the only method for the effectiveness of a method in inactivation of the pathogens. *E.coli* O111 and O26 are the most resistant strains in the Big Six in acid treatment.

CHAPTER V

ACID STRESS INDUCES DIFFERENTIAL ABUNDANCE OF METABOLITES IN *E.COLI* O26:H11

Overveiw

Shiga toxin producing non-O157 *E.coli* strains such as *E.coli* O26 are responsible for a growing number of food-related illnesses in the US and around the world. From food production to consumption the microorganisms in the food experience dramatic pH fluctuations either by organic acids introduced during food processing or by inorganic acids in the stomach. An important characteristic of pathogens associated with oral-fecal routes of transmission is the ability to survive both extremely acidic and moderately acidic environments. It is proved that exposure of microorganisms to different acids induce acid stress resistance in them. The study objective was to identify the metabolomic biomarkers in *E.coli* O26:H11 as a function of acid (pH 3.6) exposure. Synthetic buffers at pH 7.5 and pH 3.6 were used to identify the metabolites accumulating in the cells during acid exposure. Untargeted metabolomic analysis identified 293 metabolites out of which 145 were differentially ($P < 0.01$) expressed between pH 7.5 and pH 3.6 in *E.coli* O26:H11.After 24 hours of acid exposure, there was over 7-log decline in cell culturability. However, 21 different metabolic pathways appeared to be functional even after 24 hours of acid exposure, suggesting that the cells were still metabolically active. Among all identifiable pathways, the key differentially expressed pathways were peptidoglycan biosynthesis, purine metabolism, D-

Glutamine/D-glutamate metabolism, nitrogen metabolism, unsaturated fatty acid biosynthesis, inositol phosphate metabolism and few amino acid metabolisms. Transmission electron microscopy and microbial cell viability staining confirmed the structural integrity of the acid exposed cells. The results demonstrate that acid exposure elicits a unique set of metabolic biomarkers in *E.coli* O26 cells presumably to protect their structural integrity and maintain their intracellular pH levels.

Introduction

Exposure to acidic conditions is a commonly used "hurdle approach" of ensuring microbiological safety by the food industry (Leistner & Gould, 2002; Shayanfar et al., 2014). Organic acid sprays or immersion of meats in low acid solutions is commonly used by the meat industry to reduce and eliminate *E.coli, L. monocytogenes* and *S.Typhimurium* contamination of meat carcasses. The USDA-FSIS has approved the use of 2% solution of lactic acid as a critical control point in HACCP plans in slaughter houses (Hwang & Beuchat ,1995; USDA-FSIS, 2010). Similarly, in the canning industry, pH levels below 4.6 are routinely employed as a pathogen control step (Heflebower & Washburn, 2010). *E.coli* cells also endure extreme low pH (1.5-2.5) in the gastrointestinal tract that is linked to inorganic acid such as HCl in the stomach (Harris et al., 2006; Foster, 2004), yet surviving the hostile environment for hours (Foster, 2004) and outbreaks continue to occur. Thus, the microbiological safety of low acid foods is now becoming an increasing concern (Arnold & Kaspar, 1995; Harris et al., 2006).

When exposed to acid stress, *E.coli* cells trigger stress inducible proteins that endow them with the capability to maintain internal pH homeostasis and survive acidic conditions and to prepare the cell to survive future exposure to more extreme pH conditions (Bearson et al., 1996; Lu et al., 2013; Large et al., 2005; Leyer et al., 1995; Abdul-Raouf, 1993; Zhao et al., 1993; Bearson et al., 1997; Maurer et al., 2005). It is also reported that low pH enhances expression of numerous virulence factors in the pathogenic strains (Maurer et al., 2005). The main regulatory gene of *rpoS,* (an alternative sigma factor) is involved in acid resistance and regulating the expression of a variety of stress proteins (Lin et al., 1996).

It was previously reported that *E.coli* cells are capable of surviving extremely low pH conditions (pH1.5 –pH 2.5) for hours in the GI tract (Foster, 2000). Foodborne outbreaks associated with non-O157 Shiga Toxin *Escherichia coli* (non O157 STEC) strains are now being reported regularly (Gould et al., 2013; FSIS, 2012; Werber et al., 2002). The most common non-O157 STEC is *E.coli* O26 (Gould et al., 2013; CDC, 2012). This pathogen has recently been implicated in an outbreak associated with Chipotle Mexican Grill leading to 52 infected cases reported from 9 states (CDC, 2015). There are previous reports detailing the genomic responses in *E.coli* during acid exposure (Bearson et al., 1996; Maurer et al., 2005; Zhao et al., 1993; Slonczewski & Foster, 1996). The results of the previous studies suggest the activity of regulatory features including sigma factor, 2-component signal transduction systems and the major iron regulatory protein Fur in acid resistance of *E.coli*. However, specific survival mechanisms including pH homeostasis by inducible amino acid decarboxylases and

probable roles for DNA repair, chaparonins, membrane biogenesis and others are still poorly defined (Maurer et al., 2005). Furthermore, there is only limited information on the metabolites and small molecules produced as the final product of acid resistance related gene expression. The underlying hypothesis of this research was that *E.coli* cells would exhibit unique metabolomic biomarkers when exposed to acid (pH 3.6) conditions as compared to pH 7.5 that define the main characteristics of *E.coli* as the final product of gene expression. How non-O157 *E.coli* strains such as *E.coli* O26 survive during acid stress is a fundamental question of biology and understanding this mechanism is crucial to the development of hurdles and technologies to confront pathogens and ensure safety in food. Furthermore, the discovery that the pathogens with impaired stress responses are less virulent (Bearson et al., 1997) has provided new insight into microbial pathogenesis. We therefore profiled the metabolites in *E.coli* O26 cells in pH 7.5 and pH 3.6 using GC-TOF MS in an untargeted metabolomics approach to identify the metabolomic biomarkers that are indicative of acid stress (pH 3.6) response in a candidate non-O157 STEC strain, namely *E.coli* O26:H11.

Materials and Methods

Microbial strain and culture conditions

Shiga toxin producing *E.coli* (STEC) O26:H11 (TW01597) was obtained from the USDA-ARS culture collection sourced from cattle faces (USDA-ARS-FFSRU, College Station, Texas). The isolate was maintained on TSA plates at 37°C. For broth cultures, the cells were grown in Trypticase Soy Broth (TSB) (Difco, USA) maintained

at 37°C in a shaking water bath to stationary phase. For high titer cell preparations, overnight TSB cultures were concentrated by centrifugation (4000 × *g;* 5 min), washed with PBS multiple times and re-suspended in PBS and the optical density (A_{260} nm) measured to verify cell titers. Cell titers approximating 10^8 CFU/mL (confirmed by plating) at their stationary phase were used in the laboratory experiments.

Acid culturability studies

The culturability patterns of the non-O157STEC strain in pH 3.6 and 7.0 buffers were monitored using TSA plates. The pH 3.6 buffer was prepared by acidifying a 0.1 M solution of potassium hydrogen phthalate with 0.1M hydrochloric acid. The prepared pH buffer was filtered sterilized using a 0.22 µm PES filter (CORNING, USA). The stability of the pH in this buffer was also verified using pH meter at 12h intervals. In parallel to the acidified buffer, pH 7.5 buffer using Phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) were used as "control". The buffer samples (10 mL) were prepared in triplicates in 15 mL polypropylene tubes (VWR, USA). Equal aliquots of the cells were inoculated into these pH buffers and the samples were stored at room temperature (20°C) for up to 24 hours. After the incubation period, the samples were centrifuged (3X; 4000 x *g* for 10 min) to remove the acidic buffer and the cells pellet was re-suspended in PBS buffer prior to plating. The TSA plates were incubated for 24 hours at 37°C. After 24 h incubation in the buffers, aliquots of the samples were plated on TSA plates. Aliquots of the pH 3.6 and pH 7.5 exposed cells were also immediately

frozen (-80°C) and stored for the metabolomic biomarker analysis. The experiment was independently repeated three times and each experiment had 3 biological replicates.

Metabolomic analysis

The metabolites in the test samples were extracted from the -80°C frozen samples following the Fiehn's protocol (Fiehn et al., 2010). Aliquots (30 µl) were extracted by 1 mL of degassed acetonitrile: isopropanol: water (3:3:2, v/v/v) at –20°C, centrifuged and decanted with subsequent evaporation of the solvent to complete dryness. A clean-up step with acetonitrile/water (1:1) was used to remove membrane lipids and triglycerides. The purified extract was aliquoted into two equal portions and the supernatant dried down. Internal standards C08-C30 FAMEs were added and the sample was derivatized by methoxyamine hydrochloride in pyridine and subsequently by N-methyl-N-trimethylsilyltrifluoroacetamide for trimethylsilylation of acidic protons. The metabolomic data was acquired using the chromatographic parameters as applied in similar studies (Fiehn et al., 2008). A 30 m long, 0.25 mm internal diameter Rtx-5Sil MS column with 0.25 lm 95% dimethyl/5% diphenyl polysiloxane film and an additional 10 m integrated guard column was used. An average volume of 0.5 µL was injected to the mobile phase of helium gas with the purity of 99.99%. The column flow and temperature gradient were 1 mL min^{-1} and 50-330 °C respectively. The gradient was 50°C for 1 min, then ramped at 20°C min-1 to 330°C, and held constant for 5 min. The analytical GC column was protected by a 10 m long empty guard column which was cut at 20 cm intervals whenever the reference mixture QC samples indicated problems caused by

column contamination. Validations were performed that at these sequence of column cuts, no detrimental effects were detected with respect to peak shapes, absolute or relative metabolite retention times or reproducibility of quantifications. This chromatography method yielded excellent retention and separation of primary metabolite classes (amino acids, hydroxyl acids, carbohydrates, sugar acids, sterols, aromatics, nucleosides, amines and miscellaneous compounds) with narrow peak widths of 2–3 s and very good within-series retention time reproducibility of better than 0.2 s absolute deviation of retention times. There were three biological replicates for each experimental treatment, and each biological replicate was run three times on the GC-MS as technical replicates.

Data analysis

The mean peak values from the untargeted metabolite runs were normalized and then subjected to univariate analyses such as significant feature identification using Student's t-test. The mean peak value was considered to be related to the mean metabolite concentration. The key components were defined using PCA by MetaboAnalyst, a web-based metabolomics data processing tool (http://www.metaboanalyst.ca). Pathway analysis was performed using MetaboAnalyst's web-based utility. This utility uses the KEGG metabolic pathways as background knowledgebase and integrates univariate analysis method as well as pathway topology analysis. The software uses "node centrality" measures to estimate node importance in an identified metabolic pathway (Xia et al., 2015). The pathways that were either

statistically significantly different (P<0.01) or had a high biological impact (Impact>0.5) were reported as the identified key metabolic pathways in the treatment groups.

Results

Microbialculturability in pH 3.6

The *E.coli* O26:H11 cells incubated in pH 7.5 buffer for 24 hours did not exhibit any significant reduction in cell numbers (Figure. 8). However, when these cells were exposed to pH 3.6 for 24 hours, there was approximately a 7.4± 0.24 log reduction in viable cell numbers. The pathogen population declined from 8.66 to 1.50 log CFU/mL.

Metabolomic biomarkers in pH 3.6

The complete listing of the metabolomic biomarkers observed in pH 7.5 and pH 3.6 conditions are provided as Supplemental Materials. As many as 293 metabolites were identified in *E.coli* O26:H11 cells when exposed to both pH 7.5 and pH 3.6 buffers. Out of 293 metabolites, 130 of these metabolomic biomarkers were unidentifiable (based on currently available databases such as KEGG).

The concentration of a majority of the metabolomic biomarkers (145 metabolites) were significantly different (p<0.01) between the two pH treatments when peak intensities were analyzed using the Student t tests (Figure 9).

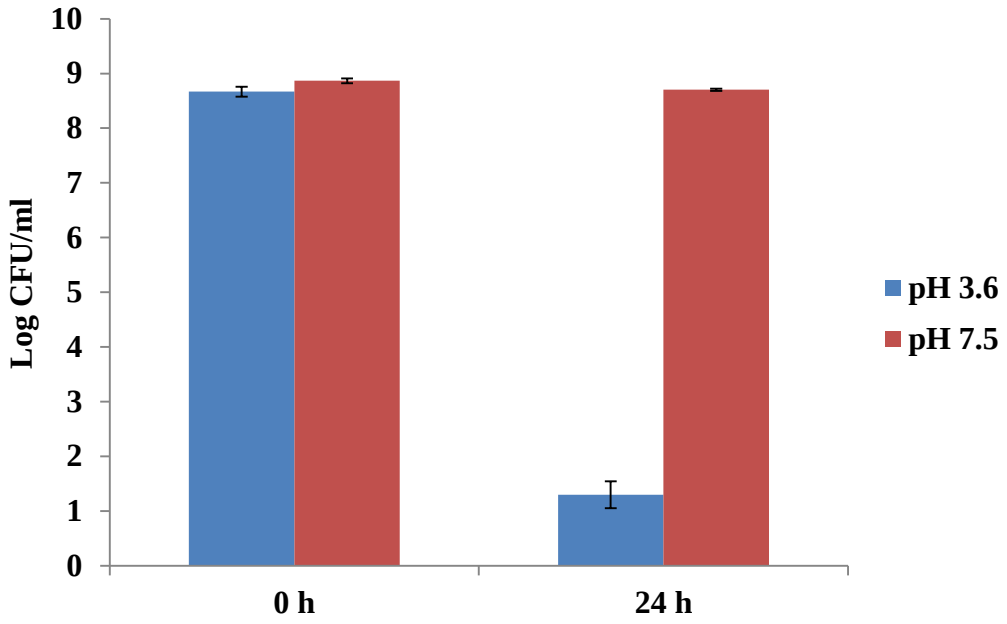

Figure 8- The plate count of *E.coli* O26 on TSA after 24 hours of exposure to PBS buffer (pH 7.5) and pH buffer of 3.6.

Based on PCA, there was a clear difference in the metabolite concentrations between the two pH treatment groups ($p<0.01$) (Figure 10). Based on PCA, the key metabolites accumulating in *E.coli* O26:H11 cells when exposed to pH 3.6 and pH 7.5 were phosphate, stearic acid, hydroxylamine, metabolite # 39, metabolite # 5471, and metabolite # 137 (Figure 11). As it is graphed in Figure 4 the concentration of stearic acid, #5471, # 137 and #39 in acid stressed cells is elevated; whereas the concentration of phosphate and hydroxylamine is higher in neutral pH matrix.

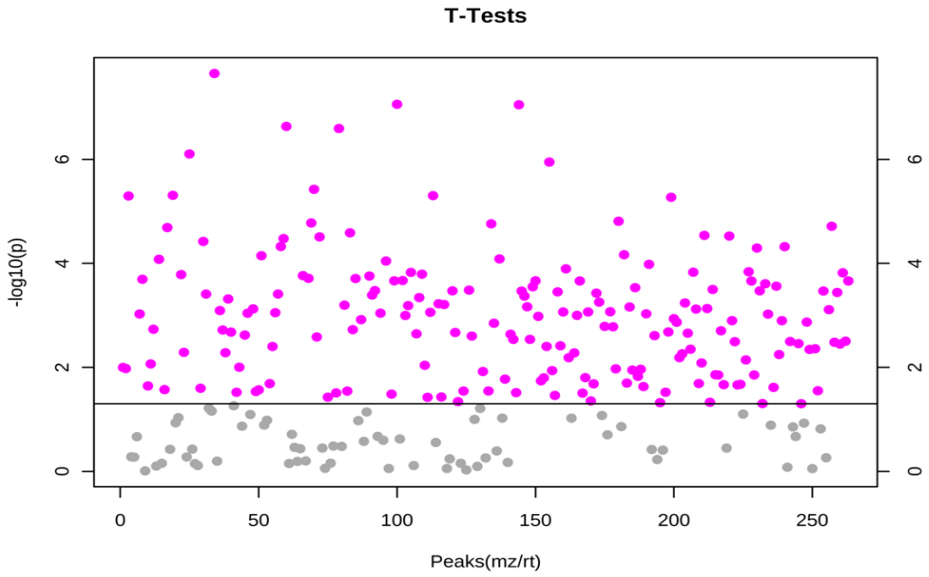

Figure 9- The Student's t-test analysis of the metabolites formed in both treatment groups. The y axis shows –log10 of p value and the x axis the peak intensity of each of the metabolites. The threshold is set at α=0.01.

Metabolomic pathway analysis

Pathway analysis was performed by making use of the "metabolome view" utility of the MetaboAnalyst software. The pathways were plotted as a function of the metabolites' impact (pathway impact) on their respective pathways and the metabolites' statistical significance (p value) (Figure. 12). Twenty-one (21) metabolic pathways were significantly ($p<0.01$) different between pH 7.6 and pH 3.6. Each of these pathways has its own specific impact value based on the position of the identified metabolites in their respective pathway. The pathways that were analyzed in detail were limited to pathways with either a pathway impact value > 1.0, or their p value was $< 10^{10}$ (Figure 12). Nine metabolic pathways met these criteria (Table 16).

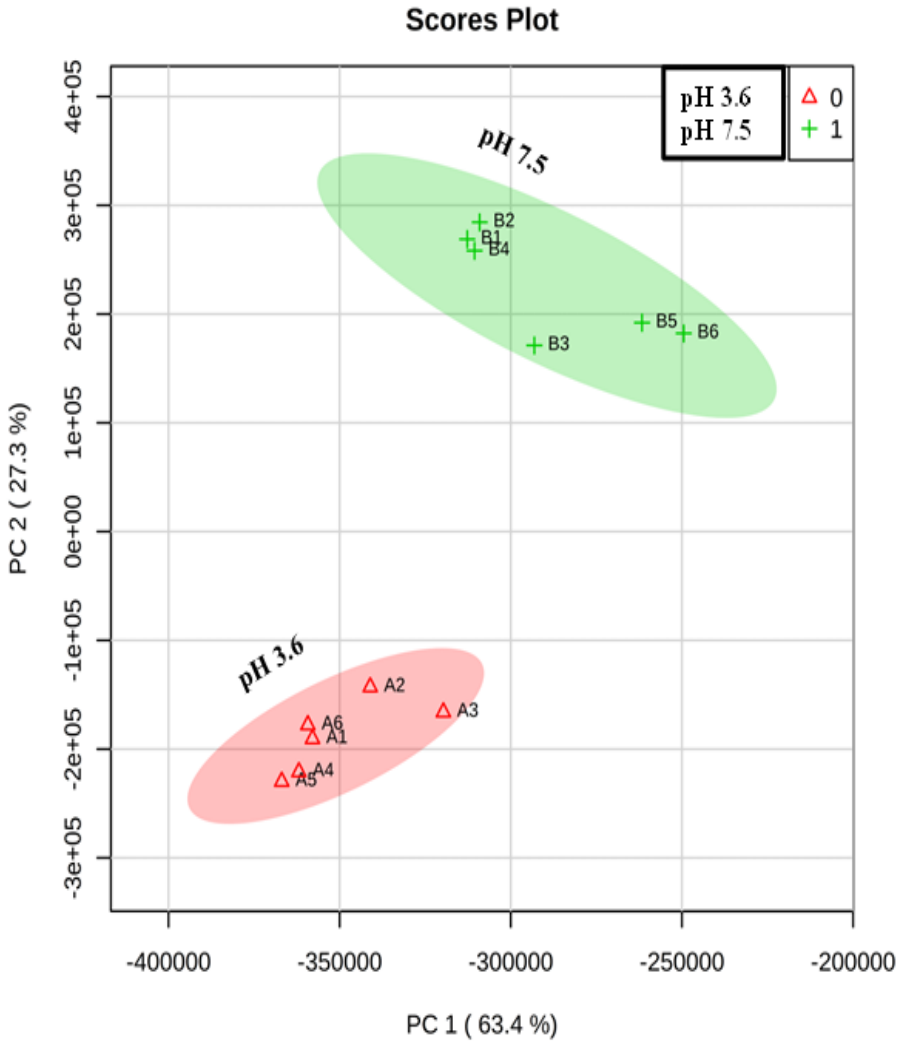

Figure 10- Scores plot between the selected PCs. The explained variances are shown in brackets.The two experiment groups of pH3.6 (A1-A6) and pH 7.5 (B1-B6) buffers are plotted including three biological and two technical replications.

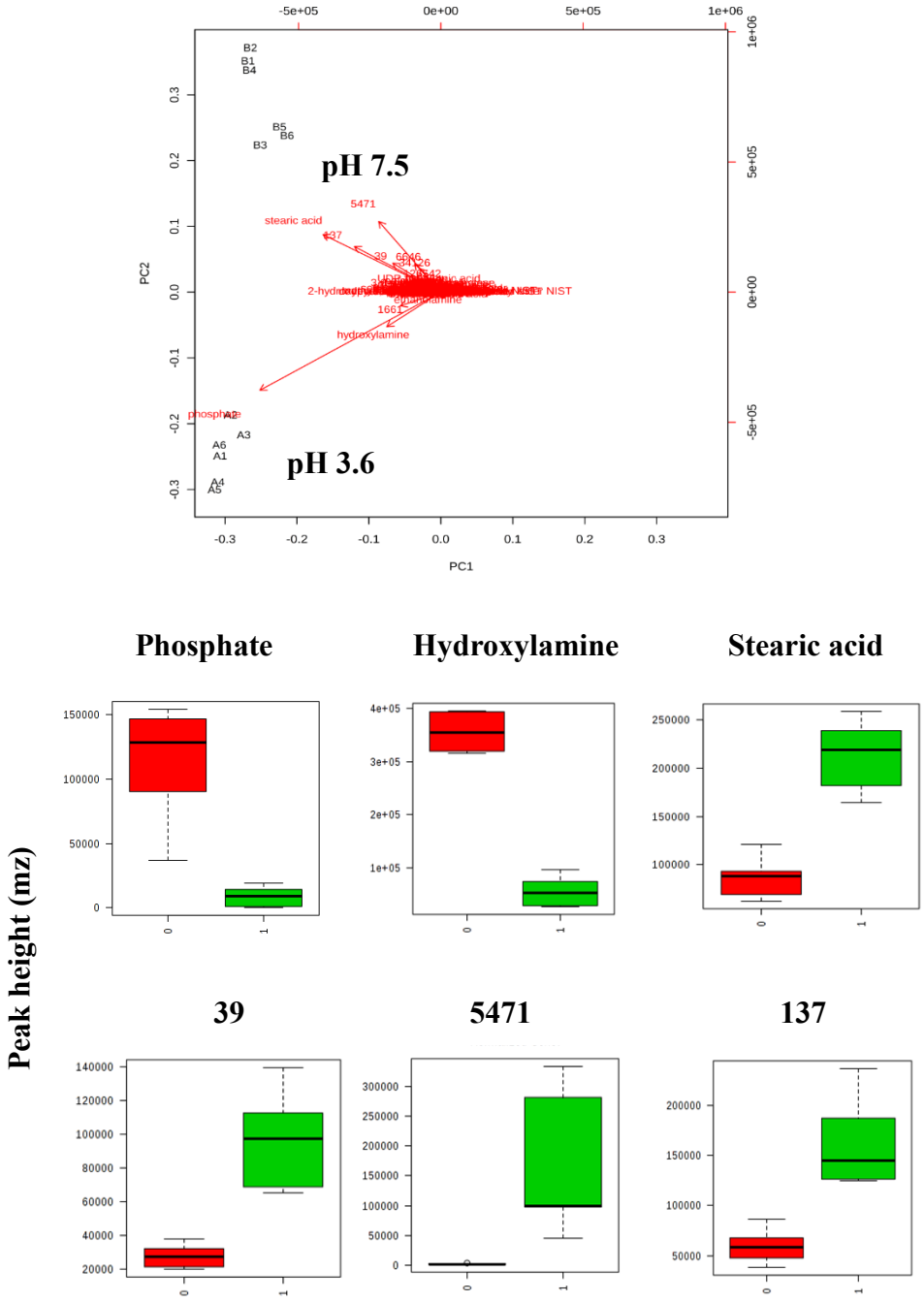

Figure 11- The concentration of key metabolites identified by PCA analysis as a result of exposure to pH 7.5 (0- red color) and pH3.6 (1 – green color) for 24 hours at room temperature.

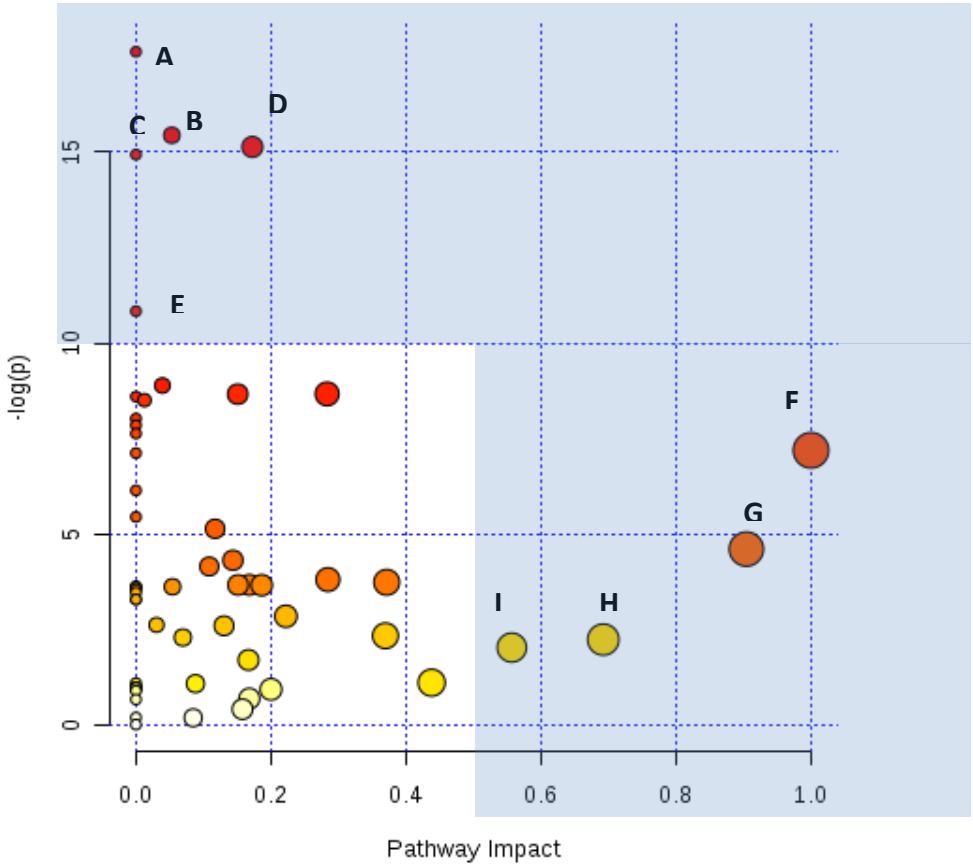

Figure 12- The metabolome view showing all matched pathways according to p values from pathway enrichment analysis and pathway impact values from pathway topology analysis. A) Peptidoglycan biosynthesis, B) Purine metabolism, C) Nitrogen metabolism, D) D-Glutamine and D-glutamate metabolism, E) Biosynthesis of unsaturated fatty acids, F) Inositol phosphate metabolism, G) Alanine, aspartate and glutamate metabolism, H) Beta-alanine metabolism, I) Glycine, serine and threonine metabolism.

Table 16- Statistically significant metabolic pathways (p<0.01) identified in *E.coli* O26:H11 as a result of acid stress exposure for 24 hours.

Pathway Name	Total	Hits	p	Impact
Peptidoglycan biosynthesis	19	1	2.2255E-8	0.0
Purine metabolism	73	8	1.9801E-7	0.053
D-Glutamine and D-glutamate metabolism	7	1	2.5433E-7	0.0
Nitrogen metabolism	18	3	2.926E-7	0.0
Biosynthesis of unsaturated fatty acids	6	3	1.9723E-5	0.0
Inositol phosphate metabolism	8	1	7.5028E-4	1.0
Alanine, aspartate and glutamate metabolism	18	7	0.0099317	0.9
beta-Alanine metabolism	16	5	0.10592	0.69
Glycine, serine and threonine metabolism	32	8	0.13057	0.55

Discussion

Bacterial cell membranes are critical to the viability of the cells since they selectively control the permeation of substances in and out of the cells. Organic and inorganic acids increase the permeability of bacterial cell membranes (Large et al., 2005). Previous studies had shown that extremely low pH levels outside the bacterial cell membranes will force the H+ protons to leak into the cells and acidify the internal pH (Foster, 2000). Other studies have shown that even though a majority of the cells may experience loss of viability, a sub-population of the cells will survive acid exposure for relatively long periods of time (Lin et al., 1995). Even more extreme pH values below the growth range (pH 1.5) *E.coli* can retain viability for hours as a result of the induction of a couple of genes including the complex gad system, RpoS, etc. (Maurer et al., 2005) .The *BacLight* images in Chapter IV clearly illustrated after exposure to pH

3.6 for 24 hours, 40.83% of the cells appeared to be still viable (based on viability staining) though not growing as expected on TSA plates, suggesting that they are metabolically active but not culturable. Hence, the metabolic pathways are mainly the ones that are affected in stationary phase cells that are in viable but non-culturable status. This is critical to bear in mind the cells in their stationary phase are more resistant to any type of stress and their resistance mechanisms are different than those in log phase (Bearson et al., 1996). Since a minimum of 10^7 CFU/mL cells are required for metabolite detection per the methods used, it is highly unlikely whether the surviving cells would have contributed to any of the detectable metabolites (Feihn, *personal communication*). Transmission electron microscopy (TEM) images illustrate the how the cell membrane in *E.coli* O26 has been affected by changes of pH (Chapter IV).

Stearic acid, phosphate, and hydroxylamine were 3 known key metabolites being differentially expressed in different experimental groups. The decrease in concentration of phosphate in the cells exposed to acid stress could be justified by de-phosphorylation of the phosphate groups on the cell membranes. Previous studies have shown that phosphate and cAMP help in acid adaptation (Rowbury et al., 1999). It is possible that the reduced concentrations of the hydroxylamine in the pH 3.6 cells could be the result of its conversion to ammonium by hydroxylamine reductase (Bernheim & Hochstein, 1968).

The analysis in this study was focused on the pathways mentioned in Table 16 that appear to have a direct role in the acid stress response of *E.coli* cells. There are three main inducible decarboxylase mechanisms associated with acid resistance in

E.coli, whose activities are medium dependent. The accumulation of glutamine in acid stressed samples (Table 28) clearly justifies activation of Glutamate-Glutamine cycle that transforms the glutamate to glutamine acid and as a result glutamine is released. It is known that *gadC* encodes a putative glutamate/ϒ-amino butrate antiporter which is required for the glutamate-dependent acid resistance system in *E.coli*. The glutmate-glutamine cycle is trigged by upregulation of *glsA* that activates L-glutamine anminohydrolase (EC 3.5.1.2) that catalyzes degradation of L-glutamine to L-glutaminc acid and ammonium ion (KEGG, eco00471). Furthermore, this enzyme supplies the nitrogen required for the biosynthesis of a variety of metabolic intermediates (Sinsuwan et al., 2012). The amino acids glutamine and glutamic acid are known enhancers of *E.coli* survival in acidic conditions (Foster, 2000; Lu et al., 2013; Lin et al., 1995). The increase in these nitrogen containing amino acids could facilitate the action of glutaminase, which results in release of gaseous ammonia which would ultimately neutralize the increasing proton levels within such cells (Lu et al., 2013). There is also murD gene triggered in D-Glutamine and D-glutamate metabolism that catalyzes the intermediate metabolites to UDP-MurNac-L-Ala-D-Glu, which subsequently enters peptidoglycan biosynthesis (KEGG, *Escherichia coli* K-12 MG1655) and supports cell wall integrity (Brown et al., 1995).The other two key decarboxylase systems of arginine and lysine dependent do not seem to be involved in acid resistance mechanism here since their metabolites are not identified (Table 28), and hence glutamine cycle remains the main inducible decarboxylase mechanism in this study.

The biosynthesis of peptidoglycan is highly regulated in bacterial cells (Sinensky, 1974). Peptidoglycan is an essential cellular component made of long amino sugar strands cross-linked by short peptides. The cell wall in bacteria is mainly formed by peptidoglycan that is comprised of repeating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) disaccharides. This structure maintains the shape of cell and protects it from osmotic shock lysis (Brown et al., 1995). Therefore, the increase in peptidoglycan biosynthesis during acid exposure could be the result of the cells attempting to maintain its structural integrity to resist acid stress. According to the pathway analysis on KEGG it appears to main genes of *MurA* and *dacA* to be upregulated in acid stress cells and the phosphate as one of the main metabolites in the peptidoglycan pathway to decrease in stressed samples (KEGG, eco00550). Earlier MurA had been also identified as the essential gene in peptidoglycan biosynthesis in *E.coli* (Brown et al., 1995).

In addition to serving as the nitrogenous bases of nucleic acids, purines are also critical in energy carrier molecules such as ATP, GTP, cyclic AMP, NADH and coenzyme A (Vogels & Van der Drift, 1976). This intensive energy enables the stressed cells to survive and repair themselves. However, considerable number of genes such as *nudF*, nude, pgm, *purF*, *paoC*, etc. are involved in purine metabolism pathway that complicates its analysis (KEGG, eco00230). The enhanced nitrogen metabolic cycles in acid stressed cells is regulated by *narK*, *narU*, *glnA*, etc. that could also contribute to triggering purine metabolism, the glutamine and glutamate metabolic and subsequently peptidoglycan synthesis pathways (KEGG, eco00910). The nitrogen cycle is a complex

interplay among many microorganisms catalyzing various reactions, where nitrogen is found in different oxidation states from +5 in nitrate to -3 in ammonia and thus its analysis requires more targeted studies.

The biosynthesis of unsaturated fatty acids could be linked to the membrane fluidity that *E.coli* cells have to regulate to survive environmental stress conditions such as acid stress (Sinensky, 1974; Janssen & Steinbuechel, 2014). In this study, we postulate that during exposure to pH 3.6 acid stress, the *E.coli* cells attempt to incorporate more saturated long chain fatty acids to decrease the viscosity and stiffen the cell membrane. The fatty acid composition of the cell membrane of acid habituated *E.coli* cells was replaced with saturated fatty to resist the induced stress (Brown et al., 1997). Another study has reported that stiffening the cell membrane reduces its permeability to small molecules (Cooper, 2000). The increased levels of stearate (Figure 11, Table 28), fully saturated fatty acids in the pH 3.6 exposed cells supports this hypothesis. The enhanced peptidoglycan biosynthesis also supports this conclusion. In this study, the biosynthesis of palmitic acid, stearic acid and oleic acid from palmityl-CoA, stearoly-CoA and oleoyl-CoA respectively were clearly identified by upregulation of TesA and TesB (KEGG, eco01040), which are also identified as genes in charge of fatty acid synthesis in other studies (Davis et al., 2000).

The enhanced activity of inositol phosphate metabolism is noteworthy. Inositol is a cyclic carbon with six hydroxyl groups on the ring structure and is capable of being phosphorylated to affect a variety of functions and being characterized as carbohydrate synthesis pathway. Inositol phosphate is an important signaling molecule in yeast cells

(Wilson et al., 2013; Shears, 2004). The increased accumulation of inositol phosphate in *E.coli* cells exposed to low pH (Table 28) is interesting and worthy of further investigation since inositol signaling pathway on the cell membrane is a major method of cell-cell signaling (Shen et al., 2003). The genes involved in this pathway include *suhB, appA* and *tpiA* (KEGG, Pathway: eco00562) that are also confirmed in other studies (Matsuhisa et al., 1995).

When cells are stressed various amino acids are accumulated in the stressed cells (Jozefczuk et al., 2010). The reason is for amino acid accumulation is either increased protein degradation to eliminate the abnormal proteins formed during stress or increasing in the concentration of the amino acids required to synthesize new protective proteins (Jozefczuk et al., 2010; Mandelstam, 1963; Willetts, 1967). Beta-alanine is a substrate for pantothenic acid synthesis in microorganisms, which is subsequently transformed to coenzyme A (Williamson & Brown, 1979). Coenzyme A is the cofactor involved in the biosynthesis and breakdown of fatty acids, plyketides and nonribosomal peptides in bacterial cells (Brown, 1959). The results of these studies illustrate that when *E.coli* O26:H11 cells are exposed to pH 3.6 conditions, it results in a differential abundance of metabolites as indicated by their mean peak intensities. Most of the metabolites that are differentially accumulating at pH 3.6 are thought to participate directly or indirectly on cell membrane fluidity and the structural integrity of cell walls. Additionally, nitrogen and purine metabolism is differentially expressed under acid stress conditions suggesting that the cells are also attempting to neutralize the accumulating proton levels within the cells. Thus, *E.coli* O26:H11 cells appear to marshal their metabolomic responses to

preserve their structural integrity as well as maintain their intracellular pH levels in an attempt to survive acid conditions. While numerous responses to pH stress are known, the mechanisms by which *E. coli* maintains its internal pH at 7.6 remain poorly understood. Correlating the results of the current study with the transcriptome analysis can contribute to a better understanding of the mechanism behind the triggered pathways. The results of several studies show the importance of low pH in the pathogenesis of enteric bacteria including *E.coli* (Maurer et al., 2005). The studies indicate that the infectious dose for the pathogens is significantly decreased if stomach acidity is buffered; suggesting that when the pathogen can tolerate stomach acid better it is more likely to survive and cause disease. Furthermore, the surface attachment and motility enhance acid resistance in *E.coli* (Soutourina et al., 2002; Maurer et al., 2005). However, the current study is not focused on virulence of *E.coli* after acid stress, which can be considered in future studies. The results of the current study is hoped to build up a basis for more targeted studies in order to identify the effect of each of key metabolites on the virulence of *E.coli* O26.

CHAPTER VI

METABOLOMIC ANALYSIS OF ACID STRESS RESPONSE IN SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O26:H11 EXPOSED TO STRAWBERRY PUREE

Overview

In spite of our understanding that low pH foods are detrimental to bacterial pathogen survival, a number of foodborne outbreaks associated with low pH foods still occur. The research objective was to identify the metabolomic biomarkers in *E.coli* O26:H11 associated with long duration (24 h) low pH exposure. Strawberry puree (pH 3.6) was used as the test matrix. *E.coli* O26 cells were incubated in strawberry puree (pH 3.6) at room temperature for 24 hours to identify the metabolites accumulating in the cells as a result of acid stress. After 24 hours of exposure to the strawberry puree, there was a 1.77 log decline in cell culturability. Using untargeted metabolomic analysis (using GC-TOF-MS), 293 primary microbial 166 metabolites were identified, out of which four were differentially ($P < 0.01$) expressed after 24 hours. Sixty different metabolic pathways appeared to be functional even after 24 hours of acid exposure, suggesting that the cells were still metabolically active. Among the identified pathways, the key differentially expressed pathways after 24 h exposure to acid stress were related to nucleic acid (purine and pyrimidine), D-Glutamaine/D-glutamate metabolism, various amino acids metabolism, beta-alanine metabolism and inositol phosphate metabolism. Transmission electron microscopy and microbial cell viability staining confirmed the

structural integrity of the pathogen even when exposed to low pH conditions for 24 hours. The metabolomic analysis reveal that exposure to the organic acids for 24 h in strawberry puree elicits a unique set of metabolic pathways in *E.coli* O26 cells to protect their structural integrity, protect their DNA, and maintain their intracellular pH levels. These findings highlight the versatility of bacterial cells to withstand the non-thermal processing conditions that the food industry routinely employs to control microbial pathogens. The food industry needs to validate its "hurdle steps" to be capable of overcoming this pathogen versatility.

Introduction

Escherichia coli O26 is one of the most common non-O157 Shiga toxin producing *Escherichia coli* (STEC) serogroups in a variety of foods (CDC 2015; Palumbo et al., 2010) Strawberries are of economic value to the US agriculture (USDA, 2014). However, since they are not routinely washed prior to consumption, there is a high likelihood of them becoming a vehicle for the transmission of pathogens such as STEC such as *E.coli* O26 serogroup (De Roever, 1998). In July 2006 there was an outbreak of *E.coli* O26 serogroup in Massachusetts that was linked to contaminated strawberries (CDC, 2015). This outbreak resulted in 5 cases of illness and 1 hospitalization). The acidic conditions in strawberries are associated with the presence of citric, malic, and ascorbic acid, which contribute to a low pH value between 3.0 and 3.5 (Castro, 2002). Low pH in foods is thought to be a natural hurdle against microbial pathogen survival and viability (Delbeke et al., 2015; Knudsen et al., 2001). Previous

studies have shown that STEC *E.coli* O157:H7 titers reduced between 1.9 to 3.9 log on bruised strawberries (Nguyen et al., 2014; Delbeke et al., 2015). However, in spite of these findings, a number of outbreaks have been associated with low pH foods (Asplund & Nurmi, 1991; Dingman 2000; Arnold & Kaspar, 1995). The response of Shiga-toxin producing *E.coli* to acid exposure has implications in terms of its virulence (Waterman & Small, 1996; Gould et al., 2013). Therefore, there is a need to have a better understanding of the underlying mechanisms involved in acid stress response in pathogens such as STEC. Our understanding of virulence and survival mechanisms in STEC has improved over the past few years with proteomic and transcriptomic advancements (ref needed). The ability to employ advances in metabolomics offers the opportunity to have a deeper understanding of how microbial pathogens respond to a variety of "stressors" that are often employed as "hurdle technologies" by the food industry. To our best knowledge there is no published report on the metabolomic profile of STEC when exposed to acidic conditions. The overall objective of this study was to identify the primary metabolites and the metabolic pathways that are differentially expressed when a strain belonging to the STEC O26:H11 serogroup is exposed to the acidic conditions (pH 3.6) present in strawberry puree for extended periods of time (24 hours). The underlying hypothesis was that there are unique metabolic pathways that are selectively expressed in STEC when exposed to long duration acid exposure in strawberries puree. An untargeted metabolomic approach screening for primary metabolites using GC-TOF MS was used in this experimental approach.

Materials and Methods

Strawberry puree preparation

Fresh strawberries (1 kg) were purchased from a local farmer's market in College Station, Texas and were blended in a standard kitchen blender before being centrifuged at 10000 rpm to separate the large pulp and seeds from the rest of the puree. The pH of the strawberry puree was measured using a calibrated pH probe (Corning, Corning, NY). The puree was frozen in small aliquots (10 ml) and stored frozen at -80 ºC prior to being used in the experiments. The pH of the sample was verified prior to freezing and after thawing prior to the experiments.

Microbial strain and culture conditions

A Shiga toxin producing *E.coli* (STEC) strain, TW01597 belonging to the O26:H11 serogroup was kindly provided by the USDA-ARS (FFSRU, College Station, Texas). The culture was grown overnight in Trypticase Soy Broth (TSB) (Difco, USA) at 37°C to a stationary phase. The cells were centrifuged (4000 × *g;* 5 min), washed multiple times (3X) with PBS and re-suspended in PBS at an optical density (A_{260} nm) that corresponded to approximating 10^8 CFU/mL (verified by plating on TSB).

Strawberry puree incubation study

The PBS buffer washed bacterial cells (as described earlier) were mixed with 10 ml of strawberry puree in a 15 ml clinical tube and incubated for 24 hours at room temperature in a shaking incubator. Three replicates were prepared. Three un-spiked

tubes of 10 ml strawberry puree were used as controls to measure the background metabolites associated with the strawberry puree. At t=0 and t=24, aliquots were removed from the samples, serially diluted and plated on TSA. We did not on purpose wash the bacterial cells off the strawberry puree (prior to plating) to eliminate any possibility of loss of cells associated with the washing step. Preliminary experiments showed no difference between strawberry samples that were washed and those that were diluted without a washing step. The plates were incubated for 24 h at 37 °C before enumeration. Preliminary studies also showed that there was no improvement in colony counts even if plates were incubated longer. The surviving population was expressed as CFU/ml.

Metabolomic analysis

About 1 ml subset of each both unspiked strawberry puree (control) and spiked strawberry puree were frozen prior to the metabolomic analysis. The frozen samples were extracted for the primary metabolites following Fiehn's protocol (Fiehn et al., 2010). The extracted metabolites were separated and detected using the GCTOF-MS. The chromatographic parameters were set as previously applied in similar studies (Fiehn et al., 2008). There were three biological replications considered for each treatment group and each biological sample was analyzed three times (as technical replicates) on the GC-TOF-MS.

Data analysis

The mean peak values obtained from GC-MS were normalized and used for univariate analysis using Student's t-test by MetaboAnalyst (www.metaboanalyst.ca). Pathway analysis was performed using MetaboAnalyst's web-based utility based on KEGG metabolic pathways. The node importance in the identified metabolic pathways is estimated using "node centrality" (Xia et al., 2015). The pathways that were either statistically significantly different ($P<0.01$) or had a high biological impact (Impact>0.5) were reported as the identified key metabolic pathways in the treatment groups.

Results

Microbial culturability

The Shiga toxin producing *E.coli* (STEC) strain TW01597 belonging to the O26:H11 serogroup exposed to the acidic conditions in the strawberry puree showed a 1.77± 0.38 log reduction as compared to the unexposed conditions (Figure 13).

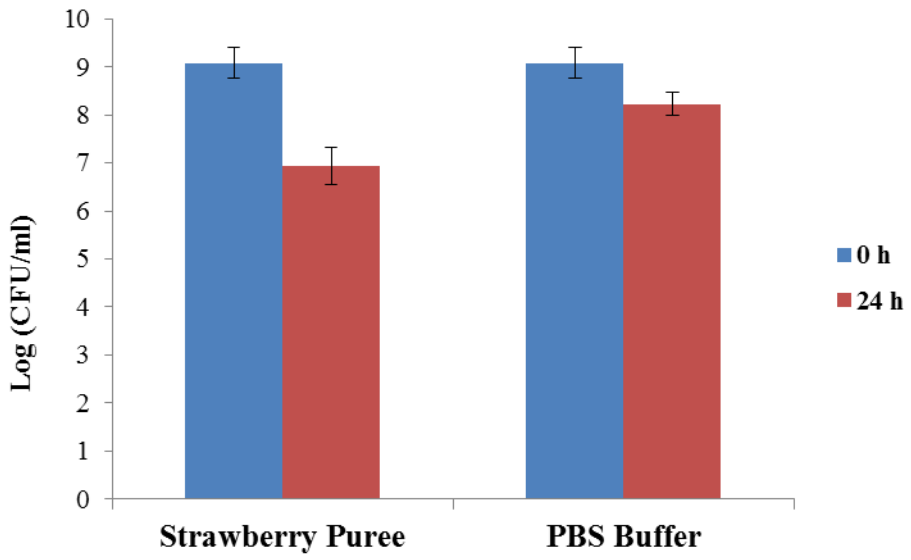

Figure 13- Inactivation of *E.coli* O26 after 24 hours of exposure to strawberry puree at room temperature as compared to the PBS buffer. Inactivation is defined as culturability on TSA plates.

Metabolomic biomarkers

As many as 293 primary metabolites were detected in *E.coli* O26:H11 cells (in both control and treatment groups) out of which 166 primary metabolites were unidentifiable (based on currently available databases such as KEGG). However, only the concentration of the four metabolites of glutamine, glutamic acid, sucrose and 68 were significantly different ($p<0.01$) between the two experimental groups (Figure 14, Figure 15, Table 17). The concentrations of glutamine, glutamic acid and sucrose declined after 24 hours of incubation of *E.coli* O26 cells in strawberry puree; whereas the concentration of metabolite 68 increased (Figure 15).

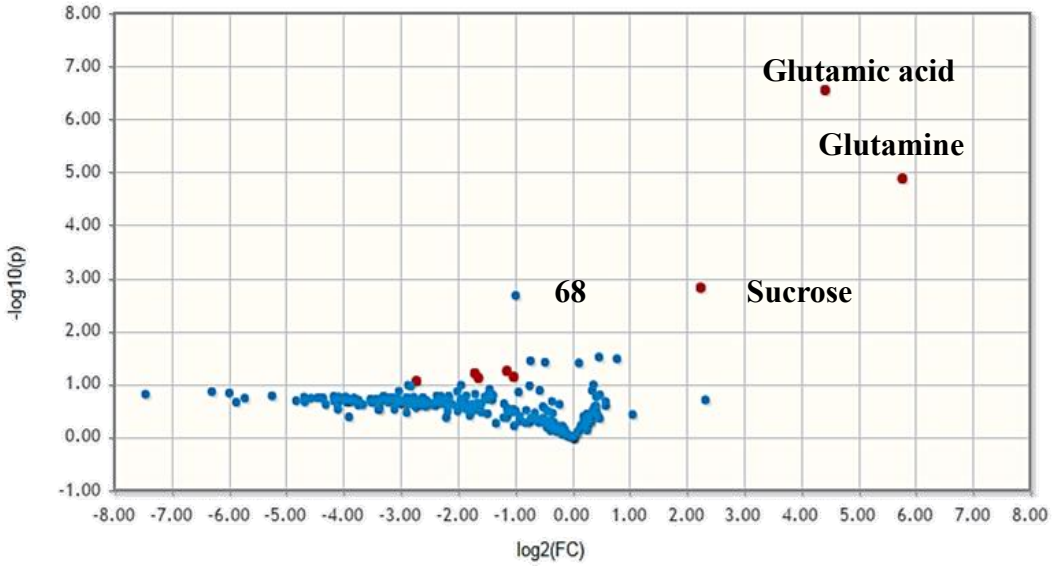

Figure 14- The statistically different (P<0.01) expressed metabolites of *E.coli* O26 cells incubated in strawberry puree for 24h at room temperature.
FC= Fold change.

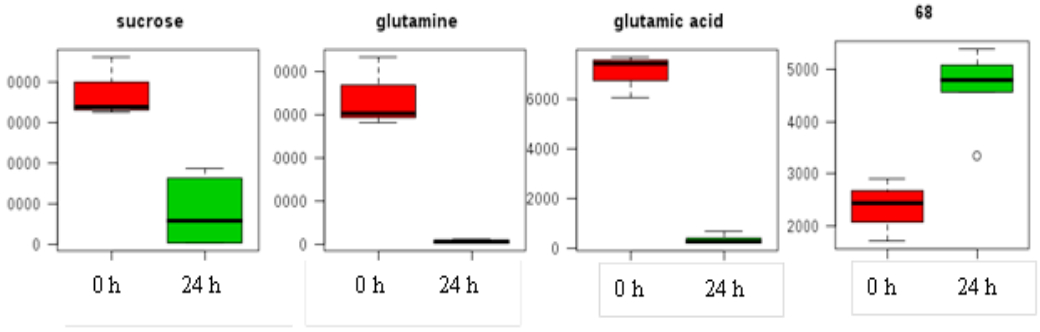

Figure 15- The changes in the amount of key metabolites expressed by *E.coli* O26 cells after 24 hours of incubation in strawberry puree at room temperature.

Table 17- The statistically different ($p<0.01$) metabolites expressed after 24 hours of exposure of *E.coli* O26 cells to strawberry puree at room temperature.

Name	p Value	FDR
glutamic acid	2.82E-07	7.40E-05
glutamine	1.32E-05	0.0017302
sucrose	0.0015031	0.13178
68	0.0021135	0.13896

Metabolomic pathway analysis

Pathway analysis was performed by making use of the "metabolome view" utility of the MetaboAnalyst software. The pathways were plotted as a function of the metabolites' impact (pathway impact) on their respective pathways and the metabolites' statistical significance (p value) (Figure. 16). Only three metabolic pathways were significantly ($p<0.01$) different between experiment groups. Each of these pathways has its own specific impact value based on the position of the identified metabolites in their respective pathway (Table 18).

Table 18- Statistically significant metabolic pathways ($p<0.01$) identified in *E.coli* O26:H11 as a result of exposure to strawberry puree for 24 hours at room temperature.

Pathway Name	Total	Hits	P Value	Impact
Porphyrin and chlorophyll metabolism *	33	1	**2.82E-07**	0.00
Pyrimidine metabolism *	44	3	**1.23E-05**	0.05
D-Glutamine and D-glutamate metabolism *	7	2	**1.25E-05**	0.17
Purine metabolism *	73	5	**1.31E-05**	0.04
Lysine degradation	11	1	0.1368	0.00
Nicotinate and nicotinamide metabolism	13	3	0.13713	0.14
Lysine biosynthesis	13	2	0.13713	0.00
Pantothenate and CoA biosynthesis	23	6	0.13719	0.17

Table 18- Continued.

Pathway Name	Total	Hits	P Value	Impact
Arginine and proline metabolism	41	9	0.13752	0.37
beta-Alanine metabolism*	16	5	0.13822	**0.69**
Cysteine and methionine metabolism	34	6	0.13835	0.22
Glycine, serine and threonine metabolism*	32	8	0.13852	**0.56**
Nitrogen metabolism	18	6	0.13861	0.00
Alanine, aspartate and glutamate metabolism*	18	7	0.13948	**0.90**
Cyanoamino acid metabolism	8	4	0.14045	0.00
Aminoacyl-tRNA biosynthesis	66	17	0.14468	0.13
Butanoate metabolism	18	5	0.15343	0.28
Glycerophospholipid metabolism	23	1	0.17047	0.03
Peptidoglycan biosynthesis	19	1	0.17727	0.00
Selenoamino acid metabolism	18	1	0.17775	0.00
D-Alanine metabolism	3	1	0.17775	0.00
Galactose metabolism	37	8	0.18117	0.17
Pyruvate metabolism	26	3	0.18417	0.40
Inositol phosphate metabolism *	8	1	0.18938	**1.00**
Streptomycin biosynthesis	9	4	0.18952	0.37
Glycolysis or Gluconeogenesis	29	3	0.19008	0.08
Pentose phosphate pathway	26	4	0.1901	0.04
Phenylalanine metabolism	23	4	0.19119	0.00
Benzoate degradation via CoA ligation	10	3	0.19183	0.00
Glutathione metabolism	21	5	0.19318	0.16
Valine, leucine and isoleucine degradation	23	3	0.19352	0.00
Sulfur metabolism	13	3	0.19483	0.07
Methane metabolism	11	2	0.19484	0.17
Starch and sucrose metabolism	31	8	0.19643	0.38
Amino sugar and nucleotide sugar metabolism	42	7	0.19718	0.20
Valine, leucine and isoleucine biosynthesis	26	6	0.19774	0.08
Propanoate metabolism	20	2	0.19845	0.05
Tyrosine metabolism	10	2	0.20152	0.00
Pentose and glucuronate interconversions	33	3	0.20412	0.00
Citrate cycle (TCA cycle)	20	4	0.21114	0.19
Glyoxylate and dicarboxylate metabolism	29	6	0.21123	0.22
Glycerolipid metabolism	14	1	0.22472	0.00
Ubiquinone and other terpenoid-quinone biosynthesis	15	1	0.22821	0.00

Table 18- Continued.

Pathway Name	Total	Hits	P Value	Impact
Thiamine metabolism	19	2	0.22923	0.00
Novobiocin biosynthesis	3	1	0.23365	0.00
Phenylalanine, tyrosine and tryptophan biosynthesis	23	4	0.23613	0.09
Ascorbate and aldarate metabolism	16	3	0.23823	0.01
Tryptophan metabolism	11	2	0.23855	0.20
alpha-Linolenic acid metabolism	8	1	0.29515	0.00
Benzoate degradation via hydroxylation	9	1	0.3313	0.00
Biphenyl degradation	4	1	0.3313	0.00
Toluene and xylene degradation	6	1	0.3313	0.00
1,4-Dichlorobenzene degradation	10	1	0.3313	0.00
Fluorene degradation	6	1	0.3313	0.00
Carbazole degradation	4	1	0.3313	0.00
Ethylbenzene degradation	7	1	0.3313	0.00
Styrene degradation	6	1	0.3313	0.00
C5-Branched dibasic acid metabolism	6	1	0.3313	0.00
Terpenoid backbone biosynthesis	18	1	0.3313	0.00
Polyketide sugar unit biosynthesis	5	1	0.54099	0.00
Naphthalene and anthracene degradation	7	1	0.61649	0.00
Biosynthesis of unsaturated fatty acids	6	3	0.76897	0.00
Fatty acid metabolism	41	1	0.7984	0.00

* The key statistically significant pathways ($p<0.01$) identified in *E.coli* O26 as a matter of acid stress.

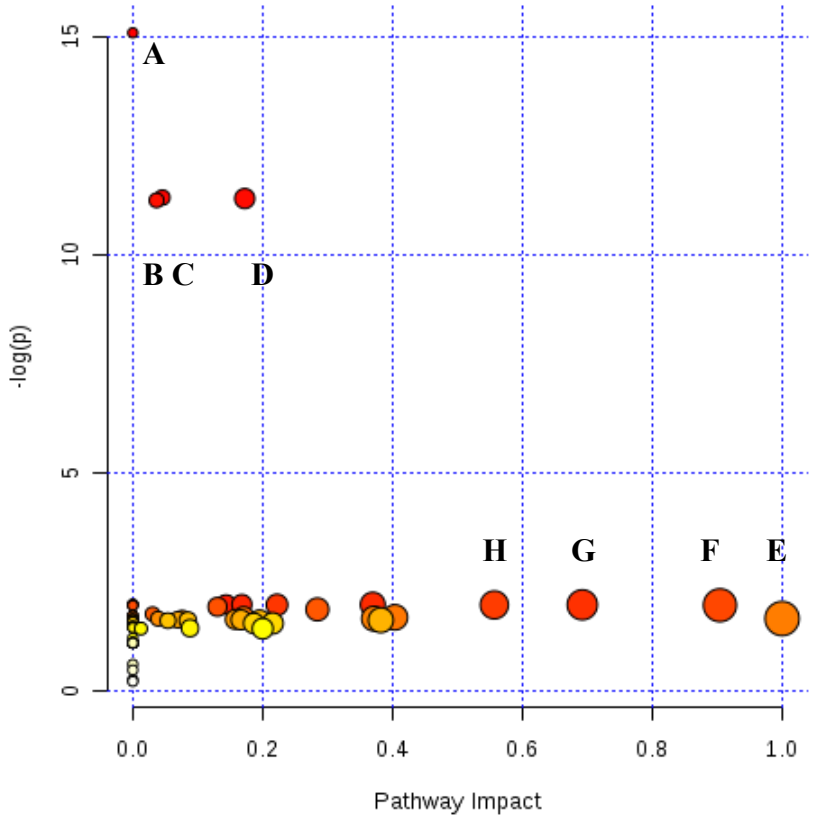

Figure 16- The metabolome view showing all matched pathways according to p values from pathway enrichment analysis and pathway impact values from pathway topology analysis.
A) Porphyrin and chlorophyll metabolism, B) Pyrimidine metabolism, C) Purine metabolism, D) D-Glutamine and D-glutamate metabolism, E) Inositol phosphate pathway, F) Alanine, aspartate and glutamate metabolism, G)beta-Alanine metabolism, H) Glycine, serine and threonine metabolism.

Discussion

The loss of culturability of *E.coli* O26 cells in the low pH strawberry puree can be linked to lowered internal pH (Brudzinski & Harrison, 1998). Exposure of bacterial

cells to low pH environment makes the H^+ ions enter the cell, increasing the intracellular acidity and leading to cell inactivation (Smith et al., 2014). The metabolomic analysis of the primary metabolites suggests that the cells are metabolically active as well. After exposure to a low pH matrix (here strawberries) the cells appeared to be still viable (based on viability staining explained in Chapter IV) though not growing as expected on TSA plates.

The organic acids naturally present in strawberries are known to reduce the culturability of *E.coli* cells to by as much as 3 logs (Kalchayanand et al., 2012), while imparting acid tolerance to *E.coli* cells (Brudzinski & Harrison, 1998). Even after 24 h of incubation in the low pH strawberry puree, there are still surviving *E.coli* cells (Figure 13), which can be justified by acid tolerance response (ATR) in bacterial cells (Brudzinski & Harrison, 1998). *E.coli* cells can retain viability for hours as a result of the induction of a couple of genes including the complex gad system, RpoS, etc. (Maurer et al., 2005), which is in agreement with results achieved in the current study (Figure 13). Different serogroups of STEC have been confirmed for their ability to survive pH values of as low as 2.5 through regulation of genes associated with acid resistance Shiga toxin by Sigma factor (Rpos) (Waterman & Small, 1996). It is also essential to bear in mind that the bacterial cells here are at their stationary phase, which is considered the most resistant growth phase of the bacteria, with about 1,000-10,000 times more resistant than exponentially growing cells to acid (Waterman & Small, 1996).

A number of regulatory networks of *Escherichia coli* respond to specific cellular stresses including heat, oxidants, starvation and DNA damage through expression of protein products that are required to resist that particular stress condition (Gustavsson et al., 2002). According to the metabolomics analysis, the *E.coli* O26 cells could retain their metabolic activity; although their replication ability was to some extend impaired (Figure 14, Table 18). The key metabolites triggered by acid stress in this study are glutamic acid, glutamine, sucrose, and 68 (Table 17, Figure 14), while the main metabolic pathways are porphyrin and chlorophyll metabolism, pyrimidine metabolism, purine metabolism, and D-glutamine and D-glutamate metabolism, inositol phosphate, glycine, serine and threonine metabolism, alanine, aspartate and glutamate metabolism and beta-alanine metabolism (Table 18, Figure 16). These metabolic pathways appear to have a direct role in the acid stress response of *E.coli* O26 cells in strawberry puree.

The significant decrease in the concentration of glutamine and glutamic acid in the strawberry puree matrix spiked with *E.coli* O26 cells (Figure 3, Table 22) clearly justifies activation of Glutamate-Glutamine cycle that transforms the glutamate to glutamine acid through expression of *gadC,* which is required for the glutamate-dependent acid resistance system in *E.coli* (Cornet et al., 1999*)*. The amino acids glutamine and glutamic acid are known enhancers of *E.coli* survival in acidic conditions (Foster 2000; Lu et al., 2013; Lin et al., 1995). Strawberries contain approximately 0.6 µmol of free glutamate per g strawberries (Blanch et al., *2012*). Considering the amount of strawberries used for puree preparation (1000 g), the glutamate present in strawberry puree is approximately 0.6 mM. The decrease in the concentration of these nitrogen

containing amino acids in the strawberry puree spiked with *E.coli* O26 could indirectly indicate the glutaminase activity, which results in release of gaseous ammonia which would ultimately neutralize the increasing proton levels within such cells (Lu et al., 2013). The other possible gene triggered in this pathway is *murD* that catalyzes the intermediate metabolites to UDP-MurNac-L-Ala-D-Glu, which subsequently enters peptidoglycan biosynthesis (KEGG, *Escherichia coli* K-12 MG1655) and supports cell wall integrity (Brown et al., 1995). Therefore, glutamine/glutamate cycles remains as the main inducible decarboxylase mechanism in this study.

The majority of *E.coli* strains are known to be normally unable to utilize sucrose (Schmid et al., 1988; Sabri et al., 2013); however, in this study *E.coli* O26 cells could utilize and decrease the initial concentration of sucrose in the strawberry puree (Figure 3). Sucrose metabolism is suggested to be associated with the regulation of two regulons of scr and csc that their mechanism is less understood (Sabri et al., 2013; Schmid et al., 1988; Schmid et al., 1982). The genes in scr regulon encode a sucrose phosphotransferase system (PTS), while the genes in csc regulon encode a sucrose non-PTS utilization system (Sabri et al., 2013). Most of the sucrose-positive bacteria phosphorylate sucrose through PTS system and transform it to sucrose-6-phospate. While the PTS genes are mainly found either on plasmids, transposons or chromosoal DNA; csc genes are found only on the chromosome (Sbari et al., 2013). The phosphate in sucrose-6-phosphate is then hydrolyzed by an invertase enzyme (e.g. sucrose-6-phosphate hydrolase) into D-glucose, D-fructose and D-phospahte and the sugars undergo sub metabolic pathways to utilize them (Bockmann et al., 1992). It is

hypothesized that due to exposure of the *E.coli* O26 cells to acid stress they switch to sucrose metabolism as an alternative source of carbon and energy to resist against the induced stress.

Purines and pyrimidine play a critical role as the nitrogenous base of nucleic acid and energy carrier molecules such as ATP, GTP, cyclic AMP, NADH and coenzyme A (Vogels & Drift, 1976). Thus, activation of the pathways associated with the biosynthesis of purines and pyrimidine in acid stressed cells is not surprising in a stressed environment; where intensive energy is required for cell adaptation to stress. There are a considerable number of genes such as *nudF*, *nude*, *pgm*, pur*F*, *paoC*, etc. involved in purine metabolism pathway that complicates its analysis (KEGG, eco00230).

Among all the pathways that are activated upon exposure of *E.coli* cells to strawberry puree porphyrin and chlorophyll metabolism seems irrelevant. There are 33 metabolites involved in this pathway from which only one metabolite of L-glutamic is identified in the current study. Porphyrin is functioning as a pigment in different microbial, botanical and animal cells (Cox & Charles, 1973) and since its biological impact in this study is 0.0 it is out of the focus of this study. Glycine, serine and threonine metabolism, alanine, aspartate and glutamate metabolism and beta-alanine metabolism are other key metabolic pathways in acid stressed *E.coli* cells (biological impact > 0.5) that are associated with amino acid metabolism (Table 17). As a result of stress (in this case acid stress) different strategies are utilized by the bacterial cells to confront the stress (Weber et al., 2005). When cells are stressed various amino acids are accumulated in the stressed cells (Jozefczuk et al., 2010). That can be justified by either

increased protein degradation to eliminate the abnormal proteins formed during stress or increasing in the concentration of the amino acids required to synthesize new protective proteins (Jozefczuk et al., 2010; Mandelstam, 1963; Willetts, 1967). Beta-alanine is a substrate for pantothenic acid synthesis in microorganisms, which is subsequently transformed to coenzyme A (Williamson & Brown, 1979). Coenzyme A is the cofactor involved in the biosynthesis and breakdown of fatty acids, plyketides and nonribosomal peptides in bacterial cells (Brown, 1959).

The enhanced activity of inositol phosphate metabolism is noteworthy. Inositol is a cyclic carbon with six hydroxyl groups on the ring structure and is capable of being phosphorylated to affect a variety of functions and being characterized as carbohydrate synthesis pathway. Inositol phosphate is an important signaling molecule in yeast cells (Wilson et al., 2013; Shears, 2004). The increased accumulation of inositol phosphate in *E.coli* cells exposed to low pH (Table 22)is interesting and worthy of further investigation since inositol signaling pathway on the cell membrane is a major method of cell-cell signaling (Shen et al., 2003).

The results of these studies illustrate that when *E.coli* O26:H11 cells are exposed to a low pH food matrix such as strawberries; their acid resistance mechanism is activated to survive the stress. Most of the metabolites that are differentially accumulating in strawberry matrix are thought to participate directly or indirectly on acid resistance and providing energy for resistance. Unlike the former studies in inorganic acids (CHPTER 6 – Part I) the pathways associated with cell membrane maintenance and repair are not triggered which can be due to the weakness of the

organic acids present in strawberries. Thus, *E.coli* O26:H11 cells appear to marshal their metabolomic responses to preserve their structural integrity as well as maintain their intracellular pH levels in an attempt to survive acid conditions. While numerous responses to pH stress are known, there is limited information available on the resistance or sensitivity of the pathogens in real food matrices with low pH and the available results are limited to plating methods. Correlating the results of the current study with the transcriptome analysis can contribute to a better understanding of the mechanism behind the triggered pathways. The results of the current study is hoped to build up a basis for more targeted studies in order to identify the effect of each of key metabolites on the virulence of *E.coli* O26.

CHAPTER VII

ELECTRON BEAM EXPOSURE INDUCES DNA REPAIR AND VIRULENCE-RELATED METABOLIC PATHWYAS IN METABOLICALLY ACTIVE YET NON CULTURABLE (MAYNC) IN SHIGA TOXIN PRODUCING *Escherichia coli* O26:H11 CELLS

Overview

Electron beam (eBeam) processing is an effective non-thermal food pasteurization technology inactivating microbial pathogens by causing a number of double stranded DNA breaks in the genome. An eBeam dose of 3kGy results in greater than 8-log reduction of Shiga toxin-producing *E.coli* such as the O26 serogroup to below detection limits. However, the cellular membrane is still intact and the cells have residual metabolic activity. Our underlying hypothesis was that the metabolome of the eBeam inactivated cells changes during post irradiation incubation. The metabolome of un-irradiated (control) STEC 026 serogroup cells was compared to the metabolome of the eBeam (3 kGy) irradiated and the metabolome of the cells that were eBeam irradiated and stored for 24 hours post-irradiation. Untargeted metabolomic analysis for primary metabolites was performed for these metabolome comparisons. The metabolome of the freshly irradiated cells was completely different from the other two treatment groups. There metabolome of the irradiated cells that were stored for 24 hours was more closely similar to the un-irradiated (control) cells. Metabolic pathway analysis indicated that DNA repair pathways being triggered and that virulence pathways of C5-

brached dibasic acid metabolism were also significantly ($P<0.01$) activated in the eBeam irradiated cells. These results suggest that eBeam irradiation inactivated microbial pathogens. However, the irradiated cells are actively attempting to repair their DNA damage and that with time, the metabolome of the irradiated cells appear to be similar to that of the un-irradiated cells. These results highlight the importance of investigating the metabolic state of irradiated cells under varying storage and possible growth conditions.

Introduction

The number of food borne outbreaks associated with non-O157 Shiga toxin producing *Escherichia coli* (non O157 STEC) are increasing in numbers to equal outbreaks linked to the O157 STEC serogroups (Gould et al., 2013; Rounds et al., 2012; Stigi et al., 2012). The food industry employs a variety of pathogen intervention steps; however, pathogens appear to be able to overcome majority of these man-made "hurdles" (Shayanfar et al., 2016; Calicioglu et al., 2003; Garren et al., 1998).

Ionizing radiation and electron beam (eBeam) irradiation in particular, is an effective pathogen inactivation technology that is in use all around the world (FDA, 2015; USDA, 1999). Previous studies in our laboratory have shown how eBeam pasteurization can be used to reduce the potential of infections associated with bacterial and viral pathogens in fresh produce and raw oysters (Shayanfar et al., 2016; Praveen et al., 2013; Espinosa et al., 2012). Electron beam irradiation inactivates microorganisms by causing a number of lethal double strand breaks (DSBs) caused by the electrons' direct breakage of the DNA molecule or by indirect damage caused by free radicals

formed during the radiolytic breakdown of the water molecules (Lung et al., 2015; Tahergorabi et al., 2012). Studies in our laboratory and others are now documenting that even when bacterial cells are exposed to lethal doses of ionizing irradiation such as eBeam (when the cells have lost their ability to multiply), the bacterial cell is still intact, their membrane is not damaged, and that the cells are still metabolically active (for up to 9 days) in terms of ATP synthesis, and electron transport systems within the cells (Jesudhasan et al., 2014; Hieke, 2015; Praveen , 2014; Secanella-Fandos et al., 2014; Caillet et al., 2008; Caillet et al., 2005; Pollard et al., 1958). The term, "metabolically active yet non-culturable" (MAyNC) has been coined for this phenomenon (Praveen 2014; Magnani et al., 2009). Inactivated bacterial cells possessing the characteristics of live cells (except for their ability to multiply) is intriguing because it raises the question about the long-term fate of these MAyNC cells and whether these cells can ever revert to a viable state. It must be emphasized that MAyNC cells are distinct from VBNC cells that have been extensively reported in the literature (Oliver, 2009; Oliver & Bockian, 1995; Pommepuy et al., 1996). VBNC cells can revert to viable cells (Oliver, 2009). However, to date there is no report of MAyNC cells ever reverting to a viable state even in immunocompromised experimental animals (Praveen, 2014). The survival of bacterial cells after exposure to ionizing irradiation such as eBeam is the net result of three endogenous mechanisms of prevention, tolerance and repair (White et al., 2014). Our underlying hypothesis was that in spite of the extensive double stranded DNA damage, the irradiated cells are attempting to repair themselves which is manifested by a changing metabolomic profile of eBeam inactivated cells during post irradiation storage.

Recent research in our laboratory has documented a specific transcriptomic pattern in eBeam irradiated *Salmonella* spp. cells during storage (Hieke, 2015). To the best of our knowledge, there are no reports on the primary metabolite changes occurring in irradiated bacterial cells especially Shiga toxin-producing *E.coli*. The primary metabolites of un-irradiated (control) STEC 026:H11 serogroup strain TW01597was compared to the primary metabolites of freshly eBeam (3 kGy) irradiated cells and the primary metabolites of cells that were eBeam (3 kGy) irradiated and stored for 24 hours post-irradiation. These untargeted (primary metabolite) metabolomic studies were performed on the STEC strain that were maintained in phosphate (PBS) buffer (pH 7.5) at room temperature.

Materials and Methods

Microbial strain and culture conditions

The Shiga toxin producing *E.coli* (STEC) strain TW01597 belonging to the O26:H11 serogroup was obtained from the USDA-ARS in College Station, Texas. The cells were grown in Trypticase Soy Broth (TSB) (Difco, USA) in a shaking water bath at 37°C to their stationary phase. The cells were harvested by centrifugation (4000 × *g;* 5 min) and washed with PBS (3X) and finally re-suspended in PBS at a concentration of approximately 10^8 CFU/mL, confirmed by plating. The cells were irradiated in the PBS buffer.

Study design

Nine double sealed pouches containing 1.5 ml of 10^8 CFU/ml *E.coli* O26 were prepared and divided into three different experimental groups in this study. They were Group A: un-irradiated control, Group B: eBeam (3kGy) irradiated and analyzed immediately and Group C: eBeam (3 kGy) irradiated and stored for 24 hour at room temperature prior to metabolomic analysis. Three biological replications were included for each experimental group.

Electron beam (eBeam) treatment

The eBeam irradiation treatment was performed using the 10 MeV, 18 kW linear accelerator at the National Center for Electron Beam Research at Texas A&M University. The actual received dose in the samples was verified using alanine (L-α-alanine pellet) dosimeters (Harwell Dosimeters, Oxfordshire, UK). The target dose was 3.0 kGy (measured dose 3.02 kGy).

Bacterial enumeration and viability staining

To confirm that the *E.coli* O26 serogroup cells were inactivated after exposure to 3 kGy, aliquots from the irradiated cells were plated on TSA plates and incubated for 24 h at 37 °C. The cells were enumerated and reported as CFU/ml. (Preliminary studies were performed to confirm that 3 kGy was a lethal dose capable of achieving greater than 8-log reduction) The Live/Dead *BacLight* bacterial staining kit (Invitrogen, Inc.)

was used in order to determine the viability (based on membrane integrity) of *E.coli* O26 cells when exposed to 3 kGy of eBeam. The cells were visualized using a fluorescence microscope (Olympus BX50, Japan) equipped with a digital camera (Olympus Qcolor3, Olympus, USA).

Transmission electron microscopy (TEM)

Aliquots (1 mL) of the eBeam irradiated *E.coli* O26 cells and un-irradiated cells treatment samples were centrifuged at 11,000×*g* for 10 min. The supernatant was poured off and the pellet was suspended in 1 ml of Trumps fixative buffer (McDowell & Trump, 1996). The sample was washed (3X) in this buffer prior to storage at 4°C until further processing. Agarose gel plugs (~ 1 mm^3 cubes) containing these cells were prepared, fixed in in osmium tetroxide. Sections of 100mm were prepared from the fixed gel cubes and stained using copper grids (400-mesh).) The sections were rinsed and stained for about 5 minutes in Reynold's lead citrate (Wright, 2000). The imaging was performed at 100 kV on JEOL 1200 Ex microscope equipped with SIA 15C CCD camera (SIE, Duluth GA) at Microscopy & Imaging Center – Texas A&M University. TEM images were adjusted for contrast in ImageJ software (Rasband, 1997).

Metabolomic analysis of primary metabolites

Aliquots of the samples from the 3 treatment groups were frozen and the primary metabolites were extracted following the Fiehn's protocol at the West Coast Metabolomics core facility at UC Davis (Fiehn et al., 2010). The primary metabolites

were separated and detected using GC-TOF MS. The chromatographic parameters were as previously described (Fiehn et al., 2008). A volume of 0.5 µL of different sample extract was injected to the mobile phase of helium gas with the purity of 99.99%. The column flow was 1 mL min^{-1} and the temperature gradient was 50-330 °C. The gradient was 50°C for 1 min, then ramped at 20°C min-1 to 330°C, and held constant for 5 min. The analytical GC column was protected by a 10 m long empty guard column which was cut at 20 cm intervals. Validations were performed to ensure of absence of any detrimental effects associated with peak shapes, absolute or relative metabolite retention times or reproducibility of quantifications. There were three biological replications for each treatment group (as mentioned above) and each biological sample was run three times on GC-TOF-MS (technical replicates). The technical replicates were included to ensure the validity of the GC-TOG-MS analysis.

Data analysis

The mean peak values obtained from GC-TOF-MS were normalized and used for univariate analysis using Student's t-test. The key components were defined using PCA by MetaboAnalyst (www.metaboanalyst.ca). Pathway analysis was performed using MetaboAnalyst's web-based utility based on KEGG metabolic pathways. The node importance in the identified metabolic pathways is estimated using "node centrality" (Xia et al., 2015). The pathways that were either statistically significantly different ($P<0.01$) or had a high biological impact (Impact>0.5) were reported as the identified key metabolic pathways in the treatment groups.

Results

Bacterial culturability

The initial population of *E.coli* cells in the experimental groups was 10^8 CFU/ml. After exposure to 3 kGy eBeam dose, there were no survivors among the irradiated samples (group B and group C). The detection limit of the assay was 10 CFU/ml. Based on the initial starting titer, the 3 kGy eBeam dose was able to achieve greater than 8-log reduction (data not provided).

Membrane integrity

The cell membrane integrity staining results are shown in Figures 17A and 17B. The cell membrane was still intact after 3 kGy eBeam exposure (Figure 17A). The membrane was intact even in cells that were incubated at room temperature for 24 hours post irradiation (Figure 17B). The un-irradiated control cells with their intact membrane are shown in Figure 17C. Bacterial cells with damaged cell membranes (from exposure to isopropanol) appear red (Figure 17D).

Transmission electron microscopy

The cellular morphology of the control and eBeam treated cells appear the same (Figure. 18A and 18B). The cell membrane is similar in both the irradiated cells and the control cells.

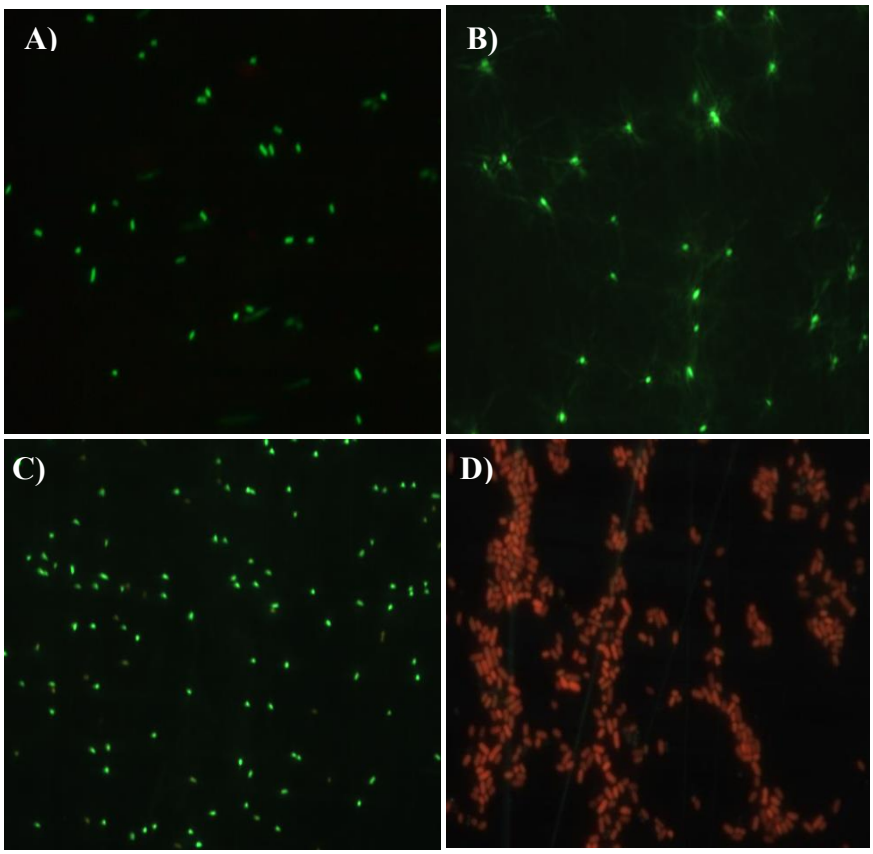

Figure 17- *BackLight* images of *E.coli* O26 under microscope.
A) *E.coli* O26 cells in PBS buffer (control) B) *E.coli* O26 cells in PBS buffer and eBeam treated with 3 kGy C) *E.coli* O26 cells in PBs Buffer and eBeam treated with 3 kGy and incubated for 24 hours at room temperature D) Killed *E.coli* O26 cells with isopropanol.

Metabolomic analysis of primary metabolites

The complete list of the primary metabolites detected in the 3 treatment groups is provided as Supplemental Materials (Table 23). As many as 349 primary metabolites were detected in all of the experimental groups out of which only 175 of these metabolites were identifiable (based on currently available databases such as KEGG) (Table 23).

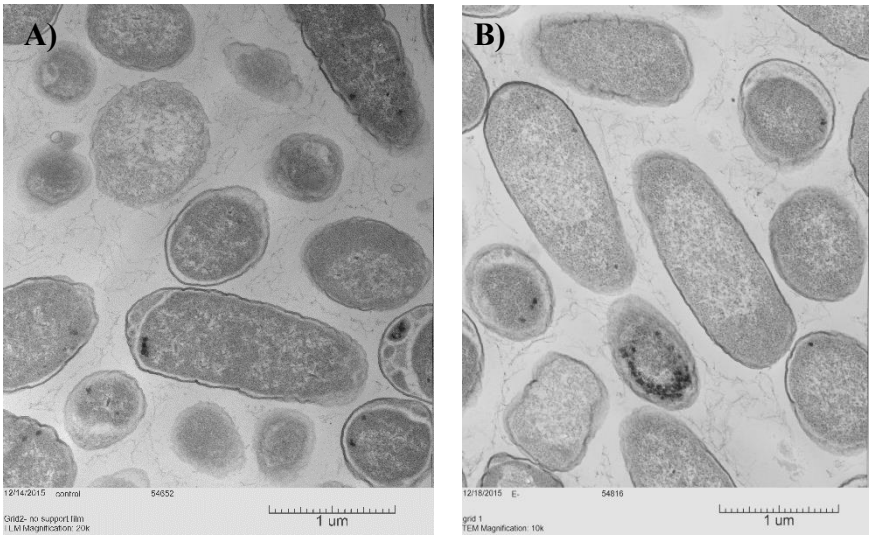

Figure 18- TEM images of *E.coli* O26 A) un-irradiated in PBS (control) B) eBeam treated in PBS at 3 kGy.

The concentration of 47 of these metabolites were significantly different ($p<0.01$) between the three experimental groups (Table 31). According to PCA analysis the primary metabolites of the control (un-irradiated) cells (group A) is very different from that of the post-irradiation incubated one (group C). The primary metabolites of the samples collected immediately after irradiation (group B) are also very distinct from the other two groups (Figure 19).

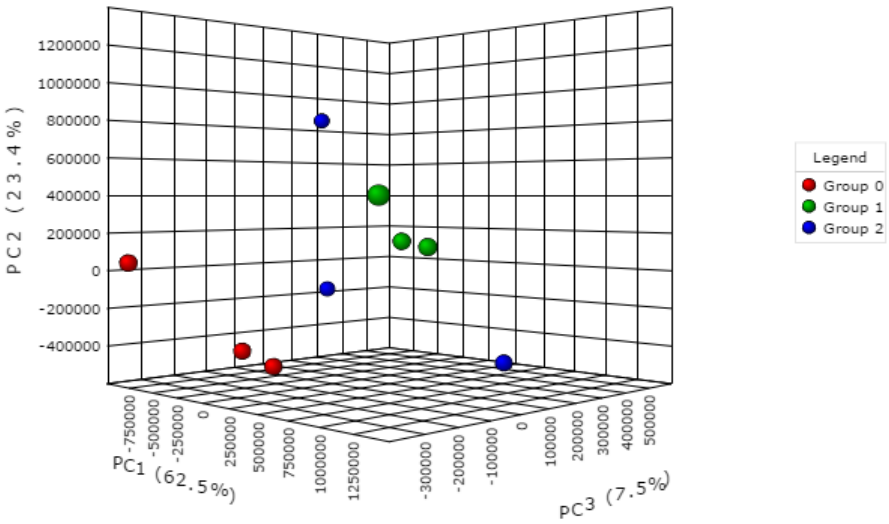

Figure 19- PCA analysis of the *E.coli* O 26 transcriptome in three treatment groups. Group 0) Un-irradiated cells (control) Group 1) Immediately after eBeam treatment (3kGy) Group 3) 24 hours after eBeam treatment (3 kGy) at room temperature (25°C).

Metabolomic pathway analysis

Pathway analysis was performed using the "metabolome view" utility of the MetaboAnalyst software (Xia et al., 2015). The pathways were plotted as a function of the metabolites' impact (pathway impact) on their respective pathways and the metabolites' statistical significance (p value) (Figure 20, Figure 21, Figure 22). Each pairwise comparison of the treatment groups showed different set of pathways being triggered. The pathway analysis of different treatment combinations (control versus eBeam treated; control versus eBeam treated and incubated and eBeam treated versus

eBeam treated and incubated) resulted in the identification of 16, 8 and 9 significant ($P < 0.01$) metabolic pathways respectively. The identification of the metabolic pathway was limited to those with statistically significant difference ($P<0.01$). The comparison of the control group (group A) and post-irradiated sample (group C) resulted in identification of the highest number of pathways (Table 19, Figure 22). The common and unique metabolites seen in group wise comparisons are illustrated in Figure 7. Comparing all the metabolic pathways triggered in each pairwise comparisons, group A-group C comparison and group B- group C have the highest number of pathways in common (Figure 23). The groups A-C and A-B had only the C5-branched dibasic acid metabolism in common; whereas, groups A-B and groups B-C had no pathway in common. The only pathway in common in the group B-C comparison is purine metabolism (Table 19). The pathways that are most significantly different when groups A and group B are compared is arginine and proline metabolism (Figure 20).

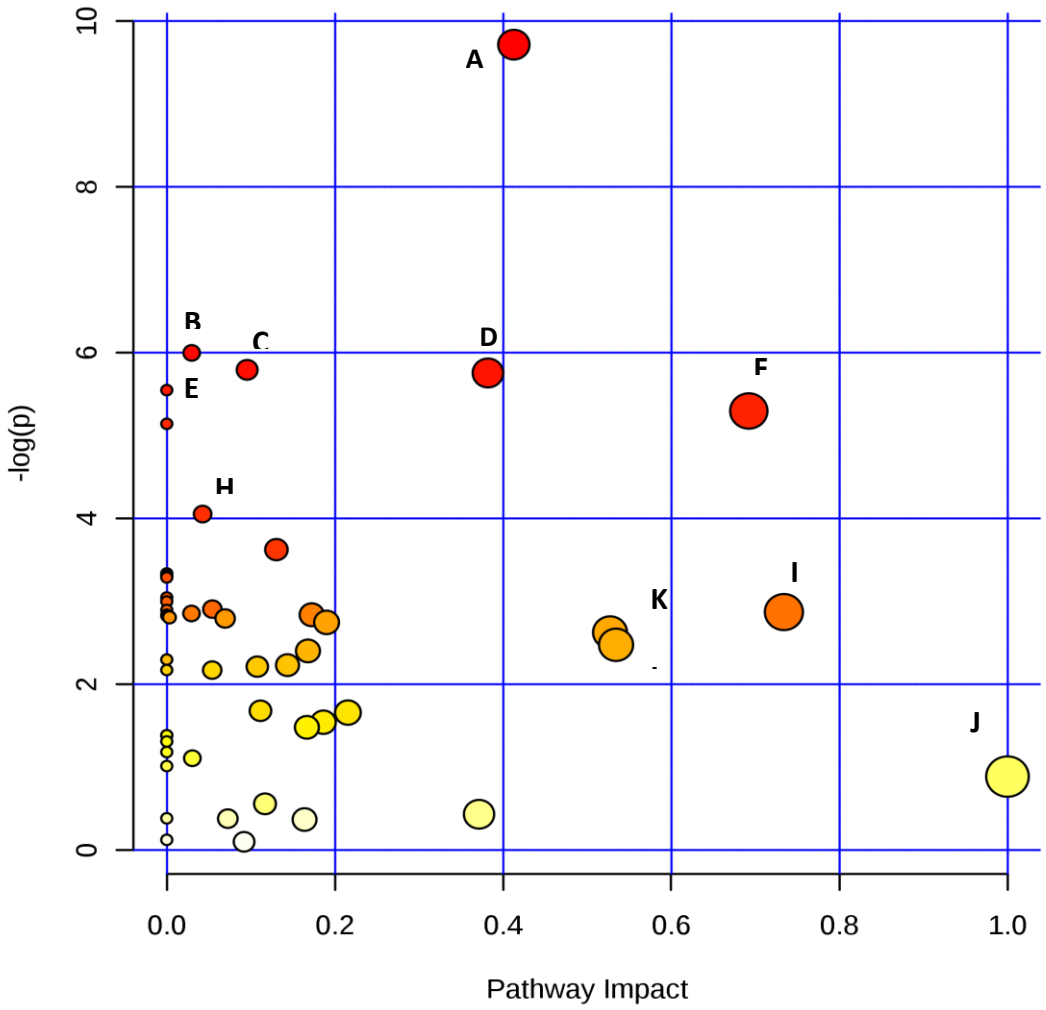

Figure 20- The metabolome analysis of the main pathways of the *E.coli* O26 cells when comparing control sample (A) and eBeam treated at 3 kGy sample (B).
A) Arginine and proline metabolism, B) Pentose and glucuronate interconversions, C) Amino sugar and nucleotide sugar metabolism, D) Starch and sucrose metabolism, E) C5-Branched dibasic acid metabolism, F) Beta-Alanine metabolism, G) Glycerolipid metabolism, H) Histidine metabolism, I) Alanine,aspartate and glutamate metabolism, J)Inositol phosphate metabolism, K)Glutathione metabolism, L) Glycine, serine and threonine metabolism.

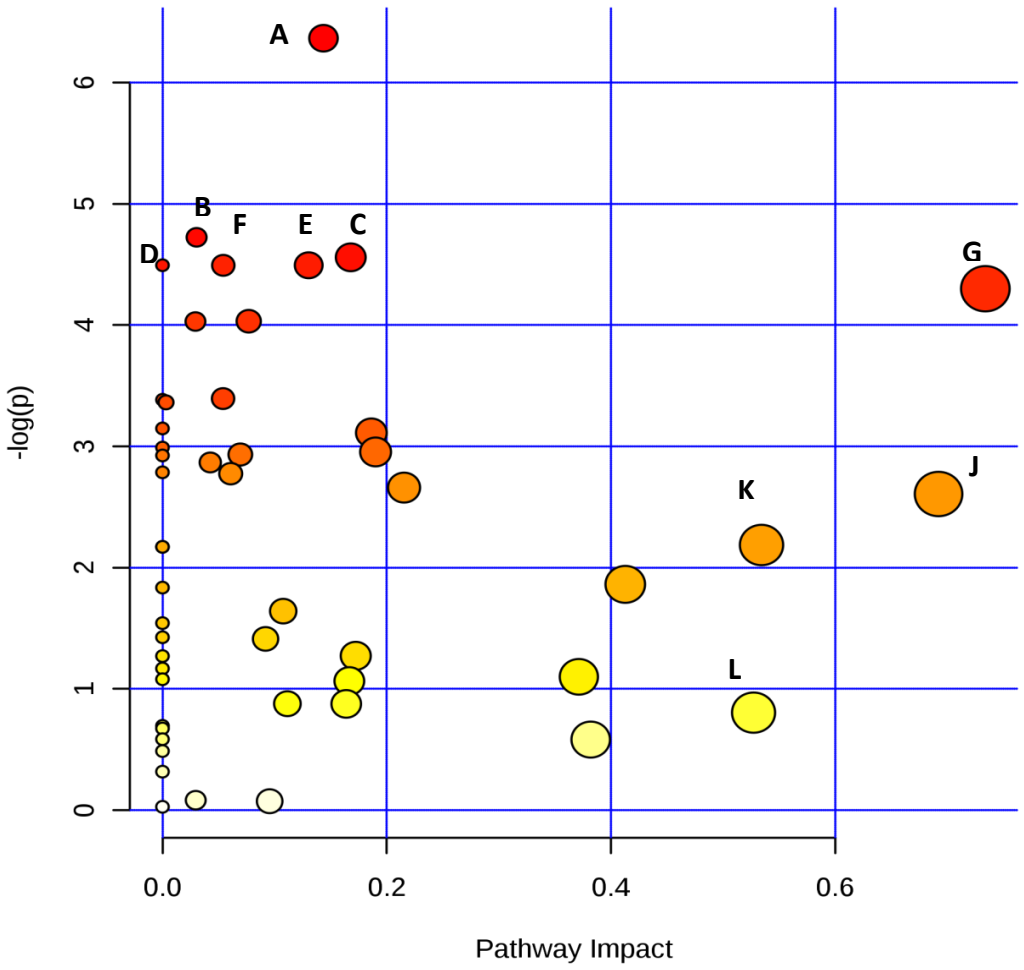

Figure 21- The metabolome analysis of the main pathways of the *E.coli* O26 cells when comparing eBeam treated at 3 kGy sample (B) with the post irradiated incubated sample for 24 hours at room temperature (C).
A) Nicotinate and nicotinamide metabolism, B) Glycerophospholipid metabolism, C) Pantothenate and CoA biosynthesis, D) Valine, leucine and isoleucine degradation, E) Aminoacyl-tRNA biosynthesis, F) Valine, leucine and isoleucine biosynthesis, G) Alanine, asparate and glutamate metabolism, H)Purine metabolism, I) Butanoate metabolism, J) beta-Alanine metabolism, K) Glycine, serine and threonine metabolism, L) Glutathione metabolism.

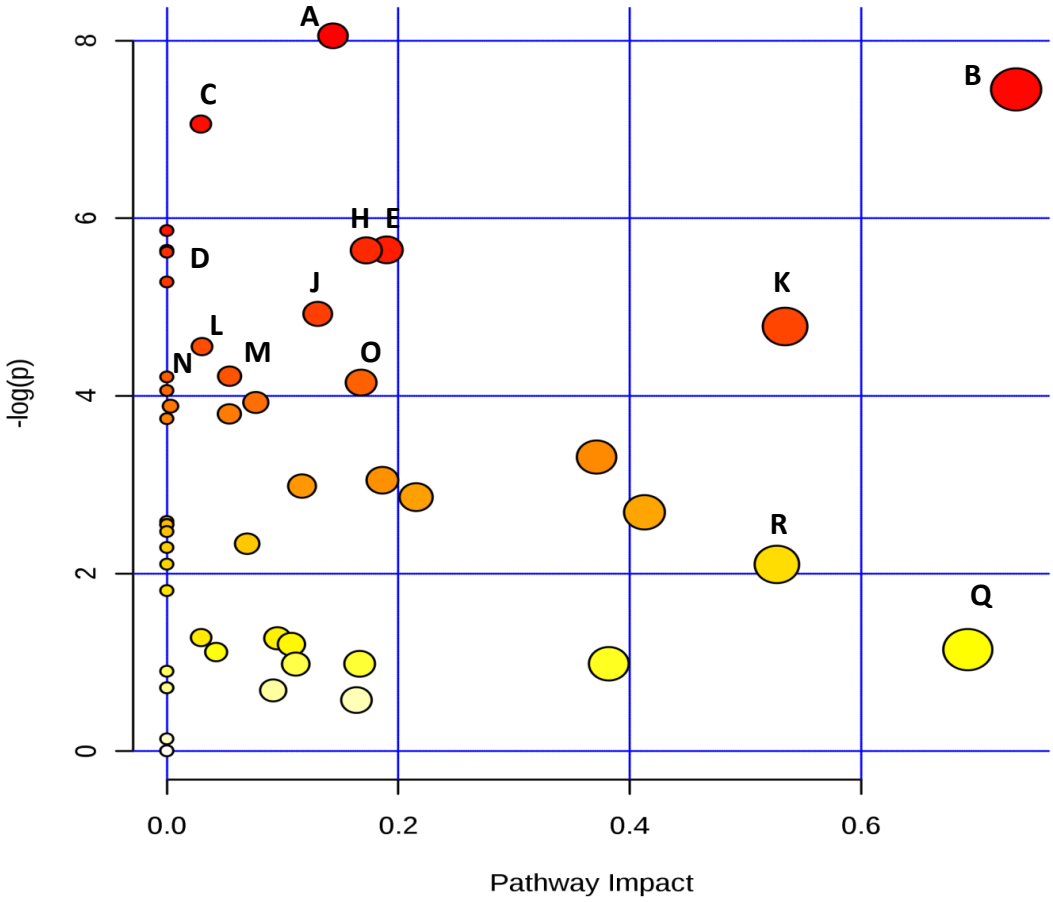

Figure 22- The metabolome analysis of the main pathways of *E.coli* O26 cells when comparing control sample (A) with the post irradiated incubated sample for 24 hours at room temperature (C).
A) Nicotinate and nicotinamide metabolism, B) Alanine, aspartate and glutamate metabolism, C) Butanoate metabolism, D)Beta-Alanine metabolism, D) Nitrogen metabolism, E) Cystein and methionine metabolism, F) Prophyrin and chlorophyll metabolism, G) D-Glutamine and D-glutamate metabolism, H) Cyanoamino acid metabolism, I) Tyrosine metabolism, J) Aminoacyl-tRNA biosynthesis
K) Glycine, serine and threonine metabolism, L) Glycerophospholipid metabolism, M)Valine, leucine and isoleucine biosynthesis, N) Valine, leucine and isoleucine degradation, O)Pantothenate and CoA biosynthesis, P)C5-Branched dibasic acid metabolism, Q) beta-Alanine metabolism, R) Glutathione metabolism.

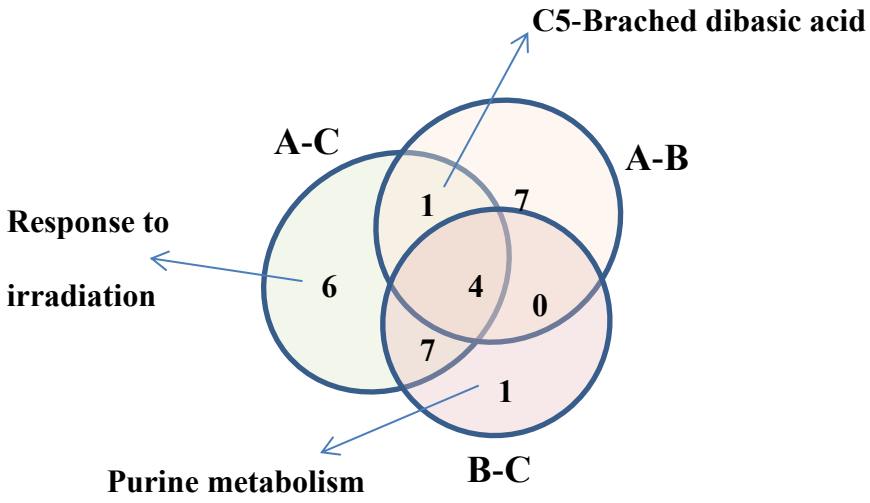

Figure 23- Venn diagram of the number of common primary metabolic pathways of *E.coli* O26:H11 serogroup strain TW01597 as a function of eBeam exposure (group B), no eBeam exposure (group A) and eBeam exposure followed by post-irradiation room temperature incubation (group C)

Table 19- The metabolic pathways that are differentially triggered in *E.coli* O26 when exposed to eBeam dose (3 kGy) and incubated post-irradiation.

Metabolic pathway	**Comparison of treatment groups**			**Function**
	A-C pValue (Impact)	**A-B pValue (Impact)**	**B-C p Value (Impact)**	
Nicotinate and nicotinamide metabolism	3.2E-04 (0.14)		1.7E-03 (0.14)	Metabolism of cofactors and vitamins
Alanine, aspartate and glutamate metabolism	5.8E-04 (0.73)*	0.05 (0.73)*	1.4E-02 (0.73)*	Amino acid metabolism
Butanoate metabolism	8.6E-04 (0.02)		1.8E-02 (0.02)	Carbohydrate metabolism
Nitrogen metabolism	2.8E-03 (0.00)			Energy metabolism
Cysteine and methionine metabolism	3.5E-03 (0.19)			Amino acid metabolism
Porphyrin and chlorophyll metabolism	3.6E-03 (0.00)			Metabolism of cofactors and vitamins

Table 19- Continued.

Metabolic pathway	Comparison of treatment groups			Function
	A-C pValue (Impact)	A-B pValue (Impact)	B-C p Value (Impact)	
D-Glutamine and D-glutamate metabolism	3.6E-03 (0.17)			Amino acid metabolism
Cyanoamino acid metabolism	3.6E-03 (0.00)			Amino acid metabolism
Tyrosine metabolism	5.1E-03 (0.00)			Amino acid metabolism
Aminoacyl-tRNA biosynthesis	7.3E-03 (0.13)		1.1E-02 (0.13)	Translation
Glycine, serine and threonine metabolism	8.4E-03 (0.53)*	0.08 (0.53)*	0.11 (0.53)*	Amino acid metabolism
Glycerophospholipid metabolism	1.1E-02 (0.00)		8.9E-03 (0.00)	Lipid metabolism
Valine, leucine and isoleucine biosynthesis	1.5E-02 (0.05)		1.1E-02 (0.05)	Amino acid metabolism
Valine, leucine and isoleucine degradation	1.5E-02 (0.00)		1.1E-02 (0.00)	Amino acid metabolism
Pantothenate and CoA biosynthesis	1.6E-02 (0.16)		1.0E-02 (0.16)	Metabolism of cofactors and vitamins
C5-Branched dibasic acid metabolism	1.7E-02 (0.00)	3.9E-03 (0.00)		Carbohydrate metabolism, pathogenicity
Arginine and proline metabolism		6.0E-05 (0.41)		Amino acid metabolism, stress response
Pentose and glucuronate interconversions		2.5E-03 (0.02)		Carbohydrate metabolism
Amino sugar and nucleotide sugar metabolism		3.0E-03 (0.09)		Carbohydrate metabolism
Starch and sucrose metabolism		3.2E-03 (0.38)		Carbohydrate metabolism
beta-Alanine metabolism	0.31 (0.69)*	5.0E-03 (0.69)*	0.07 (0.69)*	Amino acid metabolism
Glycerolipid metabolism		5.8E-03 (0.00)		Lipid metabolism
Histidine metabolism		1.7E-02 (0.04)		Amino acid metabolism
Alanine, aspartate and glutamate metabolism				Amino acid metabolism

Table 19- Continued.

Metabolic pathway	Comparison of treatment groups			Function
	A-C pValue (Impact)	A-B pValue (Impact)	A-C pValue (Impact)	
Purine metabolism	0.019 (0.07)		1.8E-02 (0.07)	Nucleic metabolism
Inositol phosphate		0.41 (1.00)*		Carbohydrate metabolism (signaling)
Glutathione metabolism	0.12 (0.52)*	0.07 (0.52)*	0.44 (0.52)*	Amino acid metabolism

A) Control sample B) eBeam treated with 3 kGy C) Post eBeam treated sample incubated for 24 hours at room temperature.

Discussion

Exposure of the *E.coli* O26:H11 cells to 3 kGy eBeam dose results in at least a 8-log reduction (from 10^8 CFU/mL to below detection limits). This can be considered a lethal dose since there were no surviving culturable cells. This level of reduction was expected since we had previously shown that an eBeam dose of 0.11 kGy can achieve a 90% (1-log) reduction of *E.coli* O26:H11 cells (Shayanfar et al., 2016). None of the irradiated cells were able to form colonies on TSA. However, despite this inability of the irradiated cells to multiply, viability staining (to determine cell membrane integrity) and transmission electron microscopy revealed that the cell membrane in the irradiated cells were still intact (Figure 17B, Figure 17C, Figure 18B). Studies from our laboratory and others have observed this earlier in irradiated cell (Hieke, 2015; Caillet et al., 2005). Similarly, studies from our laboratory and others have shown that eBeam inactivated cells are metabolically active even after exposure to lethal doses of eBeam irradiation

(Praveen, 2014; Caillet et al., 2005; Caillet et al., 2008). The unique profile of primary metabolites in the eBeam irradiated cells (group B) as compared to un-irradiated cells (group A) (Figures 19, 20, 21, 22) further supports our hypothesis that the irradiated cells metabolically active yet non-culturable" (MAyNC) (Jesudhasan et al., 2015; Praveen, 2014). *Escherichia coli* cells are known to respond to specific types of stress such as heat, oxidants, starvation and DNA damaging agents through expression of specific protein products that are required to resist that particular stress condition (Caillet et al., 2008; Caillet et al., 2005; Gustavsson et al., 2002). Increasing the dose of gamma irradiation to lethal doses significantly ($P \leq 0.05$) decreased the internal ATP concentration in *E.coli* O157 cells without significantly affecting the extracellular ATP concentration (Caillet et al., 2005). This indicated that protein translocation into the cells and phosphorylation of ADP to ATP respectively (Theg et al., 1988). The accumulation of specific metabolites in eBeam irradiated cells even after 24 hours of post irradiation incubation suggests that specific metabolic pathways are still operational in eBeam inactivated and cells during storage/incubation. Caillet et al. (2008) have reported that gamma irradiation of *Salmonella* Typhimurium and *Staphylocucus aureus* at 2·5 and 2·9 kGy followed by 24 h and 5 days of incubation still were expressing heat shot proteins respectively (Caillet et al., 2008). It is known that ionizing radiation induces DNA double-stranded breaks (DSBs), which are considered the most lethal kind of DNA damage (Liu et al., 2002; Krasin & Hutchinson, 1977; Sargentini & Smith, 1986; Hutchingon, 1985). However, the indication that metabolic pathways are still operational

24 hours after exposure to lethal doses of irradiation is intriguing because in this study, the cells were incubated in PBS and therefore had no access to external nutrients.

The metabolic pathways activated with eBeam irradiation in this study were mainly associated with DNA repair and virulence and few amino acids pathways (Table 19). DNA replication, repair, recombination, cellular transport, cell wall metabolism, and virulence-related gene expression have been earlier observed previously in cells exposed to ionizing radiation (Hieke, 2015; Liu et al., 2002; Sargentini & Smith, 1986). Our previous metabolomic studies have shown that exposure to inorganic acid stress (Chapter V) and organic acid stress (Chapter VI) resulted in the differential expression of pathways associated with cell membrane and acid resistance.

The four main pathways that are activated in all three experimental groups (Table 19) are namely:

- Alanine, aspartate, glutamate metabolism
- Glycine, serine and threonine metabolism
- Beta-Alanine metabolism
- Glutathione metabolism

All these pathways have high biological impact in *E.coli* cells (biological impact > 0.5) and are associated with amino acid metabolism (Table 19). As a result of stress (in this case ionizing radiation) different strategies are utilized by the bacterial cells to confront the stress (Weber et al., 2005). When cells are stressed various amino acids are accumulated in the stressed cells (Jozefczuk et al., 2010). The reason is for amino acid accumulation is either increased protein degradation to eliminate the abnormal proteins

formed during stress or increasing in the concentration of the amino acids required to synthesize new protective proteins (Jozefczuk et al., 2010; Mandelstam, 1963; Willetts, 1967). Beta-alanine is a substrate for pantothenic acid synthesis in microorganisms, which is subsequently transformed to coenzyme A (Williamson & Brown, 1979). Coenzyme A is the cofactor involved in the biosynthesis and breakdown of fatty acids, plyketides and nonribosomal peptides in bacterial cells (Brown, 1959).

In the earlier study (Chapter V) exposure of *E.coli* O26 to strawberries had triggered three of these four main pathways except for glutathione. Thus, it can be inferred that glutathione metabolism is specific to *E.coli* O26 cells stressed with ionizing radiation. Glutathione is known as an antioxidant in *E.coli* that by donating electrons avoids cell component damage caused by oxidative stress and free radicals (Carmel-Harel & Storz, 2000).

The C5-branched dibasic acid metabolism is the only pathway in common between the treatment groups A and group C and between group A and group B (Table 19). The C5-branched dibasic acid metabolism is a pathogen specific pathway (Barh & Kumar, 2009; Anishetty et al., 2005). In the C5-branched dibasic acid metabolic pathway, (R)-2-Methylmalate is transformed to citraconic acid, which increases upon irradiation in both groups B and C (Table 31). The accumulation of citraconic acid in the eBeam treated groups suggests the induction of virulence potential when exposed to lethal doses of eBeam irradiation. Citraconic acid also increased during post irradiation incubation (Figure 24).

When bacterial pathogenic cells are exposed to oxidative stress, their DNA structure is altered and the genes essential for survival are activated, while the genes that their products are not necessary in that particular stressing environment are turned off (Chowdhury et al., 1995). They used some *in vitro* studies not only to show how the protecting proteins expressed under different types of stress can endow the cells to survive but also they can serve as indicative of pathogenicity. Gamma irradiation of *E.coli* O157 with a lethal dose induced synthesis of DnaK, GroES and GroEL proteins right after irradiation in a dose dependent manner. These proteins, which are part of the heat shock protein (Hsps) sets are known to protect pathogenic bacteria against heat shock (Buchmeir and Heffron, 1990; Yura et al., 1993; Park et al., 2000). Heat shock proteins have also been reported in response to gamma irradiation at doses as low as 1.3 kGy (Caillet et al., 2008). The induction of these genes by non-thermal gamma rays confirmed their involvement in cellular response to stress generated by ionizing radiation too (Caillet et al., 2008; Gottesman et al., 1977).

Arginine and proline metabolism pathways were significantly ($p<0.01$) activated in the eBeam irradiated STEC cells as compared to the un-irradiated cells (Figure 4). This pathway which involves 8 enzymes is a key metabolic pathway in the synthesis of the amino acids proline and arginine from glutamate (Cunin et al., 1986). The pathway is multidirectional with arginine being metabolically interconvertible with a range of amino acids including proline and glutamate. Arginine metabolism is an important pathway for bacterial pathogenesis (Xiong et al., 2016). The hydroxyl radical produced during irradiation can oxidize the amino acid residues such as lysine, arginine, proline

and threonine are oxidized to carbonyl derivatives (Berlett & Stadman, 1997; Stadtman, 1992). Thus, it is not surprising that the arginine and proline metabolic pathways are significant in the eBeam irradiated cells.

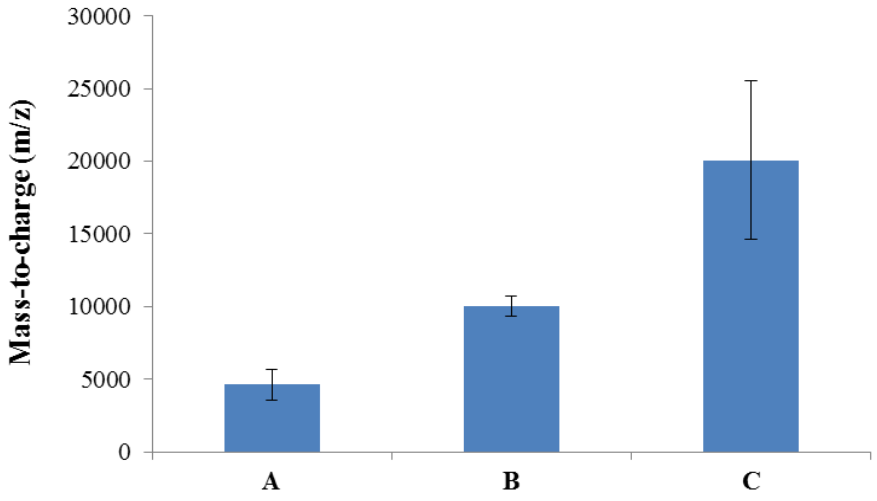

Figure 24- The concentration of citraconic acid in un-irradiated cells (group A), eBeam exposed cells (groupB) and eBeam exposed cells that were incubated for 24 hours at room temperature (group C).

Purine is the only metabolic pathway that is specific to the eBeam inactivated cells that are stored for 24 hours (B-C) (Figure 7). Purines and pyrimidine are key nucleic acids related nitrogenous bases as well as being critical to energy carrier molecules such as ATP, GTP, cyclic AMP, NADH and coenzyme A (Vogels & Drift, 1976). These pathways enable the stressed cells to obtain intensive energy required for cell adaptation to stress and DNA repair. There are a considerable number of genes such as *nudF*, *nude*, *pgm*, *purF*, *paoC*, etc. involved in purine metabolism pathway (KEGG, eco00230). The DNA repair mechanism in cells exposed to UV, X-ray or chemicals

involves incorporation of both purine and pyrimidine precursors (Cleaver, 1973). DNA glycosylase enzyme encoded by *fpg* gene in *E.coli* is known to use purines to repair the irradiated damaged DNA (Chetsanga & Lindahl, 1979; Boiteux et al., 1984).

Generally all the literature available on application of ionizing irradiation on pathogens is limited to inactivation of the pathogenic strains. There is no published information about the metabolome of the bacterial cells irradiated at lethal dose and hence the current results can provide a broad prospective of the metabolic activities of these cells.

Targeted analysis of the key metabolic pathways discussed in this study and tissue culturing can pave the way to better understanding of the effect of lethal dose of eBeam on the virulence trait of *E.coli* O26. Lethal eBeam dose creates bacterial cells that are no longer multiply but are still alive and metabolically active for an extended period of time after irradiation. Based on the fact that eBeam treatment of the pathogenic STEC induce the virulence pathways ensuring about the effective dosimetry and complete inactivation of pathogens by irradiation i is of essential importance. Considering the fact that irradiation can induce virulence pathways in pathogens, it is essential to ensure the cells are completely inactivated by eBeam or the pathogens that are treated with sub-lethal doses might be of public concern. Enhancing DNA repair capacity in bacterial cells is hypothesized to resuscitate the damaged cells (Pitonzo et al., 1999). Further studies are needed to investigate the possibility of resuscitation of the irradiated *E.coli* O26 cells at lethal dose of eBeam.

CHAPTER VIII

TRANSCRIPTOMIC RESPONSES OF *E.COLI* O26 TO LETHAL DOSE OF ELECTRON BEAM IN PHOSPHATE BUFFER AND STRAWBERRY MATRIX

Overview

Escherichia coli O26:H11 is the most common non-O157 Shiga toxin producing *Escherichia coli* (STEC) serogroup associated with food-borne illnesses. The objective of this study was to determine the differential expression of genes in *E.coli* O26:H11 as a result of exposure to a lethal dose (3.0 kGy) of electron beam irradiation in two different matrices, namely, phosphate buffered saline (PBS) (pH 7.5) and strawberry matrix (pH 3.6) A high titer of *E.coli* O26 cells (10^8 CFU/ml) was exposed to a lethal dose of eBeam (3 kGy) in PBS buffer and strawberry puree (prepared by blending strawberries). There were two experimental groups. In one experimental group the bacterial pathogen was suspended in PBS buffer while in the other, the bacterial pathogen was suspended in the puree. The samples were exposed to a lethal eBeam dose (3.0 kGy) and then incubated for 24 hours at room temperature (25°C). In parallel, un-irradiated samples were incubated under the same conditions for 24 hours at room temperature. The transcriptome of the bacterial pathogen in these two treatment groups were analyzed using RNA-Seq analysis prior to eBeam exposure, 24 hours after irradiation, as well as in the un-irradiated control samples. The RNA-Seq results indicate that almost 88% of the *E.coli* O26 genes were differently expressed (DE) after 24 hours incubation in PBS with or without eBeam treatment. The top twenty genes were all up-

regulated 30-fold. In the case of *E.coli* O26:H11 cells incubated in the strawberry matrix, only 774 genes corresponding to 12.71% of the 6089 genes in *E.coli* O26 were up-regulated. When the cells were exposed to the lethal eBeam dose and incubated for 24 hours, 5379 genes were upregulated in PBS buffer and 1166 genes were up-regulated in strawberry matrix. The majority of the genes regulated during post irradiation incubation in both PBS buffer and strawberry puree matrix were unique. The results indicate that after exposure to lethal eBeam doses, *E.coli* O26:H11 cells will not multiply. However, they are expressing its genes irrespective of whether they are suspended in PBS buffer or in a strawberry matrix. Therefore, such cells could be considered transcriptomically active yet, non culturable. These results support my previous studies which demonstrate that eBeam inactivated cells are metabolically active yet non-culturable.

Introduction

Electron beam (eBeam) processing is an FDA approved technology (FDA, 2015) with documented bacterial and viral pathogen inactivation in fresh produce and raw oyster (Shayanfar et al., 2016; Smith et al, 2016; Praveen et al., 2013; Espinosa et al., 2012). The mechanism of action of eBeam irradiation is based on double strand DNA breaks which prevent cell multiplication (Lung et al., 2015; Tahergorabi et al., 2012). However, the cellular integrity is not compromised (Hieke, 2015; Praveen, 2014; Secanella-Fandos et al., 2014; Magnani et al., 2009, Pollard et al., 1958). Electron beam irradiated cells have also been reported to retain their metabolic and transcriptional

activities presumably in order to repair their DNA (Hieke, 2015; Praveen, 2014) . Electron beam irradiated cells are therefore termed as Metabolically Active yet Not Culturable (MAyNC) state (Praveen, 2014; Magnani et al., 2009). It is of interest to identify the long-term fate of these MAyNC pathogenic cells. Studying the microbial transcriptome can shed light on the effect of varying inactivating agents as well as obtaining a deeper understanding of the metabolic state of eBeam inactivated cells Previously, the transcriptome analysis of *Salmonella* Typhimurium cells after exposure to lethal doses of eBeam (2 kGy) showed that the lethally irradiated cells focus on repairing DNA and membrane damage over a 24 hour period; while most of the long-term metabolic pathways such as citric acid cycle is downregulated to presumably direct more cellular resources toward DNA and membrane repair (Hieke, 2015). In the same study the maximum number of genes being up-regulated happened during the first 4 hours after irradiation and the amount of gene expression was influenced by incubation temperature and matrix. Incubation at 4°C in PBS for 4 hours and 24 hours resulted in about 5.6% and 0.21% upregulation of Salmonella genes respectively; while this amount was 12.7% and 10.7% for the same storage time in samples incubated in tryptic soy broth at 37°C (Hieke, 2015). The objective of the current study was to identify how the food matrix (i.e. strawberries) can influence the transcriptomic responses of *E.coli* O26:H11 after exposure to lethal doses of eBeam irradiation. Little is known about the global changes in gene expression right after ionizing radiation and the following DNA repair mechanism. To our best knowledge, there is no literature available on the transcriptomic response of any STEC when irradiated in different food matrices. It was

hypothesized that presence of sugars and the low pH (3.6) of strawberries would influence the transcriptomic responses of *E.coli* O26 as compared to cells that were irradiated in PBS and incubated, The specific objective of this study is to understand the expression patterns of the entire genome of E.coli O26:H11 in PBS buffer and strawberry matrix when exposed to lethal doses of eBeam radiation.

Materials and Methods

Microbial strain and culture conditions

Shiga toxin producing *E.coli* (STEC) O26:H11 (TW01597) was obtained from the USDA-ARS in College Station, Texas. The cells were grown in Trypticase Soy Borth (TSB) (Difco, USA) overnight in a shaking water bath at 37°C to the stationary phase. The *E.coli* O26 cells were collected by centrifugation (4000 × g; 5 min) and washed with PBS multiple times and re-suspended in PBS and the optical density (A260 nm) measured to achieve approximating 108 CFU/mL. The cell concentration was confirmed by plating.

Preparation of strawberry puree

A strawberry puree was used in order to simulate a realistic and homogenous growth environment for the bacteria. Fresh strawberries were purchased from a local farmer's market in College Station, Texas in order to reduce the chance of antimicrobials or other industrial sprays interfering in the experiment. Fresh strawberries were blended in a standard kitchen blender before being centrifuged at 10000 rpm to separate a

majority of the seeds. The pH of the strawberry puree was measured using a calibrated pH probe (calibrated with stock solutions of pH 4 and pH 7) (Corning, Corning, NY). The puree was kept at -80 °C in approximately 40 ml aliquots until needed for each experiment at which point it was thawed overnight before use.

Study design

Six different experimental treatment groups were included in this study (Figure 25).

PBS0: Un-irradiated *E.coli* O26 cells in PBS buffer (control)

PBS24EB: *E.coli* O26 cells eBeam (3 kGy) treated in PBS buffer and stored for 24 hour at room temperature (25 ºC).

PBS24: *E.coli* O26 cells in PBS buffer stored for 24 hour at room temperature (25 ºC).

STR0: Un-irradiated *E.coli* O26 cells in strawberry puree (control).

STR24EB: *E.coli* O26 cells eBeam (3 kGy) treated in strawberry puree and stored for 24 hour at room temperature (25 ºC).

STR24: *E.coli* O26 cells in strawberry puree and stored for 24 hour at room temperature (25 ºC).

Electron beam (eBeam) treatment

The eBeam treatment was performed using the 10 MeV, 18 kW linear accelerator at the National Center for Electron Beam Research at Texas A&M University. The actual received dose in the samples was verified using alanine (L-α-alanine pellet)

dosimeters (Harwell Dosimeters, Oxfordshire, UK). The target dose was 3.0 kGy; however, the actual absorbed dose was 3.1. All the experimental groups were prepared with three biological replicates.

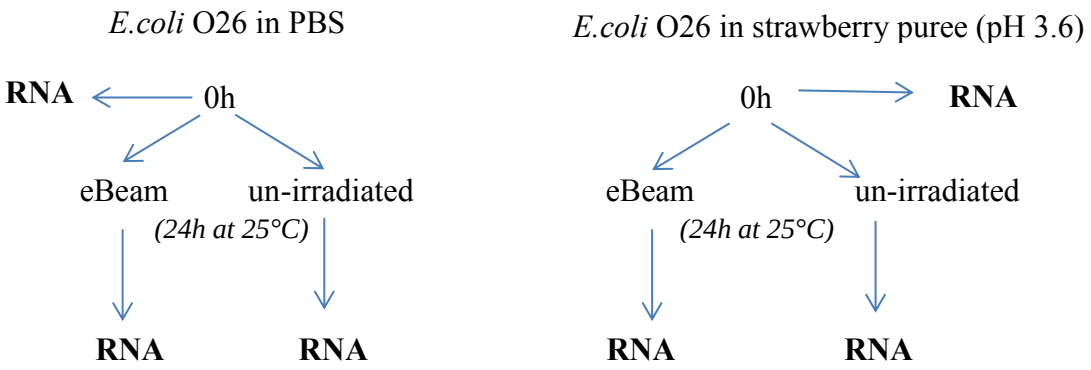

Figure 25- Study design.

Enumeration of non O157 strains after eBeam exposure

To estimate the numbers of surviving *E.coli* O26 cells each of the experimental groups they were serially diluted and plated on TSA plates and incubated for 24 h at 37 °C. The cells were enumerated and reported as CFU/ml.

RNA extraction

The RNeasy Mini Kit (Qiagen, Valencia, CA) was used in order to extract the RNA from each sample according to the manufacturer's instructions. RNA quantity was determined following RNA extraction using a NanoDrop ND-1000 spectrophotometer

(ThermoScientific, Wilmington, DE). The quality of the RNA extract was analyzed with the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer System (Santa Clara, CA).

RNA sequencing

The RNA extract samples were sequenced at the Genomics and Bioinformatics Services at Texas A&M AgriLife Research, Texas A&M University. Illumina HiSeq-2500 platform was used for sequencing 100-base-paried-end reads. All the quality parameters including sequence cluster identification, quality pre-filtering, base calling and uncertainty assessment were done in real time using Illumina's HCS 2.2.38 and RTA 1.18.61 software with default parameter settings.

Data analysis

The quality of the paired-end reads was checked using FastQC software (Andrews, 2010) to make sure all the samples had satisfactory Q30 scores. In the next step, the reads from each sample were independently mapped to the reference genome for *E.coli* O26 at NCBI (accession number AP010953) using Bowtie (Langmead et al., 2009).

Total RNA expression read counts were statistically analyzed using the edgeR package 3.3 (Zhou et al., 2014). Pairwise comparison of different treatment groups was conducted to identify differential gene expression using the Fisher’s exact test and the gene expression with the p value<0.01 were considered as differentially expressed (DE)

genes. Figure 26 illustrates all the pairwise comparisons that were performed in each matrix. Transcriptome analysis was kindly performed by Dr. Giri Athrey. Based on the log fold change (log FC), the top 12 expressed genes (with the highest log FC value) were selected as the most significantly expressed genes in each paired treatment groups. The selected genes in each paired comparison were graphed using Venn diagram (Oliveros, 2007-2015) to identify genes that overlapped in different groups. The identity of the DE genes were based on their function using NCBI (Accession JAST01000013) and *EcoGene* (Zhou & Rudd, 2013) respectively.

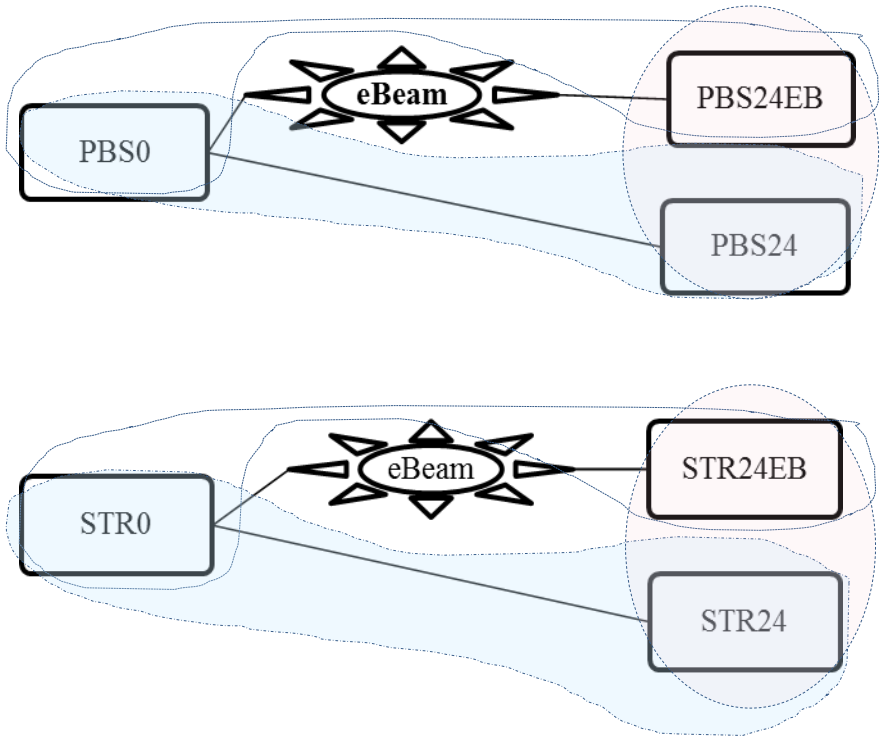

Figure 26- Illustration of the paired experimental groups in this study.

Results

Microbial culturability

The 3kGy dose used as the lethal irradiation dose was capable of inactivating an *E.coli* O26:H11 cell titer of 108 CFU/ml. The lethal dose was confirmed by plating the irradiated samples of TSA and incubation at 35C.

Transcriptome analysis

The paired comparison of different experimental groups indicated that all of the DE genes (88 %) in *E.coli* O26 were upregulated in PBS with or without eBeam (Figure 27, Table 20). There were only 12 DE genes when comparing the transcriptomes of the irradiated and un-irradiated samples after 24 hours incubation (Table 20). In strawberry matrix, 28% of the DE genes (1711 genes out of the total 6089 genes) are affected (Figure 27, Table 20). Electron beam exposure of *E.coli* cells in strawberry matrix resulted in the upregulation of 36% of the DE genes (2250 genes) (Table 20). When the strawberry matrix treatment groups were compared, the STR0-STR24EB and STR24-STR24EB comparisons with 29 and 23 genes respectively showed the highest number of downregulated genes (Table 20).

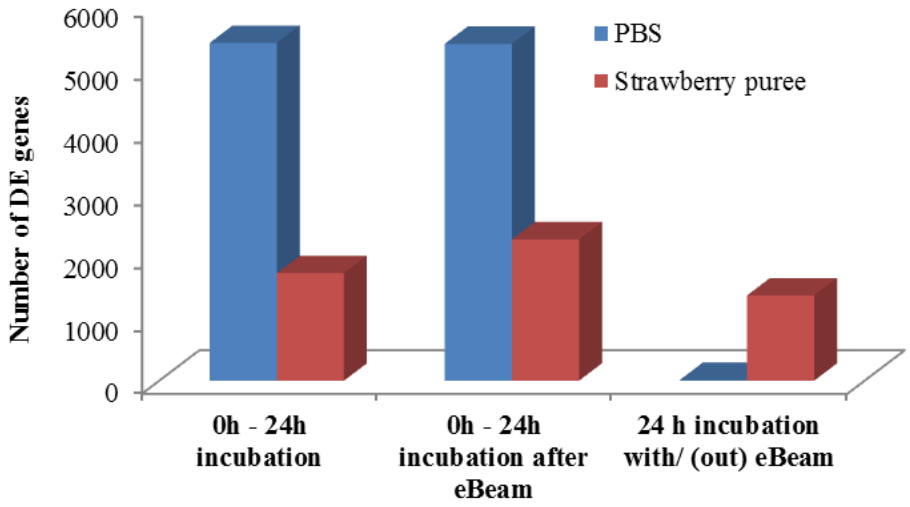

Figure 27- Comparison of time, media and treatment on differentially expressed (DE) gene counts.

Table 20- Overview of the number of differentially expressed (DE) genes in *E.coli* O26 for each pairwise comparison.

Comparison	**Total DE genes**	**% of total genes**	**Upregulated DE genes (log FC≥ 2)**	**% of total genes**	**Downregulated DE genes (log FC≤ -2**	**% of total genes**	**DE genes with log FC 2,-2**	**% of total genes**
PBS0-PBS24	5379	88.34	5379	88.34	0	0.00	0	0
PBS0-PBS24BE	5364	88.09	5364	88.09	0	0.00	0	0
PBS24-PBS24BE	12	0.20	4	0.07	3	0.05	5	0.08
STR0-STR24	1711	28.10	71	1.17	19	0.31	1621	26.62
STR0-STR24BE	2250	36.95	261	4.29	29	0.48	1960	32.19
STR24-STR24BE	1358	22.30	194	3.19	23	0.38	1141	18.74

FC: Fold change, DE: Differentially expressed.
PBS0: Un-irradiated *E.coli* O26 cells in PBS buffer (control)
PBS24EB: *E.coli* O26 cells eBeam (3 kGy) treated in PBS buffer and stored for 24 hour at room temperature (25 ºC).
PBS24: *E.coli* O26 cells in PBS buffer stored for 24 hour at room temperature (25 ºC).
STR0: Un-irradiated *E.coli* O26 cells in strawberry puree (control).
STR24EB: *E.coli* O26 cells eBeam (3 kGy) treated in strawberry puree and stored for 24 hour at room temperature (25 ºC).
STR24: *E.coli* O26 cells in strawberry puree and stored for 24 hour at room temperature (25 ºC).

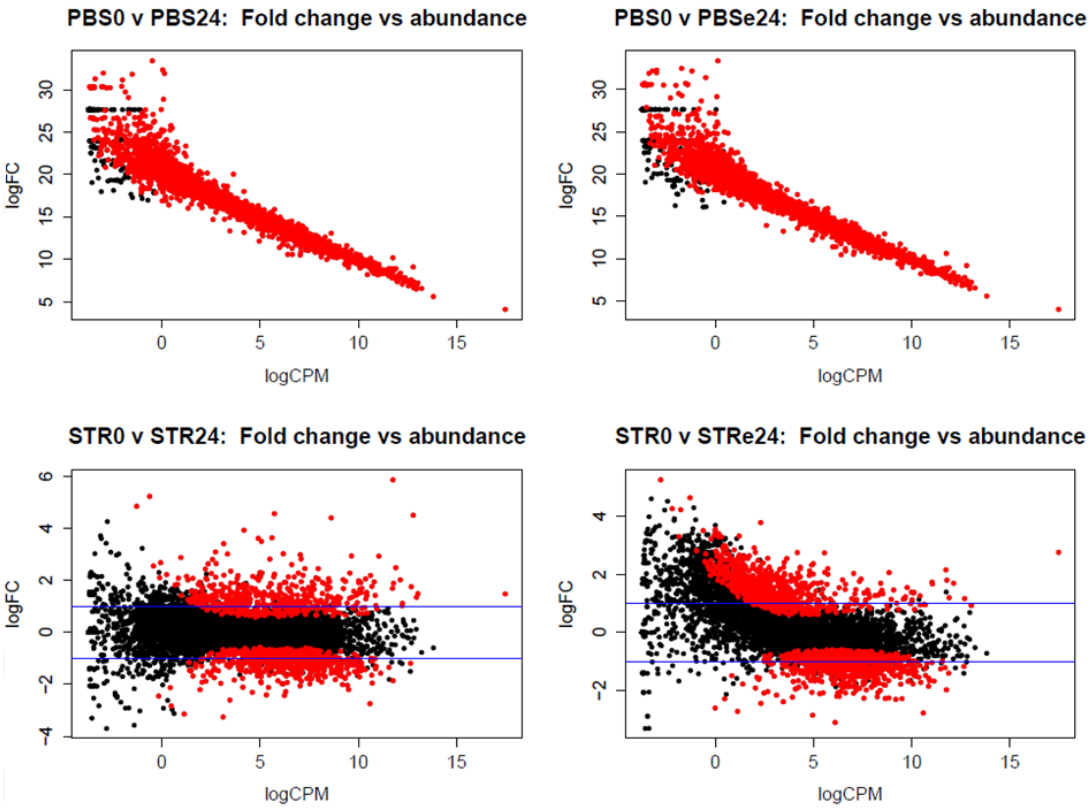

Figure 28- The log fold changes of gene expressed versus their abundance in different paired treatment groups.
BPS0) Un-irradiated *E.coli* O26 cells in PBS buffer (control), PBS24) *E.coli* O26 cells in PBS buffer stored for 24 hour at room temperature (25 ºC), PBS24EB) *E.coli* O26 cells eBeam (3 kGy) treated in PBS buffer and stored for 24 hour at room temperature (25 ºC), STR0) Un-irradiated *E.coli* O26 cells in strawberry puree (control), STR24) *E.coli* O26 cells in strawberry puree and stored for 24 hour at room temperature (25 ºC), STR24EB) *E.coli* O26 cells eBeam (3 kGy) treated in strawberry puree and stored for 24 hour at room temperature (25 ºC).

Table 21- Top 12 differentially expressed genes in paired comparison group of PBS0-PBS24.

Gene ID	Gene Name	Function	logFC	P Value
BU58_33725		hypothetical protein	33.428	1.46E-11
BU58_24215		hypothetical protein	32.346	1.48E-07
EG10274	*fabB*	Fatty acid biosynthesis	31.971	5.10E-20
BU58_27160		product: UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	31.932	2.35E-14
EG14128	*fadI*	Fatty acid degradation	31.834	8.67E-21
BU58_27885		hypothetical protein	31.299	5.50E-14
BU58_28055		hypothetical protein	31.169	1.83E-11
BU58_09505		hypothetical protein	30.412	6.65E-07
EG12103	*gapC*	Pseudogene reconstruction, Glyceraldehyde dehydrogenase	30.387	4.76E-09
BU58_32925		product: amino acid ABC transporter	30.384	4.60E-09
EG14159	*yfeR*	LysR-type transcriptional regulator	30.377	4.15E-09
BU58_32180		hypothetical protein	30.377	4.15E-09

BPS0) Un-irradiated *E.coli* O26 cells in PBS buffer (control), PBS24) *E.coli* O26 cells in PBS buffer stored for 24 hour at room temperature (25 ºC).
FC= Fold change

Table 22- Top 12 differentially expressed genes in paired comparison group of PBS0-PBS24EB.

Gene ID	Gene Name	Function	logFC	P Value
BU58_27160		product: UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	33.412	0.000
BU58_04120		Hypothetical protein	32.507	0.000
EG10274	*fabB*	Fatty acid biosynthesis	32.302	0.000
EG11742	*yddA*	Putative ABC transporter permease/ATPase	32.215	0.000
EG13504	*yeaR*	Nitrate-induced protein, function unknown;	32.200	0.000
BU58_32925		product: amino acid ABC transporter	32.003	0.000
EG13353	*abgR*	Aminobenzoyl-glutamate (Predicted regulator of the abgABT operon)	31.934	0.000
BU58_33725		Hypothetical protein	31.421	0.000
BU58_29670		Hypothetical protein	30.781	0.000
EG14128	*fadI*	Fatty acid degradation	30.736	0.000
BU58_24995		product: PTS system galactitol-specific transporter	30.613	0.000
BU58_34420		transposes	30.613	0.000

BPS0) Un-irradiated *E.coli* O26 cells in PBS buffer (control), PBS24EB) *E.coli* O26 cells eBeam (3 kGy) treated in PBS buffer and stored for 24 hour at room temperature (25 ºC).
FC= Fold change.

Table 23- Top 12 differentially expressed genes in paired comparison group of PBS24-PBS24EB.

Gene ID	Gene Name	Function	logFC	P Value
BU58_24230		Hypothetical protein	5.614	0.005
EG11794	*rlmM*	rRNA Large-subunit Methylation	4.103	0.003
BU58_26640		Hypothetical protein	2.763	0.008
EG40002	*insB1*	IS1 transposase B(Insertion Sequence)	2.010	0.003
EG13162	*ybeM'*	pseudogene reconstruction, putative CN hydrolase	1.904	0.010
EG10737	*pinE*	DNA-invertase, site-specific recombination	1.432	0.005
EG11740	*nfrA*	Bacteriophage N4 adsorption protein A, outer membrane protein	-0.894	0.010
EG13907	*puuP*	Putrescine importer	-1.117	0.003
EG40004	*insD1*	IS2 transposase B	-1.904	0.001
BU58_05865		Hypothetical protein	-2.730	0.001
BU58_29580		Hypothetical protein	-5.925	0.007
EG11511	*mog*	Molybdochelatase incorporating molybdenum into molybdopterin; chlorate resistance	-6.004	0.003

BPS0) PBS24) *E.coli* O26 cells in PBS buffer stored for 24 hour at room temperature (25 ºC), PBS24EB) *E.coli* O26 cells eBeam (3 kGy) treated in PBS buffer and stored for 24 hour at room temperature (25 ºC).
FC= Fold Change.

Table 24- Top 12 differentially expressed genes in paired comparison group of STR0 - STRS24.

Gene ID	Gene Name	Function	logFC	P Value
EG10351	*fucO*	Fucose (L-1,2-Propanediol oxidoreductase)	4.494	4.15E-29
EG13226	*ftsK*	Filamentation, temperature sensitive (DNA translocase at septal ring sorting daughter chromsomes)	4.393	4.25E-21
EG10614	*lpxM*	Lipid A expression/biosynthesis	3.921	1.48E-21
EG13570	*wcaB*	Predicted colanic acid biosynthesis acetyltransferase	3.606	1E-29
EG11324	*ubiH*	Ubiquinone	2.815	1.52E-28
BU58_15955		Hypothetical protein	2.811	4.13E-24
EG10709	*pheS*	Ubiquinone (2-octaprenyl-6-methoxyphenol hydroxylase; produces 2-octaprenyl-6-methoxy-1,4-benzoquinone)	2.599	2.51E-33
BU58_01625	*pspB*	DNA-binding transcriptional regulator	2.273	6.93E-23
BU58_20675		Product: phospho-2-dehydro-3-deoxyheptonate aldolase	2.127	4.05E-21
EG12182	*yajG*	Probable lipoprotein, function unknown; Cys conserved	-1.967	5.39E-22
EG10194	*cysN*	Cysteine	-2.307	1.06E-20
BU58_26475		Hypothetical protein	-3.254	1.19E-24

STR0) Un-irradiated *E.coli* O26 cells in strawberry puree (control), STR24) *E.coli* O26 cells in strawberry puree and stored for 24 hour at room temperature (25 ºC).
FC= Fold change.

Table 25- Top 12 differentially expressed genes in paired comparison group of STR0-STR24EB.

Gene ID	Gene Name	Function	logFC	P Value
BU58_01140		Hypothetical protein	2.728	0.000
EG11462	*yigE*	DUF2233 family protein, function unknown	2.358	0.000
EG11628	*artJ*	Arginine transport, Arginine ABC transporter periplasmic binding protein	2.190	0.000
EG11625	*artI*	Arginine transport, ligand unknown	2.112	0.000
EG13427	*rlmC*	rRNA Large-subunit Methylation	2.060	0.000
BU58_24750		Product: two-component response-regulatory protein YehT	-1.743	0.000
EG12945	*yhhY*	Aminoacyl nucleotide detoxifying acetyltransferase	-1.849	0.000
EG14419	*ybfQ*	Pseudogene, H repeat-associated protein, RhsC-linked; putative defective transposase	-1.923	0.000
BU58_22145		Hypothetical protein	-2.212	0.000
BU58_28155		Transcriptional regulator	-2.260	0.000
EG10973	*srlQ*	Sorbitol, D-arabinose 5-phosphate isomerase	-2.270	0.000
EG12182	*yajG*	Probable lipoprotein, function unknown; Cys conserved	-3.092	0.000

STR0) Un-irradiated *E.coli* O26 cells in strawberry puree (control), STR24EB) *E.coli* O26 cells eBeam (3 kGy) treated in strawberry puree and stored for 24 hour at room temperature (25 ºC).
FC= Fold change.

Table 26- Top 12 differentially expressed genes in paired comparison group of STR24-STR24EB.

Gene ID	Gene Name	Function	logFC	P Value
EG10351	*fucO*	Fucose (L-1,2-Propanediol oxidoreductase)	-5.51	4E-39
EG13226	*ftsK*	Filamentation, temperature sensitive (DNA translocase at septal ring sorting daughter chromsomes)	-5.49	5E-29
BU58_36485		Hypothetical protein	-4.95	8E-21
BU58_11740		Hypothetical protein	-3.93	5E-49
BU58_15955		Hypothetical protein	-2.71	1E-22
EG13211	*yfjY*	Hypothetical protein	-2.39	4E-16
EG10637	*nanA*	N-Acetylneuraminate lyase (aldolase)	-1.94	4E-18
BU58_24345		Hypothetical protein	-1.7	1E-17
BU58_34390		Hypothetical protein	-1.64	1E-13
EG11736	*gmhB (gmbC; gmbX; gmhX; wcbN)*	Heptose 1,7-bisphosphate phosphatase; LPS biosynthesis	-1.63	1E-16
EG11534	*ibpA*	Chaperone, heat-inducible protein of HSP20 family	-1.59	4E-15
EG14228	*yqiG*	Pseudogene reconstruction, FimD family, interrupted by IS2I; fimbrial export usher protein family	-1.47	8E-14

STR24) *E.coli* O26 cells in strawberry puree and stored for 24 hour at room temperature (25 ºC), STR24EB) *E.coli* O26 cells eBeam (3 kGy) treated in strawberry puree and stored for 24 hour at room temperature (25 ºC).
FC = Fold change.

There were top 12 DE genes (P<0.01) that were selected in each paired comparison groups accordingly to the analysis plan (Figure 26) and tabulated in Tables 21- 27. Among the top 12 selected DE genes in PBS0-PBS24 and PBS0-PBS24BE paired comparisons, there were 5 common genes encoding fatty acid biosynthesis, fatty acid degradation, UDP-3-*O*-(3 hydroxymyristoyl)glucosamine *N*-acyltransferase and amino acid ABC transporter (Table 21, Table 22). Among the 12 genes selected in PBS24-PBS24BE group, 2 known genes namely, *rlmM*, *insB1* were upregulated; while only 1 known gene, *mog* was downregulated (Table 23). Most of the DE genes had unknown function.

In the group where cells were incubated for 24 hours in strawberries among the top 12 selected DE genes there are 9 genes that were upregulated, and 2 genes are downregulated (Table 24). The genes *fucO, ftsK, lpxM, wcaB, ubiH, pheS* and *pspB* were up-regulated and genes, *cysN* and *yaiG* were down-regulated (Table 24). In the paired comparison of STR0 and STR24EB the main upregulated genes were *yigE, artJ, artI, rlmC* and among the known downregulated genes *srlQ* and *yajG* were identified. Gene *yajG* was also downregulated in paired comparison of STR0 and STR24BE (Table 25).

In the third paired comparison group of strawberry puree (STR24-STR24EB) the top selected genes were mainly downregulated. Although the genes *fucO*, *ftsK* were downregulated in STR24-STR24EB, they were upregulated in STR0-STR24 paired group (Table 24, Table 26).

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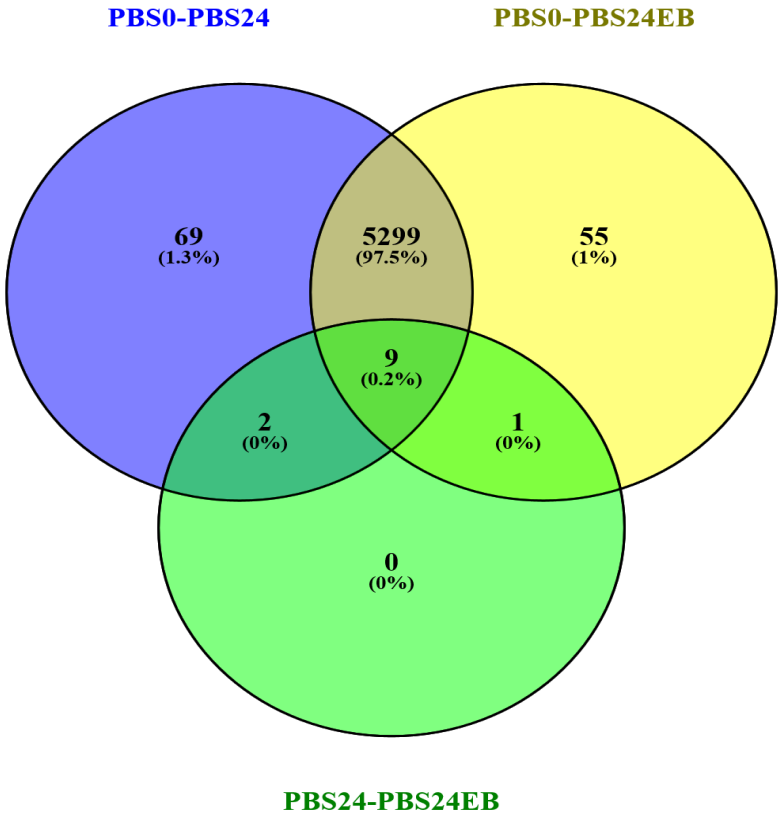

Figure 29- The Venn diagram of the top 21 differentially expressed genes in the three different paired treatment groups in PBS buffer matrix.
BPS0) Un-irradiated *E.coli* O26 cells in PBS buffer (control), PBS24) *E.coli* O26 cells in PBS buffer stored for 24 hour at room temperature (25 ºC), PBS24EB) *E.coli* O26 cells eBeam (3 kGy) treated in PBS buffer and stored for 24 hour at room temperature (25 ºC).

Figure 29 shows the DE genes that were common among the different paired comparisons. The PBS0-PBS24 and PBS0-PBS24EB comparisons had about 97% similar DE genes. There was no single gene that was unique to the PBS24-PBS24EB group. The only gene that was common between the two eBeam treated groups was

BU58_24230. However, the genes affected in strawberry puree matrix were more specific and fewer were common in different experimental groups (Figure 30).

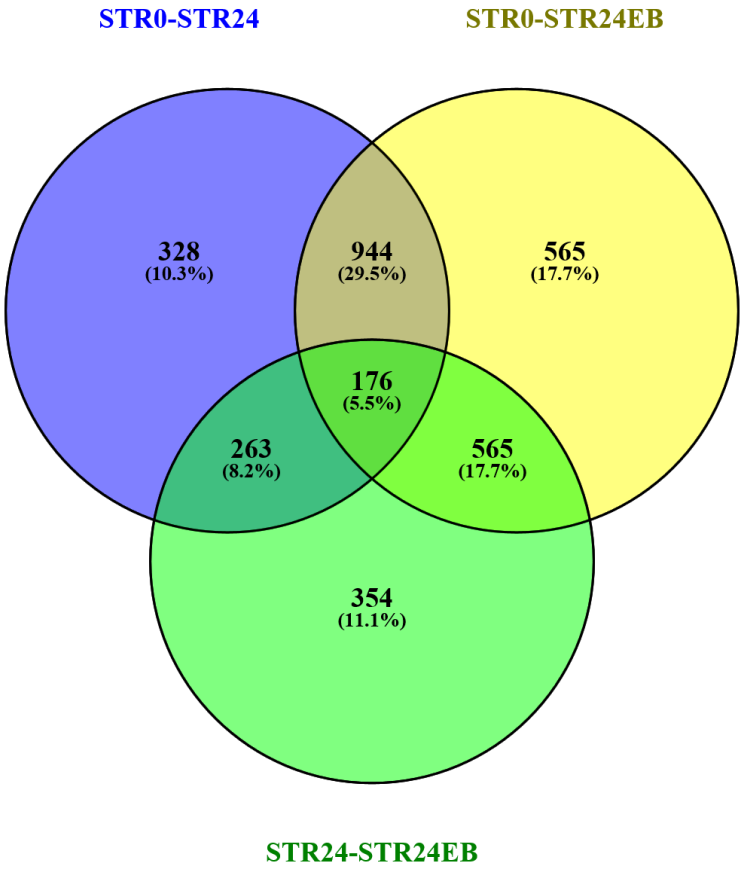

Figure 30- The Venn diagram of the top 21 differentially expressed genes in different paired treatment groups in strawberry puree matrix.
STR0) Un-irradiated *E.coli* O26 cells in strawberry puree (control), STR24) *E.coli* O26 cells in strawberry puree and stored for 24 hour at room temperature (25 ºC), STR24EB) *E.coli* O26 cells eBeam (3 kGy) treated in strawberry puree and stored for 24 hour at room temperature (25 ºC).

Discussion

The results indicate that *E.coli* O26 cells retained their transcriptional activity even though they were not capable of multiplying. These results are in agreement with the earlier experiments (CHAPTER VII) and with other studies where lethal doses of gamma and eBeam inhibited replication ability in *Brucella melitenis* (Magnani et al., 2009) and *Salmonella* spp. (Hieke, 2015) but did not affect their transcriptional activity. It is assumed the sheer number of DSBs might prevent *E.coli* O26 cells to reassemble its genome to maintain cellular multiplication ability.

A number of regulatory networks of *E.coli* respond to specific cellular stresses including heat, oxidants, starvation and DNA damage through expression of protein products that are required to resist that particular stress condition (Gustavsson et al., 2002). The result of pairwise transcriptome analysis in all the treatment groups supports the fact that the specific treatment, the matrix and the incubation all affect the transcriptome of *E.coli* O26 (Figure 27, Figure 28). There are 6089 genes in *E.coli* O26 (Figure 27), from which all the DE genes are upregulated in PBS after 24 hours with (5364 genes) or without eBeam (5379 genes) treatment (Figure 26, Table 20). Previous studies have reported the up-regulation of specific genes in viable but non-culturable (VBNC) *E.coli* O157 to PBS buffer (Liu et al., 2010; Liu et al, 2009). Incubation of *E.coli* O157:H7 in PBS buffer for 19 months induced the Stx1 and Stx2 genes more than freshly cultured cells (Liu et al., 2010). However, there is conflicting observations that in PBS buffer *E.coli* O157 cells can enter VBNC in PBS buffer at 4ºC but not at 25ºC (Rigsbee et al., 1997; Zhao and Mattews, 2000). The current study was performed at

room temperature (25ºC) in PBS and in the span of 24 hours; hence, the cells might be starved and, therefore, multiple genes are attempting to keep the cells viable. Another explanation for upregulation of various genes in *E.coli* O26 can be the fact that the cells are at their stationary phase. Upon entering into stationary phase, *rpoS* gene encodes sigma factor (σ) as the central regulator of gene expression in stationary phase (Tanaka et al., 1993). Upregulation of sigma factor will prolong *E.coli* survival and increase resistance to a variety of stress conditions including acid stress and radiation (Werber et al., 2005; Small et al., 1994).

Although the paired groups of PBS0-PBS24 and PBS0-PBS24EB have undergone different types of stress they share about 97.5% of their DE genes (Figure 29). Molecular responses to different types of stress share many common genes but have different metabolic outcomes (Amundson et al., 2001; Allen et al., 2008). Among the top 12 DE selected genes in the mentioned groups 4 genes were in common (Table 21, Table 22). UDP-3-*O*-(3-hydroxymyristoyl) glucosamine *N*-acyltransferase catalyzes lipid A biosynthesis; required for growth in Gram-negative bacteria (Bartling & Raetz, 2009). Lipid A is in the hydrophobic part of lipopolysaccharide (LPS) that constitutes the outer membrane of most of the Gram-negative bacteria (Bartling & Raetz, 2008). The biosynthesis of fatty acids supports bacterial cell growth and viability (Liu et al., 2009)

ABCs transporters are ubiquitous membrane proteins involved in importing the essential nutrients into the cell and exporting the toxic molecules through interactions with membrane (Davidson et al., 2008). They also carry ATP molecules for other

activities such as DNA repair and gene expression regulations (Goosen & Moolenaar, 2001; Davidson et al., 2008). Therefore, upregulation of ABC transporter can suggest collecting energy for extensive gene expression regulations in both 24 incubated cells and post irradiated incubated cells in PBS buffer. The results of transcriptome analysis indicate that the eBeam treated samples in PBS are not much different from the unirradiated cells after 24 hours of incubation (Figure 27, Figure 30); however, based on their primary metabolite concentrations these two groups are quite different (CHAPTER VII) . These results agree with an earlier study in our laboratory where the majority of the eBeam-induced gene expression happened within 4 hours after incubation and decreased after 24 hours (Hieke, 2015). Therefore, what is noticed in these two paired groups is mainly the effect of incubation time and starvation of the cells in PBS.

The DE genes identified in the group PBS24-PBS24BE comparisons was more specific compared to the other two paired groups of PBS0-PBS24 and PBS0-PBS24BE (Figure 29). The transcriptome in both PBS24 and PBS24EB groups was analyzed in PBS and after 24 hours, thus the only reason for transcriptome difference is the eBeam treatment. Among the identified upregulated genes in PBS24-PBS24EB, *rlmM* and *insB1* are involved in ribosomal methylation and insertion sequence (IS1) respectively (Table 23). Among the downregulated genes the only known gene is *mog*, which is involved in incorporating molybdenum into molybdopterin (Table 23). Upregulation of *rlmM* regulates methylation of the 23S ribosomal RNA (rRNA) quinine (Toh et al., 2008).

There is a body of literature on the association between IS and many virulence functions in various pathogens including *E.coli* (Collins & Gutman, 1992; Garcia et al., 1994; Hu & Lee, 1988; So et al., 1979). There is also some literature on the role of IS in accessory functions in bacteria including resistance (Mahillon & Chandler, 1998). The role of IS1 as an upregulated gene in pathogenicity of cells support the activation of C5-Branched dibasic acid metabolism pathway in eBeam treated *E.coli* O26 cells that are involved in pathogenicity (CHAPTER VII).

The results of transcriptome analysis of *E.coli* O26 cells in strawberry puree identified more specific genes being affected in all paired groups (Figure 27, Figure 28, Figure 30, Table 20). These results support the fact that matrix has a pronounced effect on the transcriptome. Auto-inducer molecules (AI-2) in *E.coli* cells that induce various virulence factors including motility, attachment and subsequent infection process are affected by multiple environmental factors such as nutrients, pH and signaling molecules as well as quorum sensing (QS) (Vikram et al., 2012; Soni et al., 2008). Exposure of bacterial cells to different media maintained different levels of Stx production in *E.coli* O157:H7 (Liu et al., 2010). The comparison of DE genes in all the paired groups of *E.coli* cells in strawberry puree indicated that the highest number of genes are DE in STR0-STR24EB with about twice as many as genes as the other two paired groups (Table 20), maybe because of the fact the both time of acid exposure and eBeam treatment are two stressors that the cells experience.

Among the top up-regulated genes in cells incubated in strawberries without eBeam treatment *fucO* gene catalyzes the utilization of L-fucose as carbon and energy

source in *E.coli* (Chen et al., 1987). Fucose is an isomer of 6-deoxy-D-fructose in strawberries (Pisarnitskii et al., 1992) and is metabolized in both aerobic and in an anaerobic condition (Chen et al., 1983). The overexpression of *ftsK* is reported as a part of the SOS response to increase resistance to DNA damage (Want & Lutkenhaus, 1998; Diez et al., 1997). That suggests incubation of cells in strawberry puree might have triggered some stress responses in *E.coli* O26 which supports the findings in CHAPTER VI, where incubation of *E.coli* O26 cells activated a variety of metabolic pathways. Both *fucO* and *ftsK* are downregulated in the paired comparison group of STR24 and STR24EB indicating both growth and fucose metabolism is impaired in eBeam treated cells suggesting cells have lost their ability to grow.

The role of *wcaB* gene (formerly called *cpsB*) is associated with the synthesis of the capsular exopolysaccharide colanic acid (Sledjeski & Gottesman, 1996). Colanic acid is a mucoid exoplysaccharide synthesized by various enteric bacterial cells including *E.coli* (Garegg et al., 1971). Specific factors that affect lipopolysaccharide synthesis and structure subsequently cause changes in the outer membrane increase colonic acid synthesis (Sledjeski & Gottesman, 1996). In another study upregulation of *wcaB* resulted in increase in colonic acid and protected *E.coli* from desiccation (Ophir et al., 1994), which suggests the protecting effect of colonic acid on *E.coli* and is upregulated by high osmolality (Prigent-Combaret et al., 2000). Regulation of *wcaB* is also considered cell-to-cell signaling in environment of high osmolality (Prigent-Combaret et al., 2000). The high concentration of sugar and viscosity of strawberry puree can explain the upregulation of *wcaB* in cells incubated in strawberries.

Ubiquionin (coenzyme Q) plays an essential role in electron transport in *E.coli* and is encoded by ubiH (Kwon et al., 2000). The gene *pheS* encodes the small subunit of phenylalanyl-tRNA synthetase (Fayat & Mayaux, 1983), which is known as one of the most complex enxymes of the aminoacyl-tRNA synthetase family in bacteria (Mosyak et al 1995) but is also involved in encoding ubiquinone (Table 24).

The genes *cysN* and *yajG* are downregulated in incubated cells in strawberries (Table 24).*CysN* encodes sulfate adenylyltrasferase subunit 1 which is involved in step 1 of the pathway that synthesizes sulfite from sulfate. This pathway is a part of hydrogen sulfide biosynthesis which is itself part of Sulfur metabolism (Leyh et al., 1988); while *yajG* encodes a hypothetical lipoprotein that supports peptidoglycan in *E.coli* (Boudet et al., 2007).

In the paired comparison of STR0 and STR24EB the main upregulated genes are *yigE, artJ, artI, rlmC* and among the known downregulated genes *srlQ* and *yajG* can be mentioned. Gene *yajG* was also downregulated in paired comparison of STR0 and STR24. Among the upregulated genes in STR0-STR24E, *yigE* function is still unknown (Table 25); while *artJ* gene is known as a virulence gene in various isolates of *E.coli* from human and animal sources (Manges et al., 2015) and is recently discovered as new members of the ArgR regulton. ArgR is known to inhibit the transcription of several biosynthesis and transport genes (Caldara et al., 2006). ArtJ and ArtI are proteins encoded by *artJ* and *artI* that act as binding proteins for polar amino acids. ArtJ and ArtI are involved in binding L-arginine and result in stimulated L-arginine uptake by the bacteria. The ArtJ protein encoded by *artJ* is strongly reduced in bacteria grown with

excessive amount of arginine (Caldara et al., 2007). ArtJ protein is a periplasmic binding protein to bind proteins for polar basic amino acids (Wissenbach et al., 1995).

Upregulation of *artJ* and *artI* here suggests the fact the the cells are trying to uptake arginine. However, the substrate for ArtI is still unknown (Wissenbach et al., 1995). In fact various amino acids have diverse physiological functions in *E.coli* and serve as the substrate for catabolism and anabolism of agents needed for osmoregulation and pH homeostasis (Furlong, 1987). Upregulation of arginine can be a mechanism to resist the acid stress in arginine-dependent systems (Richard & Foster, 2004). This is also in accordance with the metabolomics results achieved in CHAPTERS VI, where incubating *E.coli* O26 cells to strawberry puree activated arginine, glutamine metabolic pathways (Table 18).

The pH value in strawberries is about 3.6 and it is known when microorganisms are exposed pH values higher or lower than that of the cytoplasm (pH 7.6), their protective responses are induced to maintain internal pH homeostasis and to promote cell survival for later exposure to more extreme pH conditions (Castanie-Cornet & Foster, 2001; Small et al., 1994). The main metabolic pathway reported to be triggered in acid stressed bacterial cells is the Sigma factor (RpoS), which is the central regulator for a variety of stress conditions (Weber et al., 2005; Small et al., 1994). Despite the low pH value in strawberry puree (pH 3.6) the cells indicate “acid resistance” (Small et al., 1994). The acid resistance and radiation resistance in bacterial cells is fostered by sigma factor induced at stationary phase through cross-protection effect (Small et al.,

1994; Lange & Hengge-Aronis, 1991). Therefore, it is assumed the common genes expressed in paired experimental groups to be mainly associated with Sigma factor.

As mentioned earlier RNA molecules are subjected to post-transcriptional modifications including methylation which plays an essential role in protein synthesis. Each of the modifications requires its own specific methyltransferase enzyme. Upregulation of *rlmC* (previsouly known as *rumB*) also methylates 23S rRNA (Auxilien et al., 2011).

Srl gene is known for mediating metabolism of glucitol (i.e. sorbitol) (Csonka & Clark, 1979).YajG protein is also annotated as hypothetical lipoprotein that supports peptidoglycan in *E.coli* (Boudet et al., 2007). Downregulation of both Srl and YajG suggests that cells are failing to repair their cell membrane and metabolize glucitol for the required energy.

In essence since PBS buffer does not supply any nutrients the fewer number of genes expressed in post irradiation incubated sample compared to that of strawberry puree is justified (Figure 27). In case of strawberry puree since the cells are lethally treated in a nutritious matrix they have more access to nutrients during DNA repair mechanism. However, the genes affected during this process are more unique (Figure 30) which is supported by the findings of another story that the global transcriptomic response of lethally eBeam treated transcriptome of *Salmonella* when put in growth media is different from control group (Hieke, 2015).

Since there are no published reports in the literature about the global transcriptomic response of lethally irradiated *E.coli* and more specifically *E.coli* O26 the

only comparison that can be made is with lethally irradiated *Salmonella* done in our lab. The other issue is the unavailability of publicly shared annotation data on *E.coli* O26 which impairs identification of the function of the expressed genes. The results of the mentioned study found that *Salmonella* recovery (in growth media at 32°C) progressed through three stages: early phase (0-4h) in which cell growth was inhibited and there was little DNA repair; and late phase (4-24h) in which cell growth was restored and DNA repair, specifically *recA*, was repressed. In general the eBeam treated cells focused on DNA and membrane repairing over a 24 h period; while they downregulated citric acid cycle to redirect the energy to focus on DNA and membrane repair (Hieke, 2015).

The current results provide an overview of the global transcriptomic response of lethally eBeam treated *E.coli* O26 cells in PBS and a low pH food matrix. Anthology of the highly expressed genes is needed to identify the function of the genes affected in this study. It is interesting to see that level of different in the transcriptomic response of lethally treated pathogens in different matrices. These learnings can elucidate the destiny of pathogens when they occur in food matrixes and when attempted to be irradiated using eBeam technology. The lethal dose of eBeam inhibits bacterial dividing capability but the cells are still alive and metabolically active for an extended period of time, which is also supported by the metabolomics result in CHAPTER VII. According to the results in CHAPTER VII there are a number of metabolic pathways including purine metabolism and glutathione metabolism that are in charge of DNA repair and defeating the free radicals formed during irradiation that are upregulated after irradiation and thus the genes involved in those pathways can be expressed accordingly. Additionally the

upregulation of *artI* and *artJ* whose role in arginine uptake is documented supports the induction of its metabolic pathway in acid stressed cells (CHAPTER IV and V). It is of interest to understand under what conditions the lethally irradiated cells can multiply and grow again.

CHAPTER IX

CONCLUSION

Summary

Strawberries are a valuable agricultural product of the United States. Fresh strawberries are not washed because the increased moisture can cause mold growth and limit the shelf life. Therefore, fresh strawberries might harbor microbial pathogens. Because many of the available interventions such as chlorination, ozone, etc. can negatively affect the quality, such washing steps are not desired from marketing perspectives. On the other hand, most of the commercially available non–thermal interventions such as pulsed electric fields (PEF) or high pressure processing (HPP) used for microbial reduction purposes on solid fruits cannot be applied on strawberries. Electron beam (eBeam) technology/processing is an effective non-thermal technology that can be applied on any types of fruit as long as packaging configurations allows uniform dose of eBeam.

The objective of our initial study was to determine to what extend the eBeam dose approved for phytosanitary reasons in strawberries can contribute to ensuring safety as an added value of this technology. Therefore, a cocktail of six serogroups of non-O157 Shiga toxin producing *Escherichia coli* (STEC) namely, O26:H11, O45:H2, O103:H2, O111:NM, O121:H19, and O145 was prepared and inoculated into strawberry puree (pH 3.6). Application of only 1 kGy of eBeam (the maximum dose approved by FDA for fresh produce) reduced 4.5 log of the initial population of the non-O157 STEC

population. Quantitative microbial risk assessment (QMRA) showed that application of 1 kGy of eBeam can drastically reduce the health risks associated with consumption of one serving size of fresh strawberries (150g) contaminated with non-O157 STEC. QMRA analysis showed that when a typical serving size of strawberries (150 g) is contaminated with ~ 105 CFU, 2 out of 10 susceptible individuals (20%) would get sick. If such strawberries were treated with 2 kGy eBeam dose, this risk can be reduced to 4 individuals out of every 100,000 individuals. This translates to more than 99.99 % risk reduction. The current FDA regulation on ionizing irradiation limits its application to 1 kGy for phytosanitary reasons in fresh produce without any claims on microbial inactivation. As these studies have shown, there is potential for this technology to ensure safety of fresh produce. Application of higher irradiation doses (2.5-3 kGy) on berries (such as blueberries and strawberries) has shown no sensory changes in the berries. Application of higher doses in fresh produce will not only eradicate pathogens but also reduces the spoilage microorganisms that currently limit the shelf life of strawberries. There is no recall recorded for the eBeam treated items which supports the fact that this technology can be considered a robust intervention for reducing the health risk associated with consumption of fresh produce items.

One of the major interventions for ensuring food safety is the use of organic acid sprays. It is believed that there is less health concerns associated with consumption of low pH foods. However, a number of food borne outbreaks associated with consumption of low pH foods such as apple cider, berries, etc. have been reported. Bacterial pathogens especially the gastrointestinal pathogens are resistant to very low pH

conditions (pH 1.5 – 2.0) that exist in the stomach. These pathogens are known to acquire resistance mechanisms against different stressors including acid stress. This mechanism is believed to be even activated in STEC in the ruminant digestive tract before being shed into the environment and entering the human food chain. Acid stress in pathogens may also induce cross protection effect against other types of stressors. In order to identify the sensitivity of each serogroups of non-O157 STEC to different acidic matrices, each of the serogroup was exposed to different acidic matrices prepared using a) strawberry puree, b) inorganic acid as well as 3) organic acid buffer prepared using the specific organic acids found in strawberries (pH 3.6) for 24 hours at room temperature. Each of the serogroup showed a different level of sensitivity to acid stress in terms of growth on media. The results indicated that inorganic acid was the most effective at growth inactivation followed by strawberry puree and organic acid respectively. The TEM analysis and *BacLight* images indicated that exposure of *E.coli* O26 non-O157 STEC to different acids resulted to 45.83%, 97.29% and 98.18% live cells in inorganic acid buffer, organic acid buffer and strawberries respectively. Although the cells incubated in both organic acid buffer and strawberry puree could maintain their membrane integrity, there was still microbial inactivation effect reported in organic acid buffer (0.45 log CFU/ml) and strawberry puree (1.45 log CFU/ml) respectively. It can be deducted that these cells are live but unculturable. An untargeted metabolomics study was conducted to understand the mechanisms behind different responses of STEC to different acids an untargeted metabolomic study was conducted. *E.coli* O26 cells were exposed to inorganic acid and strawberry puree for 24 hours and

the metabolome was analyzed against the metabolome of the control samples. Various amino acid metabolic pathways including alanine, aspartate and glutamate metabolism, beta-alanine metabolism, glycine, serine and threonine metabolism were activated; however, some of the identified pathways were specific to each treatment group. The metabolomic analysis showed that peptidoglycan and saturated fatty acid pathways are significantly activated in inorganic acid stressed cells. That can be explained by the fact that HCl is a stronger acid (pKa < 1) and thus the cell membrane is more damaged than the cells stressed with the organic acids in strawberry puree. The unsaturated fatty acids are replaced with saturated fatty acids to endow more cell membrane resistance to the *E.coli* cells. The main metabolic pathway triggered in strawberry puree treated cells was glutamine/glutamate pathway which endows more acid resistance to the bacterial cells.

Irradiating the *E.coli* O26 cells with lethal dose of eBeam (3 kGy) resulted in changing the metabolome of the cells immediately after irradiation and after 24 hours of incubation at room temperature in different way. The pathways of alanine, aspartate and glutamate metabolism, beta-alanine metabolism, glycine, serine and threonine metabolism and inositol metabolism were activated which were also activated in acid stressed cells. Those pathways were considered as the general stress response pathways in *E.coli* O26. In addition to those pathways glutathione pathway was also activated in both irradiated treatment groups. Glutathione is a known antioxidant in cells that inhibits oxidation in cells by donating electrons. This pathway can be considered as an ionizing radiation stress specific metabolic pathway in *E.coli* cells. The comparison of the metabolome of the irradiated cells with the post irradiation incubated cells indicated that

purine metabolism is activated. Purine is one of the main components of nucleic acids and it is believed to be involved in bacterial DNA repair process. The C5-brancehd dibasic acid pathway which is involved in virulence was activated in both irradiated and post irradiated incubated treatment groups suggesting ionizing radiation can activate virulence pathways in *E.coli* O26.

The transcriptome analysis of *E.coli* O26 after exposure to 3 kGy of eBeam in different matrices of PBS buffer and strawberry indicated that background imparts a significant effect in transcriptomic responses of bacterial cells. In PBS almost all the genes were upregulated as a matter of incubation at room temperature with or without eBeam treatment; however, in case of strawberry puree fewer genes were affected. The comparison of the transcriptome of *E.coli* O26 cells 24 hours after eBeam treatment storage with an unirradiated group stored under the same conditions indicated that more specific genes are involved in post irradiation bacterial responses which are involved in rRNA methylation and DNA recombination. In the case of strawberries when comparing the cells 24h after irradiation with 24h unirradiated incubated cells fewer genes were differentially expressed, which were mainly identified for their function.

Novelty of the Research

The current study is the first study to investigate the metabolome and transcriptome of Shiga toxin producing *E.coli* when present in a food matrix. It is also the first study to investigate the effect of eBeam processing on *E.coli* cells at a molecular level. The results of the current study prove that the matrix in which bacterial cells are

irradiated has a great impact on their molecular responses. It also indicates that application of eBeam at a lethal dose completely inactivates *E.coli* O26:H11 without affecting the transcriptomic and metabolomic responses of the bacterial cells or affecting their cell membrane integrity. Consumption of an eBeam processed food in which the pathogens (if present) are completely inactivated but their cell membrane integrity and metabolic activity are maintained can trigger protective immune responses in individuals consuming such foods. Therefore, these results highlight the intriguing possibility of foods as vaccines.

CHAPTER X

FUTURE RESEARCH NEEDS

The response of the different serogroups of non-O157 STEC in the organic acid buffer (pH 3.6) as compared to the strawberry puree (pH 3.6) was different. There was on average, greater inactivation (~ 1 log difference) in the strawberry puree compared to the organic acid buffer. The quantification of live cells (based on Live/Dead Bacterial Viability™ staining) however showed that the numbers of live cells in the organic acid buffer and strawberry puree were not significantly different ($P>0.01$). It is, therefore, assumed that the presence of naturally occurring phytochemicals may be aiding in the enhanced inactivation of the STEC cells. Pelargonidin-3-glucoside, a major anthocyanin in strawberries has been reported to be contributing to the microbial inactivation in strawberries (Giampieri et al., 2012). There is a need to identify the antimicrobial compounds in strawberries that are responsible for the inactivation of the STEC serogroups. Such studies can help elucidate the higher inhibitory effect of strawberry puree against STEC serogroups. It may be possible to breed strawberry varieties with higher concentrations of such inhibitory compounds so that the survival of human pathogens in such strawberry varieties can be minimized.

Metabolomics, especially untargeted metabolomics rely on understanding the metabolic pathways based on a semi-quantitative concentrations of various metabolites at a particular snap shot of time. The metabolite concentration is the net result of the interaction of various pathways and not the net product of a single pathway. Untargeted

metabolomics in particular is a relatively new analytical tool with a variety of applications in food science. Metabolomics to unravel the mechanisms of pathogen survival and growth in natural and processed foods can help identify new strategies of pathogen control in the food industry. Metabolomics can also be used to identify the optimal storage conditions where there is enhanced accumulation of health-promoting phytochemicals. Metabolomics can also be used to identify the optimal time of consumption where the levels of phytochemicals are at a maximum. Metabolomics can also be used to optimize food processing to ensure the maximal accumulation of health promoting phytochemicals.

Application of metabolomics in ever increasing nutraceutical market where consumption of fresh produce for benefiting from the naturally occurring health beneficial compounds is promoted (Childs, 2000) is of high value. Different environmental conditions including temperature, light, storage time, etc. affect the concentration of phytochemicals in fresh produce (Schonhof et al., 2007).

This study focused on the analysis of the metabolome of *E.coli* O26:H11 during exposure to acid (inorganic acid and strawberry puree) stress. There is a need to analyze the metabolome of the *E.coli* O26 cells exposed to organic acid. That can facilitate understanding the difference in the metabolic pathways activated in the cells stressed with organic acid versus strawberry puree.

The results of this study clearly demonstrate that lethal doses of eBeam can inactivate the cells without affecting their cell membrane integrity or diminishing their metabolic activities Irradiation of *E.coli* O26 cells in PBS with lethal eBeam doses

appeared to have activated the virulence-associated metabolic pathways. . Follow-up studies are needed to understand whether any specific condition(s) could enable eBeam inactivated pathogens to resuscitate and potentially multiply.

Previous studies in our laboratory have shown that eBeam inactivated cells are metabolically active yet non-culturable (MAyNC) cells are effective vaccine candidates. Therefore, is it possible that if foods that harbor pathogens are eBeam irradiated, these foods could then function as immune modulators or vaccines and actually protect human health? Can such a strategy be used to create MAyNC forms of the regular bioburden of foods the can be used to modulate the immune responses within the gut? An extensive set of studies have to be performed to develop the concept of "food as vaccine"

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APPENDIX A

DATA FOR CHAPTER V

Table A-1 - The metabolites of *E.coli* O26 when exposed to PBS (pH 7.5) and inorganic acid buffer (pH 3.6) for 24 hours at room temperature.

No	**Sample**	**pH 7.5**						**pH 3.6**					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
1	xylulose	182	143	159	170	161	205	359	325	301	333	525	315
2	xylose	899	676	885	449	476	778	1207	1586	1297	3932	2483	1785
3	xylonolactone	276	413	211	127	115	125	367	351	273	613	538	453
4	xanthosine	153	178	158	220	252	154	377	386	364	376	473	445
5	Xanthine	385	203	206	439	812	623	427	382	434	678	565	612
6	valine	9087	2407	2212	13256	18959	9278	5696	1783	2432	8891	8032	15424
7	uridine	114	161	156	142	118	106	320	380	230	299	623	517
8	urea	1715	1027	826	1296	1357	832	365	370	310	2282	477	517
9	uracil	5089	4216	2059	2467	6049	3555	393	993	1028	1686	565	664
10	UDP-glucuronic acid	245	188	135	176	128	178	24609	27032	24678	33001	5971	37977
11	tyrosine	2584	596	773	857	1503	1292	768	581	717	1889	1887	1698
12	tryptophan	536	386	293	400	334	421	528	708	429	486	1012	999
13	trehalose	285	270	231	255	223	196	609	511	640	556	1638	962
14	tocopherol alpha-	225	264	339	179	125	116	492	327	360	568	722	696
15	thymine	569	982	244	153	494	341	407	403	414	484	814	485
16	threose	136	148	145	144	117	193	302	389	328	520	460	563
17	threonine	1452	1155	704	4266	792	500	1963	1778	791	2692	1553	1589
18	threonic acid	125	153	145	151	115	123	302	395	255	465	599	393
19	tartaric acid	137	199	177	154	131	111	341	315	276	328	535	401
20	sucrose-6-phosphate	144	131	135	154	111	100	365	378	317	364	528	427

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
21	sucrose	131	113	113	139	138	141	101	270	353	208	78	272
22	succinic acid	2068	697	408	5975	10998	6397	8186	4423	9116	25551	18315	26542
23	stearic acid	84854	121291	91530	69269	62527	93280	230573	2E+05	207721	2E+05	3E+05	2E+05
24	spermidine	2871	2852	2286	6515	8162	16815	635	674	669	6681	4442	10707
25	shikimic acid	177	207	132	185	283	277	615	834	543	560	715	676
26	serine	2109	1227	692	819	628	538	1659	1033	764	3683	1431	5014
27	sebacic acid, di(2-octyl) ester	2361	2450	2533	2380	2048	2024	4882	4965	4363	3930	27542	10098
28	salicylic acid	179	248	178	170	158	140	1637	2722	1424	1609	2991	1219
29	salicylaldehyde	634	678	383	530	507	346	861	488	1205	1523	1274	1638
30	ricinoleic acid	113	142	116	138	112	134	272	308	260	359	422	387
31	ribose	855	427	129	111	256	217	397	441	332	383	484	413
32	ribonic acid	133	153	153	153	164	108	647	659	481	455	640	508
33	raffinose	130	134	112	205	130	102	298	359	310	453	535	462
34	quinic acid	150	154	148	157	107	99	284	351	303	268	542	520
35	pyruvic acid	678	861	336	153	37	154	327	437	560	453	620	618
36	pyrophosphate	15940	18190	11781	10823	11974	16394	3425	5768	41502	6183	6339	7842
37	putrescine	3231	2811	551	8501	22877	17772	7476	8142	7443	6569	7303	12082
38	proline	242	164	173	226	639	306	5092	534	603	2105	2159	1961
39	pipecolinic acid	188	151	170	148	191	128	353	458	362	580	576	419
40	pinitol	44	170	183	233	173	234	355	408	352	611	467	384
41	phosphoethanolamine	3148	2386	1715	166	207	213	32718	25152	22189	1193	804	1017
42	phosphoenolpyruvate	366	259	200	236	413	328	325	342	375	398	705	471
43	phosphate	394674	316198	319429	377844	393426	331946	69050	74512	96389	29028	27307	36986
44	phenylalanine	2781	1426	918	923	1975	1954	2253	1012	1144	2615	1832	2285
45	phenol	898	1464	533	583	367	928	1479	3390	3081	4109	4343	2106
46	pentitol	133	139	198	135	113	89	294	194	407	350	446	430

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
47	pelargonic acid	2983	6919	4072	3169	4431	2767	8577	6309	11159	14258	7885	14457
48	pantothenic acid	144	156	136	219	295	305	377	353	301	374	647	560
49	palmitic acid	20151	23076	24353	10226	9200	12982	38697	33602	36439	28044	41303	28561
50	oxoproline	4592	2953	1004	3158	4509	4788	6103	12387	8253	19490	10508	14985
51	orotic acid	310	236	174	447	735	222	1989	18182	15533	1657	2350	2969
52	oleic acid	1602	1545	2466	503	211	821	8423	7753	4855	570	3542	1993
53	octadecanol	308	192	245	394	199	175	381	570	457	745	695	1300
54	nicotinic acid	1148	726	336	619	1296	595	506	509	289	280	657	812
55	nicotinamide	317	312	194	230	227	204	494	433	404	852	732	968
56	N-acetylmannosamine	130	135	141	138	112	99	300	378	276	448	596	407
57	N-acetyl-D-hexosamine	123	155	131	131	137	119	411	367	233	352	678	529
58	N-acetyl-D-galactosamine	136	138	149	98	108	96	361	213	230	354	562	500
59	myristic acid	20320	20828	20719	5266	7687	9850	26218	54397	26339	14662	20256	18951
60	myo-inositol	188	152	210	167	126	128	323	429	249	491	630	540
61	mucic acid	117	167	130	139	143	103	327	380	203	318	443	413
62	montanic acid	289	359	223	276	221	159	595	422	488	1130	2394	1124
63	methyltetrahydrophenanthrenone NIST	954	908	617	625	607	681	4519	3360	1463	1137	1642	1178
64	methionine sulfoxide	205	435	252	346	473	473	1272	1643	877	876	1461	1401
65	methionine	477	329	337	158	102	145	343	344	217	354	497	592
66	maltotriose	142	181	166	158	146	110	318	376	360	369	470	393
67	malic acid	192	177	194	146	177	725	806	10712	7857	378	695	979
68	maleic acid	136	144	150	136	116	33	647	1721	2102	395	463	445
69	lyxose	233	243	255	141	194	196	474	300	310	788	664	529
70	lyxitol	111	155	126	159	192	178	10659	10999	20691	31066	43254	29595
71	linolenic acid	218	270	216	215	228	370	581	750	500	759	1151	971
72	linoleic acid	147	151	151	136	149	150	484	473	518	505	879	638

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
73	lignoceric acid	160	230	269	252	183	139	562	621	515	522	787	846
74	levoglucosan	195	199	185	142	178	257	13479	14110	11168	14691	19112	18061
75	leucine	14003	4175	7849	10227	16406	475	8728	1935	1324	8570	8219	16608
76	lauric acid	6926	6040	8149	1404	1847	2860	7462	53425	16032	8075	4074	3992
77	lactitol	116	167	163	188	122	104	280	287	247	395	501	644
78	lactic acid	4207	4208	1403	1232	844	3333	951	2777	2773	6032	6240	2779
79	isoleucine	13680	10467	5257	7989	12238	6332	4043	1945	5163	25922	13750	16588
80	inositol-4-monophosphate	132	113	182	177	145	94	308	427	212	297	559	419
81	inosine	217	177	171	127	136	84	341	319	276	419	477	494
82	hypoxanthine	1345	408	208	1257	2803	2127	302	2270	3759	1348	1921	2039
83	hydroxylamine	37038	127054	129293	154109	90345	146747	1105	357	4371	13415	14330	19104
84	hydroxycarbamate	4886	4947	5153	7723	7989	4689	508	450	3404	5769	3617	14870
85	homoserine	137	167	133	127	182	156	347	418	296	450	473	647
86	hexonic acid	162	118	182	180	150	113	391	340	479	489	620	479
87	hexitol	160	179	183	135	94	139	423	587	493	433	627	430
88	hexadecane	925	1434	603	546	428	643	1338	2638	3537	2349	1863	1540
89	heptadecanoic acid	1211	1704	1632	1122	981	1617	3356	3187	3304	2895	4711	3247
90	guanosine	326	668	405	206	146	117	306	310	235	433	572	627
91	guanine	957	975	696	207	160	154	367	498	296	429	817	569
92	glycolic acid	585	537	317	360	390	1078	605	1821	673	967	1758	971
93	glycine	2272	2645	1529	10	353	213	2124	363	2007	127	685	286
94	glycerol-alpha-phosphate	5628	4787	2627	224	126	136	17524	13772	402	541	1022	979
95	glyceric acid	365	226	199	356	901	603	627	437	924	1030	882	875
96	glutaric acid	119	170	25	118	317	311	333	433	369	364	531	49
97	glutamine	318	305	359	314	255	238	5517	5495	4374	5570	7613	6675
98	glutamic acid	1061	219	143	197	525	720	419	902	258	1116	1059	416

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
99	glucose-6-phosphate	191	136	152	138	140	92	304	302	307	465	617	529
100	glucose-1-phosphate	420	368	161	218	276	341	7666	2118	1313	1868	2309	1586
101	glucose	370	286	303	354	376	425	691	853	606	876	971	982
102	glucoheptulose	148	148	118	105	122	128	381	363	244	374	487	526
103	galactonic acid	119	154	180	141	197	152	280	418	276	283	586	517
104	galactinol	400	516	466	537	379	304	1030	1174	1001	1149	1941	1482
105	fumaric acid	936	300	348	280	478	582	762	1472	947	613	545	503
106	fructose-6-phosphate	148	122	140	162	126	108	314	342	305	395	715	430
107	fructose	3605	2745	4335	1082	163	1775	2711	1535	405	8230	8887	3166
108	ethanolamine	16962	11177	68009	10240	13539	9427	8131	2765	1828	807	998	1184
109	ethanol phosphate	148	153	167	135	122	154	329	433	377	496	565	725
110	erythritol	156	188	126	136	122	200	304	420	283	402	599	511
111	dodecanol	452	324	384	252	329	250	877	541	859	1219	1226	826
112	dodecanoic acid, isopropanol ester NIST	391	82	285	381	535	577	657	927	558	307	249	546
113	deoxypentitol	191	111	170	192	134	181	314	427	323	311	634	497
114	dehydroascorbic acid	190	196	258	152	900	137	335	304	362	536	565	742
115	dehydroabietic acid	279	301	354	251	276	274	607	585	473	738	875	768
116	cytosin	1220	1691	370	160	219	100	316	604	481	893	593	612
117	cytidine	343	216	320	178	130	123	325	327	274	450	804	433
118	cysteine	126	151	179	137	110	112	290	304	355	539	569	459
119	cyanoalanine	180	178	186	214	184	124	375	435	416	421	477	448
120	citric acid	508	557	409	1024	1647	2150	1387	9337	3748	613	1124	728
121	cerotinic acid	173	211	298	230	207	165	429	439	570	733	841	742
122	cellobiose	132	132	114	150	136	92	343	353	352	342	760	503
123	catechin	129	152	155	145	146	115	284	391	274	381	521	413
124	capric acid	306	347	245	131	261	138	627	389	906	584	756	543

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
125	butyrolactam NIST	358	337	301	382	367	120	5944	2870	2977	7289	4506	7259
126	beta-sitosterol	191	188	196	212	215	3745	496	429	560	690	654	858
127	beta-gentiobiose	302	272	304	377	299	268	768	834	665	1042	1751	1794
128	beta-alanine	298	270	300	367	351	219	754	661	780	675	1318	771
129	benzoic acid	7650	5564	3983	5702	5628	3840	16295	10609	13083	15560	18931	22847
130	behenic acid	408	324	687	338	485	322	1078	655	644	1090	1202	2022
131	aspartic acid	1212	889	539	399	692	925	1486	3071	1334	1011	1328	1173
132	asparagine	1394	943	1049	1006	918	1162	2191	5092	2315	2933	3345	2845
133	arachidic acid	769	1214	1017	750	668	1063	2437	1728	2324	2378	2132	1979
134	altrose	3330	2769	2616	931	355	1735	2630	1654	466	5639	5668	2478
135	alpha-ketoglutarate	168	158	194	137	125	99	294	384	249	477	589	453
136	alanine	16153	5706	6368	4977	6118	7419	9818	10687	10865	34763	21612	21562
137	adipic acid	470	215	315	233	371	374	828	917	1245	1913	984	1017
138	adenosine-5-monophosphate	949	517	640	274	203	99	502	463	556	304	569	413
139	adenosine	807	1180	724	193	137	221	320	336	308	388	780	477
140	aconitic acid	116	151	146	142	174	126	20352	21576	17956	22198	2350	26995
141	acetophenone	402	622	484	366	340	666	1758	6344	5640	2996	3542	1889
142	5-methoxytryptamine	123	133	236	308	228	165	256	367	292	697	606	1398
143	5-hydroxynorvaline	162	239	152	486	881	844	351	492	482	520	603	659
144	5-aminovaleric acid	2788	990	310	2909	6795	7605	407	393	334	479	497	494
145	4-hydroxyphenylacetic acid	217	142	111	314	796	1009	381	296	353	572	477	589
146	4-hydroxybenzoate	170	203	225	202	140	263	478	503	380	742	392	505
147	4-aminobutyric acid	174	87	279	130	521	568	1891	1195	3879	6707	4796	7019
148	3-phosphoglycerate	1162	371	187	968	1292	1570	435	689	396	335	698	505
149	3-phenyllactic acid	552	506	147	496	1029	981	373	733	769	709	756	601
150	3-hydroxybutyric acid	1999	2171	1634	6198	3133	3464	1373	1481	1489	2045	1632	1863

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
151	3-aminoisobutyric acid	958	1038	609	237	475	264	31039	9388	5909	6087	9397	12781
152	3'-adenylic acid	737	371	580	190	195	122	363	315	425	474	508	500
153	3,6-anhydro-D-galactose	276	342	287	246	226	233	30422	31957	27820	1841	2469	2678
154	3,4-dihydroxycinnamic acid	152	141	154	169	136	104	474	427	341	599	647	560
155	3,4-dihydroxybenzoic acid	480	213	178	185	421	392	6918	12233	4586	3432	3733	5901
156	2-ketoadipic acid	7160	5195	6737	3611	2678	1147	2100	1666	4037	2546	3737	6265
157	2-isopropylmalic acid	103	135	191	144	138	84	290	422	303	469	518	534
158	2-hydroxyvaleric acid	4178	3927	1164	1569	1068	753	4305	2000	2229	3846	4091	4945
159	2-hydroxypyrazinyl-2-propenoic acid ethyl ester NIST	278	918	472	468	535	702	353	929	1645	1331	1342	1306
160	2-hydroxyglutaric acid	598	171	117	545	1072	698	409	452	319	256	753	589
161	1-monopalmitin	299	200	383	195	261	174	772	581	416	409	971	901
162	1-kestose	121	103	110	129	150	102	381	403	276	402	473	407
163	126585	132	181	149	136	100	73	306	310	296	326	453	445
164	126582	126	148	141	219	191	115	403	382	260	898	674	771
165	126542	531	234	500	264	275	172	2892	12985	76804	94469	13553	34358
166	126541	112	178	112	125	173	82	7516	6585	4604	6149	5119	6687
167	126465	510	898	459	477	7193	1706	8117	25450	20499	21456	15392	11805
168	126425	518	639	181	117	344	477	832	978	1225	1497	838	1444
169	126423	3048	1249	1853	1607	1898	3003	4731	7343	8476	10450	4605	5762
170	125960	141	316	161	187	126	150	292	336	276	412	559	505
171	125897	111	241	187	146	148	126	310	410	300	316	501	479
172	125664	5101	6191	3237	3260	5455	3798	13825	8729	16681	23307	16520	20577
173	125662	7765	7884	9962	8683	4430	7706	19663	31414	20356	24188	9898	28734
174	125154	10091	10591	9435	2204	2276	4886	10651	25446	15280	65709	45530	23255
175	124903	15793	14042	8410	8668	5257	8940	19735	19988	34181	43281	7126	34176
176	122191	431	404	220	390	382	251	1054	784	769	2421	1274	1427

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
177	121191	116	129	153	125	95	111	349	260	264	400	617	436
178	121002	836	330	674	565	445	530	4519	2773	3641	3072	3072	4081
179	120744	1572	626	636	963	794	629	1875	1721	1722	6157	3355	4919
180	120526	569	786	575	886	1006	466	697	625	2787	4152	2214	5632
181	119066	403	492	385	410	517	722	1024	1620	794	1566	1727	1259
182	117141	9929	9404	10631	10086	9481	8389	11949	19407	18091	36758	26915	23229
183	110604	327	646	526	416	175	513	705	1067	660	893	967	991
184	106936	272	207	222	154	128	200	514	1647	1932	1875	1941	1412
185	106387	187	147	181	116	122	83	310	344	305	364	392	557
186	104303	2269	2596	969	1124	912	1100	1933	2076	4103	3752	3314	4168
187	104131	495	561	1917	308	203	414	25353	17272	5007	642	1311	1297
188	103175	171	126	149	137	157	90	298	317	273	333	756	474
189	103102	8398	8169	4326	4518	4699	3941	12489	9293	16510	24109	15133	19633
190	102809	111	147	152	118	296	102	310	334	395	352	467	491
191	100666	2866	3502	1078	1248	1158	1210	397	536	943	1248	569	586
192	89383	2890	2390	1346	1503	1524	1489	2505	2741	3542	5357	2582	3255
193	88847	141	157	168	240	241	226	331	384	285	314	497	456
194	88046	567	315	227	257	399	375	635	406	305	551	705	835
195	84161	259	476	350	442	364	270	667	788	676	608	1475	2132
196	49426	600	282	260	234	162	233	657	758	1085	1324	756	1118
197	49400	828	1371	389	296	401	736	1794	4328	4347	4452	2221	2559
198	48428	2549	2938	1239	1126	949	720	1842	2203	2839	2091	2439	4850
199	46134	249	309	211	206	69	228	516	460	404	450	886	745
200	42424	4186	2266	2128	138	168	83	5015	8528	18449	503	671	404
201	42187	435	385	364	355	278	420	435	838	1024	1116	1335	1124
202	41989	160	182	162	144	128	142	248	332	321	484	487	673

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
203	41805	681	682	258	183	204	328	615	697	951	1935	2068	1170
204	41682	140	191	166	171	113	164	679	1010	549	632	886	621
205	34126	1556	1372	9655	1205	939	662	95058	2E+05	16688	2739	4465	4353
206	31460	1293	422	472	471	504	987	2544	2868	3876	3767	2473	1981
207	31408	1747	1966	860	879	768	1106	1651	1231	3494	2881	2483	2135
208	31359	1176	1783	732	799	944	1732	2820	3362	5474	5407	2418	3980
209	31285	3013	1649	893	2148	2401	2036	2989	2482	6453	5386	3934	8044
210	31273	263	853	492	469	439	412	1032	1766	1720	2799	1662	2031
211	23635	2118	2423	625	1926	751	938	2985	2414	2197	1870	4040	3353
212	22863	5107	4676	3402	4126	3907	2309	16430	6991	3474	25199	14404	16383
213	22444	162	173	178	400	185	241	339	313	292	338	596	407
214	22363	219	270	321	131	135	134	492	631	877	364	671	456
215	21885	459	460	731	449	429	738	1244	1238	881	1087	1270	939
216	21683	723	673	2033	187	221	231	1084	2030	1560	436	603	508
217	21666	435	442	558	527	495	676	4380	4839	1851	1863	2633	1537
218	18345	641	861	495	150	126	115	456	353	692	386	593	375
219	18305	1096	1049	728	1048	892	1324	740	534	595	793	967	991
220	18173	2532	2992	366	405	420	241	941	864	1327	2490	1703	2964
221	17775	103	166	150	113	138	115	236	467	291	429	426	404
222	17664	1386	1081	302	321	257	423	726	972	3325	4962	1706	2389
223	17651	402	422	449	172	282	302	1346	1369	1064	1058	865	329
224	17536	136	180	173	153	175	122	617	591	495	517	783	806
225	17186	160	131	161	126	136	80	323	264	300	477	671	505
226	17068	518	607	460	515	411	643	1377	746	762	1252	1580	1158
227	17045	140	148	146	164	125	121	268	319	298	450	855	494
228	16818	289	210	142	140	135	129	337	329	258	421	368	491

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
229	16817	123	189	185	175	139	100	387	353	317	316	439	832
230	14703	349	804	221	228	227	277	683	862	805	1032	926	1080
231	13146	105	151	159	124	125	95	331	272	305	323	521	433
232	11841	484	164	285	293	289	234	953	566	1089	1446	1104	2181
233	9320	193	462	509	419	568	591	4309	13618	7272	5239	1795	6236
234	6646	133	147	157	164	142	134	67049	76979	71064	87170	16234	1E+05
235	6435	144	133	140	147	132	104	306	275	375	378	480	321
236	5837	116	148	114	193	218	170	333	361	273	364	446	598
237	5523	236	337	557	792	455	376	1383	976	759	2119	4224	1851
238	5471	1438	3129	1898	1510	1370	1258	281960	3E+05	100636	98928	45526	97447
239	5346	1447	4700	2207	2300	2912	2791	9083	13221	8634	9037	7504	8740
240	4945	125	176	333	110	111	122	1159	974	703	333	889	563
241	4937	679	518	690	1273	1105	447	1165	906	812	4631	3222	2732
242	4850	123	158	149	121	130	106	256	315	300	318	582	479
243	4735	222	239	260	286	263	336	1008	879	556	620	1318	1126
244	4265	249	313	606	289	503	324	1058	1248	755	1755	3791	1655
245	3328	228	240	278	333	247	254	764	5895	1883	2213	2006	1747
246	3294	122	150	186	116	99	119	300	308	368	333	719	607
247	3286	995	2852	252	354	279	202	1883	1472	2541	6379	2418	5196
248	3247	107	125	130	136	126	103	337	448	303	333	429	578
249	3228	16295	20948	10611	12609	14364	9971	716	750	9025	40254	47386	38555
250	3185	127	177	138	134	118	183	316	363	402	354	552	439
251	2936	15793	14042	8410	8104	9499	8940	18649	18615	32929	41650	25471	34176
252	2706	496	505	473	308	255	521	1224	2406	1049	1964	947	916
253	2691	395	341	562	157	142	157	1213	1250	1089	386	450	410
254	2476	3254	2203	1748	1721	1687	456	8379	2192	1101	5783	9197	8108

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
255	2438	1600	1806	647	457	565	692	1165	1535	1361	1944	1638	1239
256	2233	567	315	227	143	399	375	635	562	421	891	1141	835
257	2193	119	173	151	209	120	103	1097	769	524	515	559	693
258	2061	2732	4500	1981	1806	1307	3458	6484	8780	6302	10987	6846	6707
259	1970	862	909	239	433	473	386	786	771	1042	2210	1362	1750
260	1941	3005	3857	3116	2939	3688	2834	10429	7086	11017	15893	10692	12622
261	1922	321	1988	66	451	317	688	3322	6237	5508	5958	8212	2649
262	1921	1649	1603	956	1158	941	842	1995	1611	1245	5038	1860	3657
263	1909	1459	2666	1291	727	2284	3108	6025	6625	8097	10031	5841	4396
264	1872	1730	5782	2377	2271	2951	5271	9240	9325	16627	17074	7330	11106
265	1789	1884	1036	1021	147	133	117	3870	2038	6180	484	726	702
266	1765	482	404	1219	171	145	119	5122	5326	2468	685	971	1583
267	1715	128	122	128	146	147	323	391	323	346	500	525	416
268	1709	423	760	236	505	434	334	1094	995	843	1080	1465	2423
269	1704	1225	611	449	207	219	501	1889	1491	2199	1193	1383	1135
270	1700	9448	10686	3705	3697	6781	9133	17060	25262	24048	29014	17269	25037
271	1686	7072	10336	14454	372	236	206	14585	10961	7272	702	2367	1259
272	1684	150	180	136	110	123	101	312	401	253	302	732	540
273	1675	255	698	173	394	143	235	488	855	2014	1135	3423	13382
274	1661	27164	16790	3355	70198	155128	124952	3419	1240	7681	41265	37583	80683
275	1029	10390	12284	5367	5714	4310	11656	9665	31448	24025	31957	35430	16513
276	657	1801	1081	302	321	140	423	726	1373	3836	6935	1706	2389
277	573	451	113	2339	202	195	107	5233	4277	1281	407	1087	549
278	490	514	438	415	276	296	238	1062	682	653	752	1196	1427
279	466	382	678	356	140	188	352	853	1776	2235	4167	1686	1589
280	453	6483	4601	3864	5433	3324	1856	2427	2446	1784	11523	4380	4442

No	Sample	pH 7.5						pH 3.6					
		A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
281	257	1580	1836	2276	822	461	368	2921	4066	3339	6164	2432	7371
282	203	664	992	440	442	345	1044	788	2610	1090	5014	3239	1427
283	168	338	960	880	1265	2898	127	754	4877	585	5218	1495	2152
284	137	65306	67645	47548	51960	38397	86298	144291	1E+05	146231	2E+05	2E+05	1E+05
285	134	1297	3202	4146	1492	5189	117	2729	18529	583	2502	1182	942
286	110	638	611	765	801	760	485	1248	2942	1636	2797	3784	3492
287	99	129	139	162	144	102	110	320	403	400	520	610	491
288	91	1142	1459	502	827	761	1629	1885	4366	5113	7129	10072	4399
289	68	3391	3400	5187	6375	4767	3364	26645	56353	30447	18891	37522	26308
290	62	6843	7541	5364	6907	3939	10496	10526	17530	21389	28063	37839	17247
291	54	187	190	151	171	146	207	355	454	386	340	603	569
292	47	9109	4174	5473	6906	6524	10860	10586	11510	18417	10910	14752	18336
293	39	20102	37859	26112	28927	32169	21367	65523	68811	112668	1E+05	82281	1E+05

APPENDIX B

DATA FOR CHAPTER VI

Table B-1 - The metabolites of *E.coli* O26 when exposed to strawberry puree (pH 3.6) for 24 hours at room temperature.

No	**Sample**	**Control Strawberry puree**			**Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT**					
	Label	**A1**	**A2**	**A3**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**
1	xylulose	1299	1594	1547	11557	5502	6218	616	335	558
2	xylose	337549	420854	318598	4855148	5743241	5273134	144939	170845	156393
3	xylonolactone	816	783	829	13168	6627	12404	377	409	376
4	xanthosine	159	164	134	324	386	370	171	195	244
5	xanthine	142	144	106	381	480	409	148	219	202
6	valine	6113	5468	7859	78686	76676	68351	5710	5715	4178
7	uridine	168	189	111	1819	3501	3158	223	188	163
8	urea	1610	635	161	6657	4096	2297	425	801	174
9	uracil	120	214	539	16750	8156	14667	762	726	251
10	UDP-glucuronic acid	1540	1405	1361	54503	25394	25915	802	723	951
11	tyrosine	1268	1931	1490	21718	13283	16196	587	774	662
12	tryptophan	1875	3541	2328	91395	126414	44593	958	953	830
13	trehalose	866	2314	1975	66186	9412	95056	1545	2215	1659
14	alpha-tocopherol	176	243	184	5312	2086	8022	234	218	228
15	thymine	121	159	130	293	215	225	194	188	173
16	threose	154	158	184	966	466	819	170	207	229
17	threonine	5584	3660	4676	87089	48789	74284	3155	3387	3304
18	threonic acid	1433	1553	1407	24349	13568	21379	1652	1580	1591
19	tartaric acid	103	204	205	1650	1059	1257	186	195	213
20	sucrose-6-phosphate	188	156	163	131	380	359	149	165	157

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
21	sucrose	161492	229710	168329	56899	81413	92506	1460	2038	1994
22	succinic acid	1097	1032	1487	28080	16276	29137	1242	1324	1348
23	stearic acid	104143	134125	103403	293901	312682	93070	43889	41082	45254
24	spermidine	488	662	553	6284	10024	4377	578	364	534
25	shikimic acid	745	954	738	9691	9255	8115	434	671	378
26	serine	26014	19311	27331	403504	294084	346853	16216	18005	16293
27	sebacic acid, di(2-octyl) ester	2301	8595	5597	11564	13043	14316	3297	3326	3409
28	salicylic acid	215	158	182	699	943	802	140	222	258
29	salicylaldehyde	572	405	420	3889	2360	3062	548	418	444
30	Ricinoleic acid	158	196	120	3293	5360	4124	161	202	192
31	ribose	7234	8478	7000	130062	67620	56315	4526	5135	4615
32	ribonic acid	476	634	461	17285	14903	15093	507	565	471
33	raffinose	353	1113	714	4280	2402	2107	2016	2326	474
34	quinic acid	6329	8227	6568	53925	26691	40495	3581	4251	3923
35	pyruvic acid	274	277	226	1124	697	924	217	185	60
36	pyrophosphate	4152	2330	4608	4720	3768	3563	2550	2501	2889
37	putrescine	2641	2785	2529	42798	18116	34236	1449	1681	1542
38	proline	845	777	1904	44655	23478	35622	1612	1491	1068
39	pipecolinic acid	545	474	821	2051	1054	1772	462	519	566
40	pinitol	110	200	124	301	201	174	262	197	171
41	phosphoethanolamine	172	186	141	246	347	389	163	185	182
42	phosphoenolpyruvate	110	221	113	287	166	184	195	192	181
43	phosphate	14263	15309	16079	244142	273337	267819	12344	14327	13244
44	phenylalanine	492	832	732	13639	7665	12953	592	581	681

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
45	phenol	1698	930	1197	1685	730	1089	1505	1889	1696
46	pentitol	203	209	85	1594	1113	1806	166	233	223
47	pelargonic acid	5611	3169	5359	6559	4141	3157	2567	2576	2799
48	pantothenic acid	188	165	190	1415	1153	1118	144	36	220
49	palmitic acid	17320	22416	17875	54999	27491	22982	8893	8911	9191
50	4-Oxoproline	44551	34638	39365	107335	53927	86251	1956	2117	1996
51	orotic acid	130	144	126	261	172	187	178	173	182
52	oleic acid	1412	2246	1338	4891	5712	1652	297	163	255
53	octadecanol	396	433	460	947	157	354	371	321	407
54	nicotinic acid	225	210	164	1813	1037	1753	232	249	271
55	nicotinamide	200	211	164	1749	773	1304	208	209	213
56	N-acetylmannosamine	363	509	396	14115	3858	7472	255	229	238
57	N-acetyl-D-hexosamine	940	1246	1054	36405	21701	25960	764	662	894
58	N-acetyl-D-galactosamine	182	186	178	3042	1466	2168	200	174	209
59	myristic acid	15685	18871	14710	19065	10731	20577	5025	5125	5289
60	myo-inositol	21522	29460	22482	510763	492128	402261	14875	17443	15374
61	mucic acid	317	378	281	6605	4190	4426	211	428	362
62	montanic acid	410	377	629	872	529	1105	573	1109	556
63	methyl tetrahydro phenanthrene	735	1317	1050	2360	753	1261	832	671	730
64	methionine sulfoxide	721	926	734	9393	7256	6377	477	574	437
65	L-Methionine	428	331	538	14625	7931	14288	393	403	369
66	maltotriose	130	149	89	244	179	157	176	193	173
67	malic acid	115309	127390	111750	787621	477647	1077817	68633	81104	75703
68	maleic acid	229	235	185	19373	18138	37873	110	150	290

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
69	lyxose	22589	28622	21735	348774	196214	178460	12465	14321	13010
70	D-Arabitol	436	600	446	4259	2473	4928	391	336	300
71	linolenic acid	409	488	414	7228	12131	2572	262	80	318
72	linoleic acid	301	238	237	4768	5035	2038	223	245	257
73	lignoceric acid	302	463	280	803	591	830	116	153	322
74	levoglucosan	2988	2292	1641	27865	9615	14937	1105	1141	803
75	leucine	801	867	1388	22120	15300	18862	1386	1353	981
76	lauric acid	4728	4462	3652	22142	4765	16440	1632	1736	1968
77	lactitol	301	397	276	8819	1067	3737	398	222	236
78	lactic acid	591	689	710	10333	4965	8939	974	525	1436
79	isoleucine	10234	5710	9873	38441	24866	32132	5842	5824	6252
80	inositol-4-monophosphate	70	65	202	1339	1652	1886	205	235	182
81	inosine	146	219	126	874	2770	2260	217	175	240
82	hypoxanthine	641	779	659	2754	3118	2908	810	880	852
83	hydroxylamine	175214	157946	129129	116454	132704	141003	89667	92614	96171
84	hydroxycarbamate	8104	7581	7069	16968	16881	14581	3434	3493	3333
85	homoserine	156	271	249	3833	1474	2945	256	328	326
86	hexonic acid	1384	2248	1905	27265	14956	21364	558	731	763
87	hexitol	1055	1659	1053	31008	22056	20792	650	495	899
88	hexadecane	813	977	899	1252	648	654	588	480	442
89	heptadecanoic acid	2087	2893	2250	7736	3230	6107	826	867	833
90	guanosine	113	256	147	679	1180	1420	186	183	185
91	guanine	195	204	185	435	346	274	128	178	223
92	glycolic acid	334	465	269	2534	1081	1959	78	426	615

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
93	glycine	435	424	538	8678	4331	8165	444	466	509
94	glycerol-alpha-phosphate	100	322	172	3410	2092	2586	217	235	266
95	glyceric acid	1814	1351	1538	31770	12737	27077	1240	1422	1289
96	glutaric acid	172	153	109	370	225	214	227	195	219
97	glutamine	56595	86845	60867	2407	2012	1769	411	431	460
98	glutamic acid	6029	7423	7666	403	196	697	207	218	256
99	glucose-6-phosphate	694	1182	1016	9203	12630	3673	247	164	204
100	glucose-1-phosphate	4322	5372	4354	4080	48616	12137	1706	1954	2176
101	glucose	26809	54859	62334	1285631	1641701	1574656	18835	22702	14357
102	glucoheptose	747	976	823	4984	4876	7202	344	509	637
103	galactonic acid	262	241	250	4744	3789	4017	258	306	136
104	galactinol	1500	3914	2322	29028	44192	46794	969	1296	1261
105	fumaric acid	1205	735	1012	30717	18324	37538	1208	1374	1253
106	fructose-6-phosphate	415	593	548	6783	10126	2775	331	349	276
107	fructose	1E+06	796790	1E+06	1469578	1619847	1086606	1527631	1661317	1718174
108	ethanolamine	1856	1625	2097	36670	35161	32724	1777	1901	2023
109	ethanol phosphate	244	347	298	1069	1156	1129	203	254	246
110	erythritol	317	283	329	4428	1928	3108	262	355	170
111	dodecanol	528	551	307	863	731	1001	374	313	323
112	dodecanoic acid, isopropanol ester	216	174	169	481	272	207	196	233	186
113	deoxypentitol	69	439	458	765	404	705	212	213	196
114	dehydroascorbic acid	16027	22282	16775	109483	57800	79085	2079	2383	1932
115	dehydroabietic acid	409	624	379	949	896	858	294	345	339
116	Cytosine	129	176	291	763	1012	963	160	178	239

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
117	cytidine	209	252	180	584	457	437	203	223	211
118	cysteine	231	296	159	3002	1955	3150	207	1465	227
119	3-Cyano-L-alanine	6394	2587	4338	14775	3943	6709	1230	1163	2047
120	citric acid	794068	992491	767295	1140610	998446	1620212	856141	992740	920533
121	cerotinic acid	49	80	99	382	1095	786	208	240	301
122	cellobiose	110	292	205	2617	2382	4342	89	230	268
123	catechin	578	1400	1133	24567	33282	38179	409	428	585
124	capric acid	422	191	322	562	230	293	222	124	173
125	butyrolactam	475	242	507	48379	51259	49794	2443	2886	998
126	beta-sitosterol	433	671	763	49021	27257	27668	510	469	573
127	beta-gentiobiose	472	550	726	8547	9877	18658	752	690	664
128	beta-alanine	1238	920	1119	19129	10429	20725	586	760	840
129	benzoic acid	6620	6075	6964	9779	7947	6234	4904	5109	5639
130	behenic acid	907	2712	2056	5416	4114	5148	818	677	876
131	aspartic acid	14550	10926	15818	2086211	2053824	2020221	104433	116958	108785
132	asparagine	76761	75721	76408	373655	182934	206929	13535	14303	15986
133	arachidic acid	1089	1254	943	4334	3764	3086	774	518	953
134	altrose	137406	158552	232837	827496	881043	750436	537449	195879	245502
135	alpha-ketoglutarate	169	166	120	947	489	906	211	193	203
136	alanine	75295	72550	89904	927020	885704	870999	75597	68738	70848
137	adipic acid	347	396	503	5145	2415	3162	287	385	350
138	Adenosine monophosphate	135	184	152	364	561	690	190	159	219
139	adenosine	262	271	313	944	714	1142	160	182	206
140	aconitic acid	597	701	368	21514	11175	25346	529	544	576

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
141	acetophenone	389	425	474	602	630	4533	1085	853	850
142	5-methoxytryptamine	216	166	120	509	732	802	197	191	186
143	5-hydroxynorvaline	425	607	449	8989	4155	7455	390	379	388
144	5-aminovaleric acid	179	159	142	1101	623	1134	204	199	200
145	4-hydroxyphenylacetic acid	342	385	293	3632	5110	4398	334	241	316
146	4-hydroxybenzoate	305	294	280	3069	1558	3180	170	226	287
147	4-aminobutyric acid	1738	1675	1712	527255	603164	618983	16839	15470	18002
148	3-phosphoglycerate	129	197	101	185	220	141	231	236	168
149	3-phenyllactic acid	171	247	178	362	227	184	185	182	274
150	3-hydroxybutyric acid	2456	2012	2054	7490	10253	10595	1785	2117	2276
151	3-aminoisobutyric acid	1010	625	994	1676	1092	880	895	676	707
152	3'-adenylic acid	156	155	139	208	266	261	140	184	206
153	3,6-anhydro-D-galactose	1405	1548	1407	22950	10644	19052	928	876	666
154	3,4-dihydroxycinnamic acid	821	411	264	6016	1337	2072	274	314	355
155	3,4-dihydroxybenzoic acid	547	615	411	3932	26494	6589	334	7614	7132
156	2-ketoadipic acid	317	596	404	3933	2238	2340	760	649	677
157	2-isopropylmalic acid	420	444	395	5372	3934	6342	225	250	268
158	2-hydroxyvaleric acid	1831	1021	611	805	1228	1046	1054	1129	1232
159	2-hydroxypyrazinyl-2-propenoic acid ethyl ester	828	942	615	1340	620	623	396	533	496
160	2-hydroxyglutaric acid	198	160	145	473	293	263	243	190	231
161	1-monopalmitin	194	440	327	1405	2465	2843	228	352	162

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
162	1-kestose	1255	4014	2814	51721	31825	29906	1697	2159	1690
163	126585	207	299	234	8134	12222	11154	199	388	202
164	126582	209	348	437	20312	9234	9400	161	149	53
165	126542	272	255	321	347	367	350	267	250	260
166	126541	220	158	119	533	566	516	176	190	209
167	126465	4740	3581	4971	12394	12471	10734	7716	3086	7713
168	Natamycin	561	392	600	873	259	422	336	303	312
169	126423	3965	3837	3803	5552	2785	2572	1509	1506	1598
170	125960	1023	2309	1228	28803	50400	49972	758	888	1009
171	125897	3213	4858	3561	102933	132116	111315	1809	2079	1911
172	125664	9508	6344	8884	11447	3958	6399	5200	5268	5685
173	125662	10522	11376	9321	10888	4734	2824	5805	6052	6042
174	125154	12078	12025	7990	4624	1565	6536	3548	16614	13276
175	124903	16588	9997	16223	19056	9554	10369	8714	8616	9150
176	122191	325	368	273	394	430	366	169	522	569
177	121191	203	350	379	6634	10174	11162	326	158	277
178	121002	706	1113	936	1886	771	893	411	666	715
179	120744	1394	855	1422	2108	2460	1979	962	1132	1194
180	120526	850	803	826	2569	2363	2087	495	275	376
181	119066	1036	1358	956	3304	7362	1956	567	764	797
182	117141	2161	8893	3997	13536	15859	12888	5158	8689	10241
183	110604	402	654	321	5052	16798	6342	242	195	127
184	Nitrosyl chloride ((NO)Cl)	361	477	441	230	235	249	279	470	452
185	106387	909	1265	1045	31892	40775	22182	697	921	900

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
186	104303	1419	1036	1797	7457	7103	8068	1067	855	1013
187	104131	718	767	575	1693	1742	766	271	351	322
188	103175	628	912	674	1842	1236	2090	280	274	278
189	103102	7658	5721	7819	12334	5349	7426	4842	4782	4941
190	102809	235	269	204	9466	13591	12450	322	296	301
191	100666	1494	525	747	1811	1176	1328	435	641	448
192	89383	2375	1392	2040	2509	1447	1623	1461	1328	1304
193	88847	2473	2964	3356	77105	112826	108446	1674	1872	1842
194	88046	3283	2952	3738	45049	32532	31380	2018	2587	2422
195	Halosulfuron-methyl	409	643	920	1193	987	260	908	671	610
196	Ifosfamide	1179	737	969	2042	1241	1315	335	357	433
197	49400	987	1041	987	2654	1171	1501	342	597	658
198	48428	2294	1405	2244	2641	1617	1441	990	1480	1108
199	46134	377	271	296	484	239	385	265	278	107
200	42424	117	145	106	202	143	144	206	145	206
201	42187	340	445	558	576	563	385	536	521	562
202	41989	276	303	383	779	486	403	496	465	556
203	41805	156	148	156	2013	2010	1429	203	494	332
204	41682	179	179	87	1106	852	726	276	252	209
205	34126	994	1369	688	3462	1661	1036	1438	1472	2618
206	31460	1011	1360	1194	2965	1216	1321	533	741	786
207	31408	1110	816	1020	1483	503	970	672	769	852
208	31359	2044	943	1713	4894	1920	2275	724	838	741
209	31285	1595	1172	2309	7902	4466	1238	1180	1182	1070
210	31273	1000	1302	876	10329	5980	5294	413	531	468

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
211	23635	1408	882	1707	1746	1000	1221	1250	765	1102
212	22863	6817	5812	11044	10607	4856	4827	7409	5416	4690
213	22444	135	208	110	310	218	154	164	170	168
214	22363	99	181	109	309	292	182	197	164	200
215	21885	941	739	1175	2229	2657	1904	417	446	585
216	21683	106	292	242	1497	720	1472	179	182	184
217	21666	1052	723	1329	8351	4677	4904	518	511	574
218	18345	129	207	168	236	183	204	237	202	230
219	18305	192	198	300	1300	15865	2567	285	361	247
220	18173	3565	2316	3062	59553	35123	47040	1741	2276	2084
221	17775	545	630	566	13398	6142	11512	284	371	511
222	17664	570	1061	465	453	394	605	503	1116	1789
223	17651	266	226	241	450	492	536	308	134	365
224	17536	162	256	284	3260	3942	4568	157	147	242
225	17186	249	158	206	5416	7662	8016	374	195	372
226	17068	611	568	482	691	542	334	907	1034	1195
227	17045	619	1225	1266	31562	17470	43900	937	1071	1025
228	16818	145	284	169	13748	24820	23301	462	439	387
229	16817	1265	1525	1244	13308	11974	9065	862	894	818
230	14703	728	653	667	9483	6238	7106	737	390	544
231	13146	586	774	523	15497	17463	9140	277	162	244
232	11841	521	394	522	465	371	403	434	210	375
233	9320	1270	152	3706	14075	2848	2407	417	448	523
234	6646	551	1234	972	9767	3844	6693	588	647	714
235	6435	155	304	342	5539	2802	3004	336	132	302

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
236	5837	2370	1633	1848	35717	20706	22071	575	849	1092
237	5523	439	1233	732	1166	815	1626	507	391	378
238	5471	1919	1634	1788	2156	1901	1606	1262	1024	1376
239	5346	2571	3554	5412	10995	4220	4998	2066	2242	2302
240	4945	199	183	150	266	485	312	179	212	187
241	4937	651	1442	1293	1307	795	431	826	813	880
242	4850	287	380	444	3238	3305	3224	367	444	337
243	4735	816	1007	1190	18856	25064	23482	573	677	579
244	4265	457	848	719	1337	1583	912	496	420	490
245	3328	342	331	1613	13476	6078	495	342	306	348
246	3294	352	554	501	15672	20011	22046	475	345	452
247	3286	1944	976	1592	2342	3440	1754	1063	1400	1313
248	3247	194	116	242	4717	6405	6863	246	154	312
249	3228	27764	22768	26419	20887	15208	12553	23209	22267	25043
250	3185	1004	802	836	30826	39503	35993	582	576	507
251	2936	18107	9997	16223	17773	9554	9665	8714	8616	9150
252	2706	241	398	380	705	611	657	324	279	331
253	2691	154	171	132	337	375	225	189	146	222
254	2476	2902	2850	4537	4895	1934	2216	3629	2528	2182
255	2438	769	981	682	1724	835	1100	511	537	451
256	2233	6362	6233	6829	49916	37156	31380	4130	4482	4614
257	2193	551	690	602	17108	8515	14712	636	543	540
258	2061	2948	1413	2673	2570	1659	2229	1973	1918	1702
259	1970	3648	2479	3393	64235	38475	49242	2000	2378	2210
260	1941	7015	4084	6901	8742	3353	4367	3455	3534	3738

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
261	1922	1199	1428	1329	2583	1486	1465	2375	2593	1254
262	1921	1042	701	994	1740	950	915	770	925	694
263	1909	3855	4754	3877	6845	2038	3673	1843	1853	2025
264	1872	5787	3896	5220	13473	5112	4307	2084	2439	2289
265	1789	2023	189	140	626	531	568	170	222	147
266	1765	430	356	327	2454	3315	4675	452	352	245
267	1715	974	631	354	4567	1819	4215	383	467	1037
268	1709	707	529	632	735	638	665	485	629	574
269	1704	830	713	969	277	455	683	819	553	1147
270	1700	11955	7524	11466	17340	5614	9349	5376	5464	6124
271	1686	275	468	400	1698	558	1767	331	317	326
272	1684	121	128	147	4629	2532	3241	84	123	132
273	1675	7900	327	544	667	588	197	593	1164	284
274	1661	783	1028	965	75346	50238	77501	2510	2683	2579
275	1029	13319	7098	11286	11858	1324	6678	12708	14149	6234
276	657	443	1848	465	674	394	605	503	641	1789
277	573	271	300	254	2999	6307	4370	245	495	500
278	490	728	447	836	2180	779	1357	555	612	834
279	466	401	496	601	932	852	436	339	340	391
280	453	841	441	546	12895	10238	10105	459	833	649
281	257	667	984	987	4438	1817	1622	704	575	792
282	203	664	442	354	550	529	633	415	507	329
283	168	270	586	255	846	800	432	648	184	235
284	137	77072	73205	72489	69417	67886	73437	64801	64953	71061
285	134	174	209	158	778	586	617	236	150	171

No	Sample	Control Strawberry puree			Spiked strawberry puree with *E.coli* O26 cells a for 24h at RT					
	Label	A1	A2	A3	B1	B2	B3	B4	B5	B6
286	110	947	594	809	2179	1646	979	477	614	623
287	99	26745	40204	24700	539165	351340	426939	12456	13937	13586
288	91	1273	1837	971	1687	1028	5642	2496	2297	288
289	68	2914	1704	2440	5396	5018	3344	4568	4589	5087
290	62	9745	5391	8351	10805	8685	7167	5016	5129	5885
291	54	693	1032	1055	49235	63195	28468	573	201	260
292	47	9852	5762	9426	11827	4975	6654	4879	5271	4902
293	39	55652	32694	53049	74048	34695	36155	25943	25757	27924

APPENDIX C

DATA FOR CHPATER VII

Table C-1- The metabolites of *E.coli* O26 when eBeam treated (3kGy) and incubated for 24 hours at room temperature after eBeam treatment (3kGy).

No	**Sample**	**Control cells**			**eBeam treated with 3 kGy**			**Incubated 24h at RM after eBeam with 3 kGy**		
	Lable	**A1**	**A2**	**A3**	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**
1	xylulose	1185	1444	631	1034	906	1039	2833	1620	957
2	xylose	3019	2828	2209	11835	12385	16919	32426	6171	3904
3	xylonolactone	2600	4411	874	1490	1479	1210	2254	1603	1535
4	xanthosine	402	306	301	577	475	513	6927	3399	2399
5	xanthine	5583	1493	1288	5624	3976	6954	10989	6798	4277
6	valine	139802	163347	159750	25469	98774	129818	312420	365328	235955
7	urocanic acid	130	144	73	147	92	226	262	161	75
8	uridine-5-monophosphate	2115	1054	3231	306	1867	1195	1061	520	698
9	uridine	9896	9530	3656	14370	5892	7696	7669	4583	3319
10	uracil	117767	131394	46568	154136	89891	106528	365711	264613	173067
11	tyrosol	78	41	65	39	83	28	73	64	67
12	tyrosine	67946	64365	39744	23465	29081	33304	43287	30137	25513
13	tryptophan	8656	10311	6452	4727	4847	7993	12246	9809	8058
14	trehalose	1009	4469	2847	2297	1429	2339	2178	82	1941
15	thymine	5547	1121	663	11078	11199	6381	48333	25245	18523
16	thymidine-5-phosphate	640	113	198	1926	3266	1172	3032	930	1098
17	threonine	6501	8257	6376	6311	4435	5699	7406	10105	4742
18	tetracosane	1859	1936	852	7132	2308	1859	1875	1056	715

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
19	tartaric acid	57	44	45	706	763	738	1142	955	434
20	sucrose	720	688	531	2074	1171	2732	1048	45387	207
21	succinic acid	5481	8524	6516	5531	11017	13420	62466	40113	27527
22	stearic acid	1476076	1068615	984781	1673231	1E+06	1213438	1729042	1142662	665194
23	spermidine	378240	403704	346772	169964	165553	251167	378035	365080	232151
24	serine	8120	13075	8329	9173	7769	10666	9405	8179	6467
25	salicylaldehyde	371	629	288	188	525	565	1575	982	959
26	ribulose-5-phosphate	1122	493	851	894	935	944	678	726	324
27	ribose-5-phosphate	1663	848	1042	1261	245	1814	1840	1065	684
28	ribose	46064	50069	27485	39441	35409	39337	80157	43885	30624
29	ribonic acid	187	310	332	216	337	470	548	426	280
30	raffinose	69	90	73	52	38	67	57	50	30
31	pyrophosphate	146409	145168	100218	17086	100910	122096	73234	83013	37111
32	putrescine	802014	889623	862581	285531	586786	786201	757366	733787	442523
33	pseudo uridine	1702	1184	799	1060	909	1368	6524	4296	2757
34	proline	16914	25696	29980	1744	17704	22103	10083	6536	4144
35	pipecolinic acid	485	1107	1121	116	413	626	860	801	773
36	pinitol	35510	24006	22920	5091	5843	7257	10549	7527	4071
37	p-hydroxylphenyllactic acid	384	596	561	509	612	1013	5429	4518	2976
38	phthalic acid	1128	3706	1151	882	5069	6273	8301	5386	1058
39	phosphoethanolamine	16155	9333	8771	14056	11051	19422	21040	17422	10907
40	phosphoenolpyruvate	5265	1962	2231	2277	3218	4617	2797	1726	989
41	phosphate	62712	411824	420259	183324	421212	225599	211936	450028	155501
42	phenylpyruvate	3526	1860	2461	4719	3698	6716	11951	12125	7439
43	phenylethylamine	7512	20361	6078	2352	10515	13220	10180	8097	7464

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
44	phenylalanine	35473	29536	19758	10388	14317	17144	27483	22999	18159
45	pentadecanoic acid	7173	6069	5775	2444	21769	30142	38659	30570	19808
46	pelargonic acid	2977	3430	2610	17071	2180	4968	3372	3889	1430
47	parabanic acid	831	692	390	969	15379	21601	22282	23849	10618
48	pantothenic acid	2749	1757	1456	912	983	1636	3335	2226	1330
49	palmitic acid	213214	121227	114694	399313	175469	165119	237753	133705	76796
50	oxoproline	188490	136168	105852	207890	87179	121949	87187	60729	33170
51	oxalic acid	263	141	232	606	4589	3790	3098	2805	2156
52	orotic acid	775	6364	12580	116	156	269	168	194	115
53	ornithine	26662	37160	15264	2374	13585	12527	17938	9647	8509
54	oleic acid	250	318	317	725	283	436	216	386	203
55	octadecanol	352	346	331	905	383	441	285	254	281
56	noradrenaline	191	187	187	3558	391	955	1485	576	360
57	nonadecanoic acid	1518	1396	1018	2997	1163	1534	1989	1001	653
58	nicotinic acid	2501	3127	1430	2340	1929	1791	25088	18895	12333
59	nicotinamide	18871	14502	15608	17109	12955	16077	8515	9727	3920
60	N-acetylputrescine	754585	838554	813463	1389	1529	2713	487	692828	418304
61	N-acetylornithine	1231	1493	1204	15973	13610	16323	30098	17786	9071
62	N-acetylglycine	490	79	421	1364	597	778	849	669	499
63	N-acetyl-D-galactosamine	98	244	175	713	772	856	928	517	434
64	N-acetylaspartic acid	380	272	333	344	764	776	939	724	460
65	myristic acid	3112	2471	2512	6637	2753	3540	4872	3042	2006
66	myo-inositol	289	405	389	180	597	854	791	131	145
67	methionine sulfoxide	24208	28009	21627	4013	8901	10897	18765	16330	10910
68	methionine	8143	3066	3004	1639	1863	2664	3099	4298	2923

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
69	maltotriose	769	1601	1100	977	648	1132	134	53	552
70	maltose	4941	21255	13820	11917	6806	13288	1487	636	9797
71	malonic acid	114	87	85	1254	2496	2209	3089	2563	1569
72	malic acid	1869	2701	2567	1830	3919	5456	10231	7482	4619
73	lyxose	1903	3345	670	1031	872	884	2011	1096	1308
74	lysine	171104	125248	115436	96847	84083	83154	127301	59671	49292
75	leucine	47508	49236	57473	5262	27542	29653	50214	56192	42911
76	lauric acid	5504	3188	5726	8920	3729	15402	4698	5627	9064
77	lactulose	60	80	1515	452	562	1156	728	617	755
78	lactic acid	1648	2023	2587	11962	3977	5828	4953	3866	1620
79	isothreonic acid	2122	3108	2526	629	989	1286	1699	358	695
80	isothreitol	74	14	39	54	87	90	987	457	484
81	isoribose	182	550	112	129	187	208	2370	1342	1276
82	isoleucine	107512	76826	93844	27474	49702	66466	105130	115785	77989
83	inosine	543	146	40	352	121	130	8854	3977	2232
84	indole-3-lactate	183	154	99	199	299	361	1900	1497	1036
85	indole-3-acetate	1047	1789	1349	419	842	1429	7428	6002	4778
86	hypoxanthine	4178	1157	75	4854	2445	3700	273242	185026	115840
87	hydroxylamine	25986	30795	33797	483	41041	44838	35571	38465	21212
88	homoserine	3340	1923	2509	2416	1617	1881	4660	3873	2752
89	histidine	12560	18560	20721	6270	8864	4688	17787	12981	9839
90	hexose-6-phosphate	672	1210	940	328	356	376	133	61	49
91	heptadecanoic acid	9043	6635	5747	19671	7955	10454	13129	6952	4204
92	guanosine	8323	8986	4576	7456	5736	4731	11291	6639	4851
93	guanine	9635	19498	5981	5492	4547	6594	11451	9237	6084

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
94	glycolic acid	1567	1941	1270	3536	6665	8271	17585	18001	8832
95	glycine	9678	22211	10279	4687	8531	9284	12352	9352	7508
96	glycerol-alpha-phosphate	11292	10179	5131	9532	7443	12408	13139	10746	8540
97	glycerol-3-galactoside	1825	3802	2983	1190	625	1534	2355	1903	1036
98	glyceric acid	3516	4759	3481	6529	6836	7902	35734	23701	2295
99	glutathione	4737	397	918	1538	2163	663	700	70	77
100	glutaric acid	995	461	553	517	420	348	4261	4741	3101
101	glutamine	1627	1307	1048	58	693	685	2862	955	842
102	glutamic acid	72567	100627	74208	13514	49784	56676	29293	21322	17696
103	glucose-6-phosphate	740	2127	1546	1267	1039	646	57	41	29
104	glucose-1-phosphate	2286	1802	1487	1626	1726	1898	3327	2780	1284
105	glucose	392	1688	2052	1239	1114	3292	2427	81	1287
106	galactonic acid	623	453	355	312	298	428	737	557	304
107	galactinol	214	187	121	126	118	124	250	104	113
108	fumaric acid	5571	5142	4827	4571	7175	7448	22559	15525	11419
109	fucose	1253	814	24100	2708	9428	11108	17559	8248	9497
110	fructose-6-phosphate	438	1173	666	402	407	258	235	95	47
111	fructose	183	421	274	685	2545	3194	3599	248	387
112	ethanolamine	43612	63474	38385	11368	37656	50519	371980	300711	192145
113	dodecanol	504	408	390	1565	647	756	684	453	208
114	dehydroascorbic acid	155	0	674	4391	2989	5325	5290	4694	2898
115	dehydroabietic acid	3806	3599	3497	2553	3233	2255	3821	3276	857
116	cytosin	1217	829	546	1064	1508	840	2430	716	1219
117	cytidine-5-monophosphate	2447	1590	1766	4578	2351	2749	2070	2336	816
118	cysteine-glycine	8848	5230	4733	1603	1969	2053	3177	2553	1440

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
119	cysteine	7754	12838	3670	331	481	899	2656	1296	1151
120	conduritol-beta-epoxide	1227	16903	16255	226	2670	2557	411	2941	1466
121	citrulline	6220	4988	3272	2772	2383	2411	3702	1832	1715
122	citric acid	9039	14193	13273	9718	27574	43453	41057	5192	7891
123	citramalic acid	205	309	161	599	839	1194	873	951	455
124	citraconic acid	3155	5731	4977	9083	10428	10613	26530	20534	13160
125	cholesterol	297	396	335	214	703	1286	2191	286	725
126	cellobiose	290	4435	2600	2380	840	2677	308	113	1971
127	capric acid	184	64	451	1832	332	470	383	388	272
128	butyrolactam	2046	1628	1187	2519	974	1440	1965	1301	781
129	beta-hydroxymyristic acid	143	169	157	138	84	137	339	216	114
130	beta-glycerolphosphate	472	336	224	325	86	578	183	519	400
131	beta-gentiobiose	192	412	400	55	92	73	38	63	17
132	beta-alanine	427	256	286	2476	4606	6114	6839	7476	4458
133	benzoic acid	1220	891	950	2154	847	1602	1025	1245	659
134	behenic acid	2744	5903	2262	8549	3891	2350	5214	6257	3750
135	aspartic acid	38336	34557	29145	17172	23042	31199	13681	13770	7550
136	arachidic acid	16349	12486	10692	32791	14343	16644	20843	11982	6376
137	aminomalonate	228	780	234	130	179	211	508	245	218
138	alpha-ketoglutarate	451	501	421	806	1085	1090	378	340	162
139	alpha-aminoadipic acid	139	129	92	97	218	234	366	1028	420
140	alanine-?-alanine	141848	73251	60885	11416	39425	42697	34934	21444	13836
141	alanine-alanine	4882	1746	1104	27147	679	1837	1058	630	416
142	alanine	41514	84626	55199	28649	48848	46518	106847	66945	53942
143	adipic acid	1539	1509	1248	1446	2281	3823	2405	666	456

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
144	adenosine-5-monophosphate	20593	27594	20365	10172	11518	23887	20984	28697	21287
145	adenosine	30584	39386	14697	36862	26851	21558	45408	25576	21785
146	adenine	84091	157480	46307	75149	73242	95338	176059	153409	97473
147	acetophenone	2811	3540	2224	5160	3440	3097	3264	3094	1655
148	7-methylguanine	149	155	29	78	88	163	866	615	475
149	6-deoxyglucose	2943	963	786	24533	26877	43231	57627	46935	30573
150	6-deoxyglucitol	1358	2704	2025	596	1203	1856	1521	1775	923
151	5-methoxytryptamine	8402	10602	6590	16803	2941	5505	9832	7902	4628
152	5'-deoxy-5'-methylthioadenosine	3458	2915	2140	5221	2931	3902	3219	3015	2301
153	5-aminovaleric acid	4354	3794	2280	1409	1377	1584	16155	8066	5432
154	4-hydroxyphenylacetic acid	5146	5691	6505	1664	2523	4750	26795	23264	15655
155	4-hydroxybutyric acid	639	702	689	123	86	87	871	853	687
156	4-hydroxybenzoate	941	761	541	328	585	495	3298	1801	1313
157	4-aminobutyric acid minor	30103	26335	19545	1548	7998	11353	3096	1919	1420
158	3-phosphoglycerate	22694	17208	12494	21239	15940	9667	9024	4845	4426
159	3-phenyllactic acid	4195	2183	2658	1764	2654	4096	31105	24859	17556
160	3-hydroxybutyric acid	888	739	1569	4648	1082	4699	3196	2403	2639
161	3'-adenylic acid	4421	9313	7767	3861	3523	4169	4791	3028	3325
162	2-methylglyceric acid	383	600	527	290	254	301	1232	852	580
163	2-ketoisocaproic acid	13629	2005	5997	22062	22686	23397	58835	58178	45856
164	2-ketoadipic acid	2300	2899	1687	3603	2838	3427	4140	3757	2409
165	2-hydroxyvaleric acid	120	4673	483	2404	3479	5780	4299	5016	2812
166	2-hydroxyhexanoic acid	3574	3487	3118	734	1762	1016	12943	12289	10777
167	2-hydroxyglutaric acid	945	1790	1222	511	682	977	3971	3048	1853
168	2-deoxytetronic acid	164	9202	178	2884	3270	4588	5093	4902	2332

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
169	2,5-dihydroxypyrazine	15277	11592	11996	296	5887	7443	5287	4705	1678
170	2,4-diaminobutyric acid	106	112	94	3138	2454	3847	1480	3607	1965
171	2,3-dihydroxybutanoic acid	69	38	34	502	452	409	992	955	468
172	1-monostearin	548	43	480	424	381	520	638	253	308
173	1-monopalmitin	1743	2591	1173	1600	797	731	1624	982	1152
174	1-deoxyerythritol	119500	103252	105322	96106	4791	5399	8459	5178	3938
175	1,3-diaminopropane	1822	2062	1883	1255	1597	2527	2069	2553	1690
176	704730	677	1196	257	487	149	241	1706	784	458
177	160962	5096	2014	1479	9305	3612	3006	3806	2299	1320
178	160842	5252	8117	3229	1731	3160	2965	10002	6728	4669
179	159824	2514	2557	2096	2356	2737	3187	1950	2184	1230
180	146957	3716	2897	3397	1482	3267	4167	3335	3971	1984
181	146262	352	261	220	388	253	306	1527	709	502
182	146042	661	88014	78498	7930	7267	13284	10004	14573	6776
183	145865	771	73	679	725	696	961	6988	4497	2349
184	134760	2416	963	786	23681	27980	45401	60774	45594	29091
185	134752	50	49	45	288	158	335	389	444	213
186	134122	212	367	237	414	512	258	897	527	338
187	133242	2067	2298	1990	7347	2198	2856	4072	3503	1917
188	132976	1227	4902	487	3186	408	503	584	306	649
189	131620	81069	73842	79865	48340	75917	95337	76163	87494	50703
190	130797	1669	1358	1269	3171	4276	5744	4714	5671	1984
191	130396	518	885	1011	922	526	834	625	809	456
192	129313	57	159	66	91	27	37	117	34	67
193	127277	2676	2893	4352	1844	4726	3513	9772	3282	1685

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
194	125786	45	383	341	2513	1393	2554	473	128	1902
195	124903	8761	8244	8393	26201	8149	9635	9312	10605	5222
196	124568	6126	4134	4085	492	628	1534	1597	1185	974
197	123989	58	135	57	405	558	638	807	628	341
198	121002	2071	1487	655	1884	1426	1898	1060	2442	1119
199	120789	1781	1125	969	1829	1178	1106	1952	1349	740
200	119066	1437	890	912	3232	1266	1490	1818	1168	637
201	113700	1857	3726	2004	317	1045	1359	564	7382	4814
202	112264	25916	41053	14008	9609	36025	34184	43163	30077	23245
203	111826	5886	2842	1892	12166	4740	3203	4792	2982	1572
204	111057	382	960	739	101	593	746	560	102	405
205	110359	757	563	212	789	549	700	1955	1121	977
206	110346	286	511	349	1609	483	942	1582	1107	523
207	110265	246	158	212	2686	271	356	2562	2454	951
208	110131	1524	1704	1441	588	850	868	1631	1351	1280
209	108309	488	522	426	553	263	208	1958	1678	1229
210	106742	107489	143416	289596	214826	297679	287371	322314	162358	177458
211	104906	3057	4190	3784	6061	4914	5532	5147	5276	2802
212	104022	9453	4380	3286	2965	2033	1640	4453	2167	1186
213	103857	31	139	36	394	644	448	673	52	69
214	103138	7375	5321	4762	1650	5774	5230	6983	7240	4921
215	103102	27838	24526	24344	23452	23217	30074	23535	28869	15149
216	102232	3523	3842	2798	1409	1491	1808	2612	1316	1410
217	100723	1166	12015	5042	355	123	606	642	9871	4381
218	88786	4774	4201	4461	3397	556	1294	1448	1148	837

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
219	88502	1456	3981	4030	5925	4747	4197	4832	4067	2228
220	88046	3930	5782	3605	2210	2070	2652	2007	1057	2198
221	87947	54	132	47	71	33	76	107	59	33
222	87312	1799	454	971	9631	667	1140	1318	99	655
223	87282	4452	3775	2075	2629	1963	3579	16013	14108	7946
224	84565	2626	1268	995	3087	962	1499	1096	543	228
225	84209	8066	10042	6933	17767	2782	5797	10189	7630	4386
226	66261	78	63	81	4487	559	1267	2062	1254	126
227	48608	67	66	71	1341	1374	1616	1699	1442	1062
228	47420	6575	3279	4118	836	3429	4534	9188	7266	4405
229	47170	885	985	652	2281	822	943	983	675	125
230	46357	5502	945	1532	5992	11428	12599	12837	17076	9572
231	46128	696	692	693	1702	720	808	794	699	380
232	41989	86	65	78	658	334	992	1111	582	328
233	41811	893	896	765	1201	646	1201	815	1026	550
234	41808	423	1135	955	1743	346	1561	1250	1565	745
235	33999	114	62	31	375	83	433	234	604	298
236	32148	922	265	399	694	654	840	5500	2028	2176
237	31460	1219	1107	1116	3560	966	1515	1221	1136	737
238	31408	1323	1185	1354	2968	1273	1716	1693	1528	863
239	31362	6585	3790	2020	14955	6007	3700	5995	2359	1797
240	31359	1494	1335	1390	4614	1748	2041	2391	1960	953
241	31285	3186	4047	3505	4986	4144	5095	13685	11011	6886
242	26062	307	44	113	452	428	119	337	55	55
243	21885	1097	1271	1160	3898	1261	1522	1425	1229	729

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
244	21683	3347	3123	1621	3868	538	3873	4087	3490	2810
245	21666	2519	6941	1777	7175	1830	1767	2075	1507	1404
246	21665	2267	2099	844	7504	2351	2941	3177	1656	1248
247	21664	6362	4927	4212	8165	3148	3630	5058	3155	1801
248	21511	370	211	2006	1750	1442	437	2840	1715	1799
249	20903	508	18869	136	1718	2582	5662	5129	8082	3505
250	20330	3610	2758	1403	10141	3683	2672	3300	1533	1190
251	20282	2820	5154	719	795	905	1241	3268	2890	1266
252	18588	1271	1084	1033	1162	594	991	1010	1553	893
253	18485	6361	1814	2836	3515	8660	3094	4998	1078	1264
254	18266	896	937	912	19	463	701	379	485	207
255	18248	358	255	257	63	48	149	810	891	420
256	18225	937	900	755	592	777	1199	1065	1014	690
257	18177	3754	2505	2377	991	900	1039	2578	2513	1666
258	17962	5716	2820	1879	11662	4541	3746	4561	2880	1523
259	17830	855	1390	1103	476	417	545	669	687	361
260	17775	71	38	17	460	556	1035	718	700	283
261	17651	420	407	492	1436	363	701	717	322	101
262	17463	188	134	117	101	84	234	328	0	14
263	17437	1694	4380	670	1490	853	884	2011	1603	1428
264	17245	1247	1393	945	1858	972	1922	1371	1461	983
265	17068	639	924	438	465	333	982	866	710	618
266	17002	1646	973	868	885	619	716	2243	824	568
267	14703	1791	1131	1319	2615	3164	1399	5942	2222	3465
268	14697	589	3421	1454	262	768	875	162	130	979

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
269	10176	72	261	34	167	133	209	667	426	323
270	9320	8055	10674	2924	19403	3331	4978	5908	2935	4129
271	7408	1361	735	534	2980	3032	2959	3227	2632	1870
272	7403	227	33	76	1737	6184	5180	3458	6194	2730
273	5691	3174	769	1184	3747	2190	2347	12759	14234	10422
274	5523	4245	1794	1311	8308	3185	2684	3179	1760	1110
275	5346	5412	4372	5149	14273	4859	5454	5832	5652	3054
276	4945	1024	889	357	1032	383	1136	888	802	807
277	4937	1136	1022	751	2724	1014	1161	1156	1082	674
278	4850	48	7	29	962	348	1425	1253	1400	825
279	4712	910	1167	351	1583	1680	1842	2250	1819	1136
280	4550	1062	1177	653	1153	1294	968	90	1217	932
281	4265	4723	2790	2010	11571	4189	3456	4120	2959	1500
282	4264	5807	3535	2041	15207	5300	3893	4472	1969	1712
283	4263	7486	3285	2816	15590	6174	4933	6321	3803	2293
284	3188	2346	1388	1125	3123	3160	4677	3578	3970	2980
285	3122	11026	26916	17883	3242	13921	18383	15521	871	10835
286	2847	274	447	321	1649	261	603	360	356	233
287	2706	1214	1255	1082	3728	1306	3375	1318	2271	1360
288	2543	1413	2382	994	316	202	1119	912	1241	609
289	2503	4481	1192	1388	956	1522	963	730	745	291
290	2438	12262	19770	3764	7823	5249	5101	15968	10259	7204
291	2262	4926	5096	4886	5659	6420	11010	8148	9214	5192
292	2242	12245	11715	6492	7355	7060	7756	12369	10764	5338
293	2233	3671	5462	539	2210	2047	2844	2051	1057	2250

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
294	2042	3184	2876	1561	2583	2279	6082	7659	12689	6276
295	2039	1507	2372	1726	4014	1306	1778	3234	4638	1903
296	2031	15059	24604	21484	4923	8850	8542	9423	10786	6622
297	2030	1851	1355	852	2466	436	1969	1496	1945	1148
298	2028	1093	653	309	567	408	159	1022	186	202
299	2017	268	837	816	268	148	944	1267	2883	1548
300	2001	693	6225	274	1693	167	195	241	185	380
301	1996	369	414	213	1994	3392	4964	3401	4635	1962
302	1981	1600	1801	1510	1216	1712	1446	2841	2359	1224
303	1970	2434	2468	2230	1112	671	797	926	853	526
304	1969	532	496	120	713	234	364	513	600	304
305	1941	881	715	632	162	648	906	878	861	553
306	1912	1746	1357	1077	4709	1285	1803	1595	1529	953
307	1878	2675	3705	4372	11545	3333	5579	5463	5072	3116
308	1875	4766	3755	4210	10592	4280	4683	6509	4593	2712
309	1872	5523	4869	5452	9740	5468	5860	6571	6310	3568
310	1852	48	701	506	360	261	402	1938	997	713
311	1826	1847	874	410	531	249	158	2624	389	532
312	1815	2516	1329	928	12206	1957	3354	3495	2646	2056
313	1812	1330	1323	1363	569	732	1212	783	298	869
314	1809	1354	2173	2100	636	1278	2616	1833	1666	1162
315	1806	1775	1090	13211	961	1007	987	3107	2288	1193
316	1805	3925	2757	1262	2564	3349	3389	1935	687	663
317	1803	195	515	343	494	519	891	801	879	461
318	1799	10516	9826	6476	75393	65315	75751	138931	76219	42765

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
319	1760	3082	2076	2188	413	772	1325	656	447	396
320	1753	3763	3826	4753	6120	3948	6647	9146	5383	5015
321	1751	9418	4102	5160	15089	3831	7362	9046	6761	3671
322	1746	1349	768	562	462	435	491	685	400	270
323	1744	773	610	790	1649	663	769	1103	537	413
324	1737	198	5740	4906	27	36	2342	255	2128	1760
325	1735	1023	2692	2161	415	759	823	1598	848	450
326	1725	4131	2800	2466	8624	1546	3333	3970	2226	2316
327	1721	2817	1190	1359	1349	922	887	2176	706	625
328	1719	210	244	32	106	70	77	1691	822	594
329	1717	17474	21394	21843	6966	19749	19571	15896	15369	10717
330	1713	1839	3337	2523	605	830	911	742	867	544
331	1708	3613	3379	2509	1345	983	1307	1968	1034	705
332	1702	6623	6391	6936	898	1280	1602	2084	2155	1339
333	1701	446	1852	340	0	392	123	471	666	694
334	1696	462	335	235	516	169	116	385	350	303
335	1675	3065	665	18309	511	340	803	253	283	173
336	1673	1903	3400	670	1031	872	884	2115	1155	1255
337	1666	1267	1703	756	711	1407	1986	4065	1397	1683
338	1661	3070017	2614563	2E+06	1273728	2E+06	2075500	3238949	2878343	1688226
339	1064	2155	2424	1748	3481	3184	5712	2976	2467	1384
340	816	6904	4956	4383	4159	3429	4660	9703	7725	4576
341	453	41602	21491	19127	30260	12454	20468	38756	31014	15083
342	443	11219	10516	8880	6553	9598	14292	11086	9330	5574
343	307	16469	7466	5914	8270	25607	50122	20254	18344	10035

No	Sample	Control cells			eBeam treated with 3 kGy			Incubated 24h at RM after eBeam with 3 kGy		
	Lable	A1	A2	A3	B1	B2	B3	C1	C2	C3
344	257	2063	1300	1373	4050	1689	1349	1312	1629	1056
345	168	527	1146	2511	1487	425	1321	539	1383	1852
346	137	5101	2488	552	18617	4023	4193	6788	4681	2581
347	134	4342	5785	33121	17913	3164	7641	3360	6239	13854
348	110	1517	2650	2205	2020	2682	3809	5235	5471	3534
349	47	4612	7845	10556	19219	12393	10176	10535	10227	5637