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Controlling Enterohemorrhagic E. coli O157: H7 using Selenium and Rutin

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Enterohemorrhagic *E.coli* O157: H7 (EHEC) has emerged as one of the leading causes of food borne illness and pediatric diarrheal disease in the United States. The EHEC associated life-threatening hemolytic uremic syndrome in human patients is linked to the expression of several virulence factors of the pathogen, especially verotoxins. Verotoxins (VT) or shiga like toxins (Stx) are the major virulence factors elaborated by EHEC. Even after 30 years of research on EHEC epidemiology and pathogenesis, little advancement has been made in disease prevention and treatment against the bacterium. One of the main limitations in treating EHEC infection is the contraindicated usage of antibiotics, since they have been reported to exacerbate verotoxin-mediated HUS and renal failure. Therefore, there is a crucial need for the development of alternate interventions for preventing and treating EHEC infections in humans. EHEC being a foodborne pathogen, controlling its persistence in meat processing facilities and inactivating the pathogen in high-risk foods such as undercooked ground beef products could potentially reduce food-borne disease outbreaks in humans. Moreover, identifying drugs that attenuate EHEC virulence, especially verotoxins would reduce the severity of infection and improve disease outcome in humans. This dissertation investigated the potential of a phytochemical, rutin (RT), and an essential mineral, selenium (Se), for targeting EHEC from both these aspects, thereby reducing risks to humans. Specifically, the efficacy of Se in reducing EHEC biofilms on food contact surfaces, and the efficacy of RT for decreasing EHEC in undercooked ground beef were investigated. Selenium was found to be effective in inhibiting and inactivating EHEC on abiotic surfaces. In addition, coating of stainless steel surface with Se nanoparticles exerted significant

antibiofilm effect against EHEC ($P < 0.05$). On the other hand, RT significantly increased heat inactivation of EHEC in undercooked ground beef patties without adversely affecting meat color and shelf-life. In addition, mechanistic investigations on the anti-virulence property of these natural antimicrobials revealed that Se and RT significantly decreased both intracellular and extracellular verotoxin synthesis ($P < 0.05$). In addition, Se decreased EHEC toxin receptor expression in host cells, whereas RT competed for the receptor binding sites on the toxin. Moreover, RT decreased EHEC motility and adhesion to cultured intestinal epithelial cells ($P< 0.05$). Subsequently, the protective effect of RT and Se against EHEC was validated *in vivo* using *Caenorhabditis elegans* model. Rutin significantly increased the survival of EHEC-infected *C. elegans* compared to control worms, where the survivability was increased by ~ 65% ($P < 0.05$). However, Se was lethal to *C. elegans*, and at very low sub-lethal dose failed to protect the worms when challenged with EHEC.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
CH	Chitosan
DAEC	Diffusely adherent *Escherichia coli*
DHPAA	Dihydroxyphenylacetic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
EPS	Extracellular polysaccharide
ESRD	End-stage renal disease
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal *Escherichia coli*
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gb3	Globotriaosylceramide

GMP	Good manufacturing practices
GRAS	Generally recognized as safe
GUD	Glucuronidase
HAACP	Hazard analysis and critical control points
HC	Hemorrhagic colitis
HPAA	Hydroxyphenylacetic acid
HUS	Hemolytic uremic syndrome
LB	Lysogeny broth
LSD	Least significant difference
MAbs	Monoclonal antibodies
MBC	Minimum bactericidal concentration
MEM	Minimum essential media
MIC	Minimum inhibitory concentration
MW	Molecular weight
NA	Nalidixic acid
NGM	Nematode growth medium
NMEC	Neonatal meningitis *Escherichia coli*
NOAEL	No-observed-adverse-effect level
NR	Neutral red
PBS	Phosphate buffered saline
PDA	Plant derived antimicrobial
PLEX	Plasma exchange
PMB	Premature browning

qPCR Quantitative polymerase chain reaction

ROS Reactive oxygen species

RPMI Roswell Park Memorial Institute

RT Rutin

RT-Qpcr Real-time quantitative polymerase chain reaction

SAS Statistical analysis software

Se Selenium

SeNP Selenium nanoparticles

SIC Sub-inhibitory concentration

SMA Sorbitol MacConkey agar

SOR Sorbitol

STEC Shiga toxin producing *Escherichia coli*

Stx Shiga like toxin

TBARS Thiobarbituric acid reactive substances

TEM Transmission electron microscopy

TLC Thin layer chromatography

TSA Tryptic soy agar

TSB Tryptic soy broth

UPEC Uropathogenic *Escherichia coli*

USDA United States Department of Agriculture

USDA-ERS United States Department of Agriculture Economic Research Service

US-FDA United States Food and Drug Administration

VT Verotoxin

VTEC Verotoxin producing *Escherichia coli*

WHO World Health Organization

CHAPTER I

Introduction

Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC) is a subset of Shiga-like toxin-producing *E. coli*, which causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. Because of its low infectious dose in humans (2–2,000 cells) (Buchanan, 1997; Strachan et al., 2005), EHEC poses a significant public health hazard. EHEC associated foodborne illnesses are estimated to cost the US economy ~ 200 million dollars annually (Hoffman et al., 2015). Although several foods have been implicated as vehicles of infection, the majority of EHEC foodborne outbreaks are linked to the ingestion of undercooked ground beef products (Armstrong et al., 1996; Hussein, 2007). Cattle are the most common source of EHEC, excreting the pathogen in feces, thereby contaminating foods, water and the environment (Croxen et al., 2013; Shere et al., 1998; Naylor et al., 2003).

EHEC is known to form persistent biofilms in diverse food processing environments (Doyle, 1991, Buchanan, 1997, Vogeleer et al., 2015). These biofilms are recognized as a potential and continuous source of food contamination and have been linked to foodborne outbreaks (Costerton et al., 1999, Janssens et al., 2008). Since common disinfectants and sanitizers are not fully effective in inactivating biofilms, there is a need for identifying effective treatments for controlling biofilms in food processing environments.

The United States Department of Agriculture (USDA) has established a zero tolerance policy for EHEC in ground beef and recommended that beef patties should be cooked to an internal temperature of 71.1°C (160°F) to ensure complete pathogen inactivation (USDA, 1998). However, due to the lack of homogeneity in patty composition, and temperature monitoring difficulties, the recommended internal temperature may not be uniformly attained. Additionally, the use of thermometers for cooking beef patties by consumers is limited, and most consumers determine the doneness of beef patties by observing the color and texture of cooked meat, which are not accurate

indicators of beef doneness. Therefore, it is beneficial to include an antimicrobial hurdle to ensure inactivation of EHEC in undercooked ground beef patties.

Verotoxins (VT) are the key virulence factor that defines EHEC pathogenesis, and are responsible for causing HUS in humans (Sakagami et al., 2001). EHEC produces verotoxin 1 and/or verotoxin 2, which are encoded by *stx1* and *stx2*, respectively. Verotoxins belong to AB5 toxin family with A and B subunits. The pentameric B subunit binds to the target cell glycolipid receptor, globotriaosylceramide (Gb3), and gets internalized by receptor-mediated endocytosis. After internalization, the subunit A interacts with 60S ribosomal subunit and inhibits protein synthesis, thereby leading to cell death (Weeratna and Doyle, 1991; Tam and Lingwood, 2007). Therefore, an adequate expression of Gb3 receptor on host cells is a pre-requisite for toxin-mediated pathogenesis. The total verotoxin produced by EHEC includes two components, namely extracellular and intracellular. EHEC cells release the extracellular verotoxin component directly into the growth environment, whereas the intracellular portion gets accumulated in the bacterial cells, and is released when bacterial cells are lysed (Yuk and Marshall, 2003, 2004, 2006). These Stx1 and Stx2 toxins are encoded on prophages and are integrated into EHEC genome. The lytic cycle of these prophages gets stimulated during bacterial stress, leading to increased release of the intracellular toxin from lysed bacterial cells through SOS response. Therefore, the use of antibiotics to treat EHEC infections is contraindicated (Croxen et al., 2013).

Since the use of antibiotics is linked to increased mortality (Su, 1995) by increasing intracellular VT release (Su, 1995; Suwalak and Voravuthikunchai, 2009), the current treatment options for EHEC infections are mainly supportive. These include fluid resuscitation, peritoneal dialysis, plasma exchange and appropriate emergency care strategies for complications such as renal failure and anemia (Ho et al., 2012). Moreover, antibiotics can negatively affect the normal

gut flora which in turn can lead to EHEC overgrowth (Su, 1995). Thus, there is a critical need for alternate approaches for controlling EHEC infection in humans. A relatively new strategy which is being increasingly investigated for combating infectious diseases involves targeting microbial virulence rather than growth, where a pathogen's specific mechanisms critical for causing infection or disease symptoms in hosts are inhibited (Rasko and Sperandio, 2010; Khodaverdian et al., 2013). Since anti-virulence agents are neither bacteriostatic nor bactericidal, they exert a reduced selection pressure for the development of bacterial drug resistance (Rasko and Sperandio, 2010; Hung et al., 2005; Cegelski et al., 2008; Mellbye et al., 2008; Clatworthy et al., 2007), and are minimally deleterious to the host gut microflora.

Metals have been used as antimicrobial agents for centuries. However, their usage in medicine and agriculture declined after the discovery of antibiotics. However, due to the emergence of multidrug-resistant pathogens and a shortage of new antibiotics, the use of antimicrobial metals is now undergoing a period of resurgence (Lemire et al., 2013). Many transition metals and metalloids such as copper, silver, and gallium have been reported as effective antimicrobials and anti-biofilm agents against *E. coli, Salmonella, Pseudomonas aeruginosa,* and *Staphylococcus aureus* (Lemire et al., 2013, 2015; Warnes et al., 2012; Wakshlak et al., 2015). Selenium (Se) is a naturally occurring essential microelement recommended for daily intake by the Food and Drug Administration (FDA). Selenium is present in a wide variety of foods and is available as a dietary supplement. Sodium selenite (Na_2SeO_3) is a commonly used dietary supplement containing Se, with proven antifungal and antibacterial properties (Soriano-Garcia, 2004; Kumar et al., 2010).

Phytochemicals have been an important ingredient of traditional and herbal medicine. Extensive research has documented a wide range of beneficial properties with phytochemicals,

including antiseptic, anti-inflammatory, antioxidants and antimicrobial effects (Deans and Ritchie; 1987; Baratta et al., 1998a, b; Alma et al., 2003). Rutin (RT, quercetin-3-rhamnosyl glucoside) is a natural flavone derivative present in buckwheat, asparagus, rhubarb, and berries (Kreft et al., 2006). Various pharmacological effects of RT, including anti-allergic, anti-inflammatory, antitumor, antibacterial, antiviral and antiprotozoal properties have been reported (Yang et al., 2006).

Several animal models have been proposed to investigate the pathogenesis of EHEC. Recently, the nematode *Caenorhabditis elegans* has been well recognized as a naturally infected and genetically tractable animal host to study bacterial pathogenesis (Ritchie et al., 2013), thereby making it suitable for determining host-pathogen interactions. Moreover, the nematode has been identified as an efficient model to screen for novel anti-infectives in enteric bacterial infections.

Based on published literature on the antimicrobial properties of Se and RT, and preliminary research conducted in our laboratory, it is hypothesized that Se and RT are effective in attenuating EHEC virulence by reducing the expression of verotoxins and impeding host cell receptor–toxin-mediated pathogenesis. In addition, Se is effective as an anti-biofilm agent on food contact surfaces, and RT decreases heat resistance of EHEC in ground beef. The specific objectives of this dissertation include:

1. Investigate the effect of Se in inhibiting and inactivating EHEC biofilms on food contact surfaces.
2. Investigate the effect of RT in enhancing thermal destruction of EHEC in undercooked ground beef.
3. Determine the effect of Se in reducing EHEC verotoxin production and Gb3 receptor expression on host cells.

4. Determine the effect of RT in reducing EHEC verotoxin production and toxin binding to Gb3 receptor.

5. Determine the anti-EHEC effect of Se and RT in *Caenorhabditis elegans* infection model.

CHAPTER II

Review of literature

Escherichia coli is a Gram-negative, chemoorganotrophic, oxidase-negative, commensal bacterium, which constitutes ~ 1% of the gastrointestinal population of the mammalian gut (Brenner et al., 2005). *Escherichia coli* was first described by the German physician, Dr. Theodor Escherich in 1885 (Escherich, 1989). It is a member of *Enterobacteriaceae*, closely related to *Salmonella*, *Serratia*, and *Klebsiella* (Brenner and Farmer, 2005). Physiologically, *E. coli* is a facultative anaerobe possessing the ability to sustain life with or without oxygen. Furthermore, its ability to survive short exposures of pH levels as low as 2.0 enables the successful passage of *E. coli* through the acidic stomach environment prior to its intestinal colonization (Small et al., 1994). Owing to its hardiness, versatility, and ease of handling, *E.coli* has been one among the most thoroughly studied microorganisms in the history of biological sciences (Blount, 2015; Alteri and Mobley, 2012). The findings utilizing this bacterium have shed light on understanding and illustrating fundamental genetic and biochemical evolution in bacteria. Moreover, several *E.coli* strains have been identified as platforms for genetic engineering for a number of applications, including recombinant protein production (Richins et al., 1997; Terpe, 2006), biosensor development (Chalova et al., 2009, 2010), and as indicator probes in biotechnological studies (March et al., 2003; Sanchez and Golding, 2013).

Escherichia coli is a constituent of the normal flora of the intestinal tract of humans and other warm-blooded animals (Drasar and Hill, 1974). Although most *E. coli* are believed to be normal commensals, there are pathogenic strains of *E. coli* associated with human and animal diseases. Many of the pathogenic strains cause enteric diseases ranging from mild diarrhea to severe dysentery; some colonize the urinary tract causing cystitis or pyelonephritis, or others may even cause extraintestinal septicemia and meningitis. These pathogenic strains are diverse and differ from those that predominate the enteric flora of healthy individuals. The acquisition of

multiple virulence determinants such as toxins, adhesins, and effector proteins in commensal *E. coli* can occur through the transfer of mobile virulence plasmids, phages, and pathogenicity islands (PAI), and this is believed to have led to the evolution of pathogenic *E.coli.* To date, eight *E.coli* pathovars have been identified. They are broadly classified as either diarrheagenic *E. coli* or extraintestinal *E. coli* (ExPEC). The six diarrheagenic pathovars include enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). The two most common ExPEC include uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) (Croxen and Finlay, 2010). Within each pathotype classification, *E. coli* strains are further characterized based on O-antigen (lipopolysaccharide), H-antigen (flagellar), and K-antigen (capsular) (DebRoy et al., 2011).

Enterohemorrhagic *Escherichia coli* (EHEC)

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged as the predominant cause of bloody diarrhea and hemorrhagic colitis in humans, occasionally resulting in life-threatening systemic complications, including hemolytic uremic syndrome (HUS) and renal failure. Being a food and waterborne enteric pathogen, EHEC has been associated with numerous outbreaks globally, constituting a serious public health threat. Among the 380 different EHEC serotypes, the antigenic variant O157: H7 is commonly associated with foodborne disease outbreaks in the United States. The serotype O157: H7 was first implicated in a foodborne illness reported in 1982, and during the subsequent 10 years, approximately 30 outbreaks were recorded in the US (Griffin and Tauxe, 1991). However, O157: H7 received considerable attention after a large foodborne disease outbreak traced back to the consumption of undercooked, contaminated hamburgers served at a fast-food restaurant chain in the Western United States (CDC, 1993). More than 700 people in four

different states were infected, and there were 51 HUS cases and four deaths (Feng, 1995). Since that outbreak, enhanced surveillance systems have been implemented and awareness about the pathogen has increased among physicians, clinical microbiologists, public health scientists, and consumers. There have been 554 outbreaks, 12250 illnesses, 2005 hospitalizations and 35 deaths linked to this pathogenic *E.coli* (CDC, 2017).

Evolution of EHEC

EHEC is a subset of the larger group of Shiga-like toxin producing *E. coli* (STEC), which produce Shiga-like toxins (Stx). For epidemiological reasons, the toxins are alternatively referred to as verotoxins (VT), and the organisms that produce these toxins as VTEC. Evolutionarily, the O157: H7 serotype is most closely related to an enteropathogenic *E. coli* clone of serotype O55: H7, a non-Stx-producing strain associated with infantile diarrhea, although it is a distinct clone distantly related to other Stx-producing EHEC (Feng et al., 1998). Feng and colleagues formulated an evolutionary model from genotypic and phenotypic data collected, which explained the series of steps that led to the emergence of O157: H7. Based on the model's assumption, during divergence, the gain of most functions occurred via lateral or horizontal transfer of mobile gene elements. Interestingly, the loss of function during the evolution greatly exceeded that of gain of function for metabolic genes in the serotype. This observation helped further specific predictions about the history of descent and the order of acquisition of virulence factors in the emergence of the EHEC pathotype. The evolutionary steps outlined in the model began with the ancestral or primitive EPEC-like serotype with the ability to express β glucuronidase (GUD+) and ferment sorbitol (SOR+) similar to most present day *E.coli.* Additionally, EPEC-like O55:H7 ancestor carried the gene, *eaeA* and acquired the *Stx2* gene by transduction from a toxin-converting bacteriophage, resulting in a Stx2-producing O55:H7 strain. From this ancestral strain, the somatic

antigen changed from O55 to O157, giving rise to a hypothetical O157:H7 Stx2-producing ancestor that retained the GUD+ SOR+ phenotype. Further divergence caused the lineage to lose the ability to ferment sorbitol and acquired the *Stxl* gene, probably by phage conversion. This led to an intermediate ancestral stage still possessing the primitive traits, GUD+ and Stx2+ along with the derived states (SOR-, Stx1+). Later in the evolutionary tree, the clones lost the GUD activity, producing the immediate ancestor of the common O157: H7 clone (ET1), which spread globally (Feng et al., 1998).

EHEC virulence

Karmali and colleagues in the late 19th century found that patients presented with diarrhea and HUS were positive for a toxin capable of inducing irreversible cytotoxicity in cultured Vero cells (African green monkey kidney epithelial cells), and identified EHEC as the pathogen (Karmali et al., 1985). The toxin was found to be structurally and antigenically similar to the toxin produced by *Shigella dysenteriaie* type 1, which resulted in the name, Shiga-like toxin. EHEC strains produce two immunologically distinct toxins, Stx1 or Stx2, alone or both. Nevertheless, O157: H7 strains that produce Stx2 are associated with an increased risk of systemic complications (Donohue-Rolfe et al. 2000). As mentioned, Stx1 is nearly identical to the *S. dysenteriae* toxin, differing by a single amino acid, whereas Stx2 was initially distinguished from type 1 by distinct antiserum neutralization (Melton-Celsa, 2014; Bryan et al., 2015). Both shiga-like toxins are produced via a single operon containing two genes, *stxA* and *stxB*, and at least one promoter. The Shiga-like toxin or verotoxin operon(s) are found to be embedded within the genome of integrated lambdoid prophages, and they are believed to have been acquired either by horizontal transmission via phage and/or by direct inheritance (Garcia et al., 2010; Bryan et al., 2015). Shiga-like toxins are AB5 toxins, composed of a single copy of the ~ 32 kDa A component (encoded by *stxA*) and

5 copies of the ~ 8 kDa B component (produced by *stxB*). The A component bears the enzymatic activity of the toxin, whereas each B subunit possesses three binding sites for the toxin receptor (Fraser et al., 1994, 2004). Other biotoxins, including pertussis toxin, cholera toxin, and heat-labile enterotoxin of enterotoxigenic *E coli* also belong to the AB5 family (Beddoe et al., 2010). Environmental stressors, including antibiotics induce the activation of the operon promoters, which are embedded within the prophage genome. Thus, upon induction, Stx-encoding bacteriophages can increase the toxin production. Therefore, until last decade, it was assumed that the prophage lytic cycle is strongly linked to the toxin production. However, recently there is some evidence suggesting that Stx can be delivered via outer membrane vesicles (OMVs) without cell lysis (Bielaszewska et al., 2017; Yokoyama et al., 2000). Nevertheless, the mechanisms by which the Stxs are delivered to the systemic circulation have not been understood fully. Despite the knowledge gap, Stx remains as the key virulence factor that defines EHEC pathogenesis in humans (Sakagami et al., 2001).

Shiga-like toxins or verotoxins use a carbohydrate moiety for endocytic transport into their target cells. The pentameric B subunit binds to the host cell glycolipid receptor, globotriaosylceramide (Gb3), and gets internalized by receptor-mediated endocytosis. Therefore, adequate expression of Gb3 receptor on host cells is a pre-requisite for toxin-mediated pathogenesis. However, the expression of Gb3 is restricted to only certain tissues, and the cell types expressing Gb3 vary among hosts. For example, Gb3 content is found abundantly in the microvascular glomeruli and proximal tubule cells of the kidney, which justifies the renal pathology of HUS. Gb3 is also found in intestinal microvascular endothelial cells, including colon and central nervous system. Therefore, during infection with STEC, main toxin target sites are the vascular endothelium of the colon (Omhi et al., 1998), kidneys and the central nervous system

(Ren et al., 1999). Moreover, Gb3 is expressed in platelets and constitutes the rare Pk antigen present on erythrocytes (Steffensen et al., 2000). In the immune system, Gb3 represents a lymphocyte differentiation antigen, termed CD77, which is expressed in a subset of germinal center B lymphocytes (Mangeney et al., 1991). It is also known that Gb3 expression is often increased in cancer cells (Engedal et al., 2011). However, the precise physiological role of Gb3 is not clear, and also the reason behind the restricted Gb3 expression in certain tissues is unknown.

After receptor-mediated internalization, verotoxin gets transported through early endosomes and recycling endosomes, and to the Golgi apparatus. From the Golgi, the toxin is transported retrogradely to the endoplasmic reticulum, where it is catalytically active. The A subunit is released and translocated to the cytosol (Sandvig et al., 1989; 2010), which then interacts with 60S ribosomal subunit and inhibits protein synthesis, thereby leading to cell death (Weeratna and Doyle, 1991; Tam and Lingwood, 2007). The A subunit exhibits an RNA N-glycosidase activity against 28s rRNA, which induces apoptosis (Sandvig et al., 2010). Consequently, any interventions that interfere any of these transport factors could impede proper intracellular transit of verotoxin and protect cells against the cytotoxic action of the toxin. For example, Stechmann et al. (2010) identified two low molecular weight substances called Retro-1 and Retro-2, which inhibited the retrograde transport of Stx1B from endosomes to the Golgi apparatus without affecting the compartment integrity and endogenous retrograde transport. This in turn phenotypically reduced the cytotoxicity induced by Stx1 and Stx2 in A459 and HeLa cells, when added 30 min prior to challenge with the toxins (Stechmann et al., 2010).

EHEC pathogenesis is however not solely due to toxin-mediated effects, and the infection is initiated by a number of other supporting virulence factors, including adhesins such as intimin, flagella, lipopolysaccharides and Type III secretion system. All of these contribute to effective

EHEC colonization, marked cytoskeletal rearrangements, disruption of intestinal barrier function, downregulation of host inflammatory response, and induction of host cell apoptosis (Barnett Foster, 2013). In addition, the large EHEC pO157 plasmid carries a homolog of the *lifA* gene encoding lymphostatin, genes encoding a type II secretion system, catalase-peroxidase (katP), a secreted serine protease (espP), and a hemolysin operon (Nataro and Kaper, 1998; Farfan and Torres, 2012).

Epidemiology of EHEC

Animal reservoirs

EHEC is a globally important zoonotic enteric pathogen. The first recorded EHEC infection in humans was associated with the consumption of contaminated undercooked ground beef burgers. Cattle have been recognized as the major reservoir of EHEC (Ferens and Hovde, 2011). In addition, EHEC carriage has also been reported in bison and cervids (deer, elk), and occasionally in pigs, camelids, rabbits, horses, dogs, cats, zoo mammals such as bears, large cats, and various free-living wild species such as raccoons opossums, and rats. The pathogen has been detected at times in the gut of wild or domesticated birds, including, chickens, turkeys, geese, ostriches, pigeons, gulls, rooks, and starlings. (Anonymous, 2017).

The prevalence of EHEC in cattle has been a topic of much research in the late 19th century, and the role of several EHEC factors in bovine colonization has been well characterized (Sheng et al., 2006). Healthy domesticated ruminants, mainly cattle, and to a lesser extent sheep and goats, carry EHEC transiently without manifesting any pathological symptoms (Blanco et al., 1996; Gyles, 2007; La Ragione eta l., 2009; Ferens and Hovde, 2011). This is explained by the apparent resistance to the systemic effects of verotoxins due to the absence of Gb3 receptors in the bovine intestinal tract and the vasculature (Pruimboom-Brees et al., 2000; Hoey et al., 2002). The primary

site of EHEC colonization in cattle is the terminal rectum, particularly an anatomical area within the terminal rectum referred to as the recto-anal junction (RAJ (Naylor et al., 2003). Fecal shedding of EHEC occurs for a short time (a few days) if individual animals passively shed the pathogen in their feces without establishing a colonized state, or sheds the fecal pathogen for a longer time (a month or more) if the bacteria colonize and persist in the animal gut (Grauke et al., 2002). Thus, a major contributor in animal-to-animal transmission is the high prevalence of EHEC in cattle feces. Another important source leading to carcass contamination is cattle hide. The higher the fecal shedding of EHEC, the greater the extent of hide contamination, and consequently increased contamination of the carcass. This occurs particularly in the summer months, and also in a cattle feedlot with a higher percentage of supershedders (Elder et al., 2000). Super shedders are animals which harbor a high intestinal load of the pathogen for extended periods of time (Ferens and Hovde, 2011). EHEC carriage in cattle thus leads to human infection through fecal contaminated water, meat, produce or by direct contact in petting zoos. Outbreaks have increasingly been reported through environmental contamination of water supplies from feces of grazing animals (Licence et al., 2001; Hrudey et al., 2003) and direct contact with farm animals and their feces when camping on a farm pasture (Strachan et al., 2001) or during a visit to agricultural and animal fairs (Crump et al., 2003).

Food as a source

Food has been the predominant transmission route of EHEC since 1982, constituting 52% of 350 outbreaks and 61% of ~ 9000 sporadic cases (Rangel et al., 2005). EHEC associated foodborne illnesses are estimated to cost the US economy ~ $ 200 million annually (Hoffman et al., 2015). Although a variety of foods have been implicated as vehicles of infection, the majority of EHEC foodborne outbreaks are associated with the consumption of undercooked ground beef

products (Armstrong et al., 1996; Hussein, 2007). Interestingly, in an American case–control study on sporadic EHEC cases, a strong positive association was reported with eating "pink hamburgers" (Kassenborg et al., 2004). Among the 183 foodborne outbreaks reported until 2002, the source of infection for 75 (41%) was ground beef, whereas the remaining 38 (21%) were contributed from produce, 11 (6%) from other beef products,7 (4%) by dairy products and rest unknown (Rangel et al., 2005). In addition, pulsed field gel electrophoresis showed that several human EHEC isolates were found to be indistinguishable to those from undercooked retail burgers (CDC, 1997; Strachan et al., 2006).

The USDA has established a zero tolerance policy for EHEC in ground beef, and recommended that beef patties be cooked to an internal temperature of 71.1°C (160°F) to ensure complete pathogen inactivation (USDA, 1998). Based on the degree of doneness, cooked patties can be classified as rare (60°C/140°F), medium-rare (65°C/149°F), medium (71.1°C/160°F) or well done (77°C/170.6°F) (Marksberry et al., 1993). In order to ascertain that the required internal temperature (71.1°C) is attained during cooking of ground beef patties, the USDA advised consumers to use a meat thermometer. However, because of the lack of homogeneity in patty composition, and temperature monitoring difficulties, there is the possibility that the recommended internal temperature may not be uniformly attained (D'Sa et al., 2000). Further, the use of thermometers for cooking beef patties by consumers is limited (NCBA, 1999; McCurdy et al., 2005) due to the inconvenience of the procedure, consumer uncertainty, and a lack of consumer confidence in a thermometer's ability to ensure food safety (USDA, 1998; Research Triangle Institute [RTI], 2001; McCarty, 2008).

According to a survey conducted by the USDA-ERS, approximately 20% of the US population preferred eating rare or medium-rare patties at home, restaurants, and cafeterias

(Ralston, 2002). The same study revealed that at the internal cooking temperatures specified for rare and medium rare cooked patties, EHEC numbers did not decline significantly. Moreover, most consumers determine the doneness of beef patties by observing the color and texture of cooked meat. However, color is not a good indicator of doneness because ground beef is prone to a non-typical color change associated with cooking called premature browning (PMB) (Claus, 2007), where meat appears fully cooked despite not having achieved a safe internal temperature. Thus, PMB in ground beef can lead to inadequate cooking by consumers who are misled by the cooked color (Warren et al., 1996a, b), potentially allowing EHEC survival. Killinger et al. (2000) reported that PMB incidence averaged about 47% in ground beef purchased from retail stores. When compared to steaks and roasts, ground beef is more susceptible to PMB because of accelerated oxidation of the meat pigment, myoglobin that occurs as a result of grinding. Since the infectious dose of EHEC in humans is low (2–2,000 cells), it is important to avoid undercooking of beef patties (Buchanan, 1997; Strachan et al., 2005).

Besides, ground beef, other common food products implicated with EHEC outbreaks in the United States include lettuce, alfalfa sprouts, spinach, raw milk, unpasteurized dairy products and dry cured salami (Hillborn et al., 1999; Breuer et al., 2001). Apple cider was also identified as a vehicle of EHEC in several previous outbreaks reported since late 1900's (Zhao et al., 1993; Besser et al., 1993; Hilborn et al., 2000; Mamadou et al., 2011). Recently, unpasteurized apple juice and cider have received considerable attention due to local and multistate recalls and outbreaks in different parts of the country (Anonymous, 2015). Fresh produce could be contaminated directly with feces or indirectly via contaminated irrigation and processing water (during vegetable cultivation) (Strachan et al., 2006).

The prevalence of EHEC biofilms is another major concern in the food industry. Biofilms are aggregates of microorganisms adhered to biotic or abiotic surfaces embedded by a self-produced extra-polymeric matrix, which in turn facilitates survival in a hostile environment (Costerton et al., 1999). The presence of EHEC biofilms in food processing environments, animal slaughterhouses and meat packing plants has been well documented, and these biofilms possibly act as continuous sources of contamination of food products (Kumar and Anand, 1998; Aslam et al., 2004; Rivera-Betancourt et al., 2004; Sharma and Bearson, 2013; Vogeleer et al., 2014). Studies have shown that contamination of beef carcasses with EHEC could occur during different stages of processing such as slaughtering, dressing, chilling or cutting (Bacon et al., 2003; Koutsoumanis and Sofos, 2004). The ability of EHEC to form biofilms on different equipment surfaces has been linked to the distribution and persistence of EHEC in meat processing plants (Carpentier and Cerf, 1993; Dewanti and Wong, 1995; Aslam et al., 2004; Rivera-Betancourt et al., 2004). Furthermore, many EHEC outbreaks associated with the consumption of fresh produce, such as cabbage, celery, radish sprouts, lettuce, spinach, and tomatoes have also been linked to surface colonization by EHEC biofilms (Beuchat 2002; Brandl 2006).

Human transmission

Although EHEC transmission to humans is principally via consumption of contaminated food and water, interpersonal spread can also contribute to outbreaks (Ryan et al., 1986; Carter et al., 1987). Person-to-person transmission of EHEC contributing to disease spread during outbreaks usually occurs via the fecal-oral route. Infants and young children tend to shed the pathogen although humans are not believed to be a reservoir of EHEC. According to Rangel et al (2005), until 2002, 50 outbreaks were reported via the fecal-oral route of transmission between ill and

immunocompromised individuals. These outbreaks mainly included child-care centers, individual residences, community centers, school and residential facilities.

Treatment strategies against EHEC infection

Currently, supportive care measures only are recommended for treating EHEC infections and antibiotics are contraindicated. The conventional antibiotic usage has been reported to exacerbate verotoxin-mediated cytotoxicity. Several studies observed increased chance of developing renal failure in children on antibiotic therapy for HUS associated with EHEC infection (Wong et al., 2000; Zimmerhackl, 2000; Safdar et al., 2002; Tarr et al., 2005). Antibiotics enhance the replication and expression of *stx* genes encoded within a chromosomally integrated lambdoid prophage genome resulting in increased verotoxin production. It also causes phage-mediated lysis of EHEC cell membrane, allowing the release and dissemination of the toxin into the environment (Karch et al., 1999; Matsushiro et al., 1999; Wagner et al., 2002). Moreover, antibiotics can negatively affect the normal gut flora, which in turn can lead to EHEC overgrowth (Su, 1995). Furthermore, several drugs, including antimotility agents, narcotics, and non-steroidal anti-inflammatory medications are not advised for EHEC-infected patients (Tarr et al., 2005).

Generally, HUS is manifested as acute renal failure, hemolysis, and thrombocytopenia with the disruption of clotting cascade and perturbation of fluid and electrolyte balance, potentially leading to stroke (Ruggenenti et al., 2001; Tarr, 2005). The syndrome is, therefore, better managed by a multi-targeted approach, which includes fluid resuscitation, peritoneal dialysis, plasma exchange and appropriate emergency care strategies for complications such as renal failure and anemia (Holtz et al., 2009; Ho et al., 2013). As alternate treatment modalities, studies have investigated monoclonal antibodies (MAbs) such as Urtoxazumab, antithrombotic agents, plasma exchange therapies (PLEX), tissue-type plasminogen activator, oral Stx-binding agents (Synsorb

PK), and eculizumab for their efficacy in the management of HUS (Lopez et al., 2010; Rocha et al., 2010; Trachtman et al., 2012; Goldwater and Bettelheim, 2012). Despite these studies, data supporting the use of the aforementioned treatment strategies for helping patients recover from the illness are still lacking. Thus there is an urgent need for the development of alternative treatment approaches for treating and controlling EHEC infections in humans.

Recently, new approach of targeting virulence of a pathogen for controlling infectious diseases is widely explored. Since virulence factors of pathogens contribute to the establishment of infection in a host, inhibition of these factors could prevent disease progression (Defoirdt, 2016; Rasko and Sperandio, 2010; Khodaverdian et al., 2013). Thus, a viable strategy for controlling EHEC infections is the use of drugs against the virulence factors of the pathogen. Moreover, this class of drugs presents a lesser selective pressure on development of bacterial resistance compared to traditional antimicrobial therapy (Rasko and Sperandio, 2010; Hung et al., 2005; Cegelski et al., 2008; Mellbye et al., 2008; Clatworthy et al., 2007). Therefore, novel approaches for treating EHEC infections in humans could be directed against verotoxins, toxin receptor, and their interaction, thereby reducing the cellular uptake of toxins and downstream pathogenesis.

Antimicrobial metals

Metals have been used as antimicrobial agents since antiquity. Copper and silver were used to disinfect and preserve water, milk, and vinegar by North American settlers. Japanese soldiers used these metals as antimicrobial agents during the Second World War to prevent the spread of dysentery (Alexander, 2009; Borkow et al., 2009). However, their usage in medicine and agriculture decreased gradually after the discovery of antibiotics. Owing to the emergence of antibiotic resistant pathogens and a dearth of new antibiotics under discovery, this decade is witnessing the resurgence in antimicrobial metal research. Many transition metals and metalloids

such as copper, silver, and gallium have been reported as effective antimicrobials and anti-biofilm agents against *E. coli, Salmonella, Pseudomonas aeruginosa and Staphylococcus aureus* (Lemire et al., 2013; Warnes et al., 2012; Wakshlak et al., 2015). The ability of metals to remain stable and withstand high temperature and pressure has made them ideal for application in food processing area (Murakoshi et al., 2003; Fu et al., 2005). Further, advancements in nanotechnology have enhanced the potential of using some metals or their oxides in sizes less than 100 nm. Studies have documented selective toxicity of metal nanoparticles to bacterial pathogens with minimal effect on human cells, which enhances their potential use in agricultural and food industries (Brayner et al., 2006; Jiang et al., 2009; Reddy et al., 2007; Zhang et al., 2007). Thus antimicrobial metals offer natural and safe sanitizing agents, food additives, and antimicrobial treatments to reduce foodborne pathogens in the food chain continuum. The antibacterial mode of action of metals has been elucidated through several approaches during the past decade. These studies indicate that there are no exclusive mechanisms by which metals inactivate microbial cells. However, there exist a few well-characterized mechanisms, including the production of reactive oxygen species, antioxidant depletion, inactivation of essential bacterial proteins, membrane function impairment and genotoxic effects, as discussed in the review by Lemire and colleagues (2013).

Selenium (Se) is a metalloid, well-recognized as a dietary antioxidant, and its compounds are commonly used in nutrition and chemoprevention therapy (Estevam et al., 2015). It is an essential component of several enzymes, including glutathione reductase, and is recommended for daily dietary intake in humans by the Food and Drug Administration (FDA), with an upper tolerable intake level of 400 -800 µg (Sunde, 2012). Several selenium-based formulations such as selenomethionine and sodium selenite (Na2SeO3) are available commercially as food supplements, anticancer agents, and immune stimulators. The antibacterial and antifungal

properties of Se were studied during the early 20th century (Soriano-Gracia et al 2004; Kumar et al., 2010). Xi et al (2002) reported potential antibacterial property of four different Se compounds on *S. aureus* using microcalorimetry. In 2011 Vasić et al. (2011) observed that sodium selenite affected growth and extracellular protein synthesis in multiple pathogens, including *B. subtilis*, *B. mycoides*, *E. coli*, and *Pseudomonas spp*. These researchers also showed that sodium selenite increased the inhibitory effect of antibiotics on all pathogens tested *in vitro*. Furthermore, anti-toxigenic effect of Se against *Vibrio cholerae* was reported recently (Bhattaram et al., 2017). Moreover, *in vitro* studies with Se showed that the essential mineral strongly inhibited spore germination, germ tube elongation and mycelial spread in *Penicillium expense* (Wu et al., 2014). Similar results were reported on spore germination in *Botrytis cinerea*, which causes spoilage in tomatoes (Wu et al., 2015). Recently, studies have evaluated the potential of Se to inhibit Ebola and other enveloped viruses as well (Lipinski, 2015; Lyons, 2014). During the latest Ebola outbreaks, it was identified that a deficiency in Se could lead to increased viral pathogenesis. This observation supported the earlier findings of Beck (2007) that adequate intake of Se could protect the host against viral infections.

In a study published in 2015, the biological activity and role of different selenium compounds against antibiotic resistant pathogens such as *S.aureus* were investigated (Estevam et al., 2015). The results demonstrated that that in addition to the direct toxicity effects on microbial cells, they also enhanced the antibacterial potential of antibiotics, by bioreductive formation of insoluble elemental deposits, thereby inferring with the bacterial protease machinery/proteasome. (Estevam et al., 2015)However, some of the previous studies speculated that selenite compounds exerted their antimicrobial activity by increasing oxidative stress, damaging the DNA, depleting the cellular thiostat (Jacob, 2011). Additionally, owing to its pro-oxidant property, Se generates

superoxide radicals on interactions with bacterial membranes, damaging the cellular components (Tran and Webster, 2013; Seko, 1997). Therefore, strong positive genotoxic effects, including DNA-damaging capacity and mutagenicity explained "metal toxicity" to bacteria.

During the last decade, Se has been introduced as a potential biomolecule in the field of nanotechnology owing to its excellent antibacterial, antiviral, and antioxidant activity (Shakibhaie et al., 2015). Antibiofilm activity of biosynthesized and synthetic Se nanoparticles were demonstrated against several foodborne pathogens, including *B. cereus, Enterococcus faecalis, S.* Typhimurium, and *S.* Enteritidis (Khiralla and Deeb, 2015; Tran and Webster, 2011), and against drug-resistant nosocomial pathogens such as methicillin-resistant *S. aureus* (MRSA) (Tran and Webster, 2011). Investigations on the pharmacokinetics of Se after oral administration of sodium selenite in rats revealed an absorption rate-limiting elimination in plasma profiles. The bioavailability of oral sodium selenite was about 49% and the renal excretion was identified as a major route of Se excretion after absorption (Natsuhori, 1998). Se-methylseleno-N-acetyl-galactosamine and trimethyl selenonium-ion were identified as the urinary excretory metabolites (Loeschner et al., 2014). Nevertheless, based on toxicity studies conducted in mice, the estimated no-observed-adverse-effect level (NOAEL) in mice for sodium selenite is 0.9 mg selenium/kg (16 ppm sodium selenite) (Abdo, 1994), whereas in humans, sodium selenite was found safe and tolerable when administered up to 10.2 mg/m^2 i/v (Brodin et al., 2015). The most common adverse effects associated with Se toxicity in humans were fatigue, nausea, and cramps in fingers and legs, with no identifiable biomarkers for organ toxicity (Brodin et al., 2015).

Phytochemicals

Phytochemicals have been an important ingredient of traditional and herbal medicines, and used prophylactically and therapeutically for controlling metabolic and infectious diseases.

Several studies have reported a wide range of beneficial properties with phytochemicals, which include antiseptic, anti-inflammatory, antioxidants and antimicrobial effects (Deans and Ritchie, 1987; Baratta et al., 1998; Alma et al., 2003). The majority of phytochemicals are secondary metabolites, elaborated in response to pathogen invasion or environmental stress cues. Consequently, phytochemicals play a critical role in plant health and defense rather than regulating or modulating plant physiology (Reichling, 2010). One of the main advantages of using phytochemicals for therapeutic purposes is that antimicrobial resistance to these natural antimicrobials have not been documented, apparently due to their multiple modes of action which would impede the selection of resistant bacteria from the exposed population (Van Wyk et al., 2000). In light of a wide spectrum of antimicrobial effects and margin of safety and affordability, the majority of phytochemicals are classified as GRAS (Generally recognized as safe) compounds by FDA.

Several studies have demonstrated the antimicrobial properties of phytochemicals and their effectiveness in controlling pathogenic and spoilage microorganisms. These natural molecules impede bacterial viability by degrading the cell wall, damaging cytoplasmic membrane and membrane proteins, causing leakage of intracellular contents, coagulating cytoplasmic proteins and abetting the depletion of proton motif force (Burt, 2004; Savoia, 2012; Negi, 2012). Modulating bacterial virulence by regulating gene transcription, protein expression, and quorum sensing at sub-inhibitory or sub-lethal concentrations is relatively a newer approach in the use of phytochemicals as anti-virulence agents (Upadhyay et al., 2015). Although, the exact target(s) for phytochemicals are often not well defined, based on the active ingredients present, we could hypothesize the interacting reactions taking place in the context of antimicrobial action. For instance, terpenoids and phenolics often cause membrane disruption, phenols and flavonoids

exhibit metal chelation, whereas coumarin and alkaloids are known to affect the bacterial genetic material (Cowan, 1999). Nevertheless, it is important to note that these interactions are not "all or none" since some membrane-disrupting compounds can also cause leakage of cellular content, interfere with active transport or metabolic enzymes, or can dissipate the cellular energy (Davidson et al., 2013).

Flavonoids are one of the important classes of phytochemicals distributed widely in the plant kingdom, and are largely incorporated in the daily diet (Cushnie, 2005; Sandhar et al., 2011; Romano et al., 2013). They are found in a variety of fruits, vegetables, seeds, nuts, stem, flowers tea, wine and even honey (Middleton and Kandaswami, 1994). Approximately, 4000 types of flavonoids have been reported to be present in plants (Guardia et al., 2001). They are basically polyphenolic compounds that contain benzopyrone moiety. The basic structure of a flavonoid is 2-phenyl-benzo-γ-pyrane nucleus consisting of two benzene rings linked through a heterocyclic pyran ring (Brown, 1980). There are 14 classes of flavonoids, categorized on the basis of the chemical nature (Savoia, 2012). They include mainly flavonols, flavones, flavanones, isoflavone, catechin, anthocyanidin, and chalcones. However, in plants, they are often present as pigmented compounds in fruits and flowers, and chemically as O-glycosides or C-glycosides. Based on research, the dietary intake of flavonols and flavones was found to be 23 mg/day, among which, flavonol quercetin contributed the maximum of 16 mg/day (Cushnie, 2005; Heim, 2002). Flavonoids have been reported to exert a wide range of biological activities, including anti-inflammatory, antilipidemic, antihyperglycemic, antiviral, hepatoprotective, gastric antiulcer, cardioprotective, neuroprotective, antioxidant and anticancer actions (Sandhar et al., 2011; Romano et al., 2013).

Most of the flavonoids are used as constitutive antimicrobial ingredients for centuries, and many studies identified different classes of flavonoids possessing antifungal, antiviral and antibacterial activity (Cushnie, 2005; Tapas et al., 2008; Sandhar et al., 2011; Romano et al., 2013). Quercetin and naringenin extracted from tribal medicinal plants were reported to be antimicrobial against *Bacillus subtilis*, *Candida albicans*, *E. coli*, *S. aureus*, *S. epidermis* and *Saccharomyces cerevisiae* (Taleb-Contin et al., 2003). Similarly, Rattanachaikunsopon and coworkers (2010) isolated morin-3-O-lyxoside, morin-3-O-arabinoside, quercetin, quercetin-3-O-arabinoside from *Psidium guajava* leaves and reported their bacteriostatic action against different foodborne bacteria, including *Bacillus stearothermophilus, Brochothrix thermosphacta, E. coli, L. monocytogenes, Pseudomonas fluorescens, Salmonella* Enteritidis, *S,. aureus* and *V. cholerae.* Additionally, there are literature reports on the effect of flavonoids on the binding of enterotoxins such as staphylococcal enterotoxin B (Benedik et al., 2014). Further, numerous studies have sought to elucidate the antibacterial mechanisms of different flavonoids, which include their ability to inhibit the cytoplasmic membrane function (Ikigai et al., 1993), deplete energy metabolism (Haraguchi et al., 1998) and compromise nucleic acid synthesis (Mori et al., 1987). The glycosylated flavonol, rutin, for example, was found to inhibit bacterial DNA gyrase. As the topoisomerase IV found to be essential for bacterial cell survival, the rutin-induced topoisomerase IV-mediated DNA cleavage thus led to an SOS response, thereby inhibiting the growth of *E. coli* (Bernard et al., 1997).

Rutin is chemically 3, 30, 40, 5, 7-pentahydroxyflavone-3-rhamnoglucoside, also called as rutoside, quercetin-3-rutinoside, and sophorin. It is a citrus flavonoid glycoside abundantly present in passion flower, buckwheat, tea, and apple (Harborne, 1986; Kreft et al., 1999). Similar to many other flavonoids, rutin also possesses a number of pharmacological activities, including

antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities (Ganeshpurkar and Saluja, 2016). Rutin has also been studied extensively for its antimicrobial activity against various bacteria, virus and fungi (Arima et al., 2002; Ibrahim et al., 2013). For instance, Choi et al (2012) demonstrated the antiviral activity against influenza A/WS/33 virus in mice, whereas Han (2009) used rutin to treat *C. albicans* associated septic arthritis. Antioxidant and antimicrobial activities of rutin were also identified in different food systems by Stojkovic´ et al (2013). Likewise, de Queiroz Pimentel and coworkers isolated rutin from honey and evaluated its antimicrobial activity against *S. aureus, E. coli* 0157: H7*, Proteus vulgaris, Shigella sonnei and Klebsiella spp.* (de Queiroz Pimentel et al., 2013). Rutin was also found to inhibit biofilm formation by foodborne drug-resistant *E. coli* and *S. aureus* (Al-Shabib et al., 2017).

Flavonoids, in general, have minimal toxicity due to their wide distribution in edible plant products, and rutin demonstrated no significant carcinogenic potential *in vivo* (Morino et al., 1982). According to investigations on the pharmacokinetics and bioavailability of quercetin glycosides in humans, after oral administration, rutin is absorbed in the intestine and peak plasma level reaches ~7.0 ± 2.9 hours after ingestion with a terminal elimination half-life of about 11 hours (Graefe et al., 2001; Andlauer et al., 2001). Rutin was found to get absorbed more slowly than other quercetin compounds since its gets hydrolysed by the cecal microflora (Manach et al., 1997). Rutin circulated in plasma in conjugated forms and its clearance is mostly renal (Choudhury et al., 1999; Marin et al., 2015). In studies conducted in rodents, a high concentration of conjugates was maintained in plasma with a regular supply of rutin in diet (Manach et al., 1997). After oral ingestion of rutin, 3, 4-dihydroxyphenylacetic acid (DHPAA) was found to increasingly circulate in the urine of rabbits identifying HPAA as a potential metabolite of the molecule (Booth et al.,

1956; Pashikanti et al., 2010). Meanwhile, in humans and rodents, the methylated form of rutin, namely isorhamnetin, was found to be the renal metabolite that was eliminated (Manach et al., 1997; Graefe et al., 2001).

Animal models of EHEC infection

Several animal models have been proposed for investigating the pathogenesis of EHEC. Among these, some of them served as infection models, in which animals are orally inoculated with live EHEC in order to study the colonization effects. Meanwhile, others are injection models, in which they are parenterally inoculated with vertoxins intending to evaluate the effects of toxin. However, no animal model to date reproduces the full spectrum of clinical disease induced by EHEC in humans despite 30 years of research on EHEC pathogenesis (Ritchie et al., 2015; Mohawk and O'Brien, 2011). At present, pigs, rabbits, and mice are the three most common mammalian species used to study EHEC-mediated disease (Ritchie et al., 2015). Among the mouse infection models, germ-free and streptomycin-treated models facilitate better EHEC colonization by neglecting or suppressing the effects of normal enteric flora (Mohawk and O'Brien, 2011). Other animal species occasionally considered as model hosts of EHEC-induced disease are ferrets, greyhounds, gnotobiotic calves, and primates (Ritchie et al., 2013). Recently, the nematode *Caenorhabditis elegans* has been recognized as a naturally infected and genetically tractable invertebrate host to study EHEC infection (Ritchie et al., 2013).

Several characteristics of *C. elegans* make it suitable for studying host-pathogen interactions. Its rapid life cycle, ease of conducting research, genetic screens, and sharing of conserved innate immune pathways with humans (Irazoqui et al., 2010) provide benefits in investigating EHEC pathogenesis. Moreover, reports indicate that intestinal cells of the nematode share similar anatomic features with humans (McGhee, 2007), which makes it an ideal model to

study infections caused by enteric pathogens. Furthermore, EHEC can colonize and induce the characteristic attaching and effacing lesions in the nematode gut, and shiga-like toxin 1 (Stx1) is identified to be a partial requirement for EHEC pathogenicity in *C. elegans* (Chou et al., 2013). All these observations suggest that EHEC exerts similar and conserved virulence mechanisms in *C. elegans* and humans. An additional advantage of using *C. elegans* is that the pathogenic bacterium under study can be substituted in place of the normal feed of the nematode (*E. coli* OP50) as the nutritional source, and the primary site of the infection will be the intestine. Moreover, phenotypes such as animal survival, motility, and pathogen burden can be easily and noninvasively examined in *C. elegans* (Marsh and May 2012). The nematode has also been identified as an efficient model to screen for novel anti-infective agents against bacterial infection. It has been hypothesized that these nematode-based screens would identify the efficacy of those antimicrobials in blocking microbial virulence, inhibiting bacterial growth and stimulating host immune responses (Sifri et al., 2005; Moy et al., 2006, 2009).

In summary, EHEC is a major food-borne pathogen causing severe morbidity and mortality in humans. Controlling EHEC persistence in processing facilities and inactivating the pathogen in undercooked ground beef could reduce food-borne disease outbreaks in humans. Moreover, attenuating EHEC virulence would reduce the severity of infection and improve disease outcome in humans. This Ph.D. dissertation targets EHEC from both these aspects, and is expected to yield potential new strategies for reducing risks due to this pathogen to humans.

Hypothesis

Based on published literature and preliminary research, it is hypothesized that Se and RT are effective in attenuating EHEC virulence by reducing verotoxin synthesis and impeding host

cell receptor–toxin-mediated pathogenesis. In addition, Se is effective as an anti-biofilm agent on food contact surfaces and rutin increases heat sensitivity of EHEC. The specific objectives include:

1. Investigate the effect of Se in inhibiting and inactivating EHEC biofilms on food contact surfaces.

2. Investigate the effect of RT in enhancing thermal destruction of EHEC in undercooked ground beef.

3. Determine the effect of Se in reducing EHEC verotoxin production and Gb3 receptor expression on host cells.

4. Determine the effect of RT in reducing EHEC verotoxin production and toxin binding to Gb3 receptor.

5. Determine the anti-EHEC effect of Se and RT in *Caenorhabditis elegans* infection model.

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CHAPTER III

Investigating the efficacy of Se for inhibiting and inactivating EHEC biofilms on food contact surfaces

ABSTRACT

Biofilms are groups of microorganisms enclosed in a self-generated extracellular polysaccharide (EPS) matrix and can adhere to surfaces creating a dynamic environment to utilize available nutrients efficiently. *Escherichia coli* O157:H7 (EHEC) is a potent biofilm-forming foodborne pathogen and its biofilms have been identified as a source of food contamination and foodborne disease outbreaks. Owing to the high resistance of biofilms to most of the approved chemical sanitizers and disinfectants, there is an increasing interest in identifying effective treatments for controlling EHEC biofilms on food processing equipment and environments.

Selenium (Se) is a naturally occurring dietary essential element with demonstrated antibacterial and antioxidant properties. This study investigated the effect of Se in reducing EHEC EPS synthesis, inhibiting biofilm formation at 37ºC and 25ºC on polystyrene surface, and inactivating mature EHEC biofilms in combination with hot water. Sterile 96-well polystyrene plates inoculated with EHEC (~6.0 log CFU/well) were treated with sub-inhibitory concentrations (SIC) of Se, and biofilms were allowed to mature at 4ºC and 25ºC for 96 h. Biofilm-associated bacterial population was determined by scraping and plating, whereas the extent of EPS production was determined using ruthenium red staining assay. Solid Surface Assay was used to study the effect of Se on early attachment of EHEC cells to polystyrene. The efficacy of Se in rapidly inactivating pre-formed, mature EHEC biofilm was investigated by treating biofilms on polystyrene plates with the MBC and 2XMBC of Se in combination with hot water at 80 °C with a contact time of 0, 2 and 5 min. Furthermore, the effect of Se on EHEC biofilm architecture was visualized using confocal microscopy, whereas the effect of Se on EHEC biofilm genes was determined using real-time qPCR. Finally, the potential feasibility of coating stainless steel surface with Se nanoparticles to inhibit EHEC biofilm formation was studied.

Selenium reduced early attachment of planktonic cells, biofilm formation and EPS synthesis in EHEC ($P < 0.05$). Selenium in combination with hot water (80°C) reduced biofilm-associated bacterial counts to undetectable levels at 5 min of exposure compared to control ($P < 0.05$). However, hot water treatment alone decreased biofilm-associated bacterial counts by only 1.0 log CFU/ml. RT-qPCR results revealed that Se down-regulated the transcription of critical genes associated with biofilm synthesis in EHEC ($P < 0.05$). The results collectively suggest that Se could potentially be used for controlling EHEC biofilms in food processing environment, but appropriate applications need to be validated.

Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC) is a major foodborne pathogen that causes a serious illness in humans, including hemorrhagic diarrhea and hemolytic uremic syndrome (HUS), characterized with kidney failure. EHEC poses a significant public health concern owing to its ability to produce fatal food-borne infections with a low infectious dose (2–2,000 cells) (Buchanan, 1997; Strachan et al., 2005). EHEC associated foodborne illnesses are estimated to cost the US economy ~ 200 million dollars annually (Hoffman et al., 2015). Although a variety of foods have been implicated as vehicles of infection, the majority of EHEC foodborne outbreaks are associated with the consumption of undercooked ground beef products (Armstrong et al., 1996; Hussein, 2007). Cattle serve as the principal reservoir of EHEC, excreting the pathogen in feces, thereby contaminating food, water, and the environment. Besides its presence in animal reservoirs, EHEC has the ability to persist in multiple environmental niches, including water and soil in the farm (Vogeleer et al., 2014).

Biofilms are groups of microorganisms enclosed in a self-generated extracellular polysaccharide (EPS) matrix, and such aggregates of bacteria adhere to surfaces creating a dynamic environment to utilize nutrients efficiently (Donlan and Costerton, 2002). Microbial biofilms can be formed on any surface with an abundance of moisture and nutrients, and are found to be highly resistant to common disinfectants and antimicrobials (Bower et al., 1996; Srey et. al., 2013). Biofilm formation starts with the initial attachment of planktonic bacterial cells to a surface, following which attached cells enter a maturation stage, where an extracellular polymeric substance gets secreted out and microcolonies are formed (Donlan and Costerton, 2002). The aggregates of these microcolonies subsequently form matured biofilms, which become disintegrated and dispersed to new substrates to form new biofilms (O'Toole et al., 2005).

Therefore, the presence of biofilms on food contact surfaces may act as a continuous source of food contamination, thereby constituting a food safety hazard (Cappitelli and Villa, 2014).

The presence of EHEC biofilms in food processing environments, animal slaughterhouses and meat packing plants has been reported (Kumar and Anand, 1998; Aslam et al., 2004; Rivera-Betancourt et al., 2004; Vogeleer et al., 2014). Contamination of beef carcasses with EHEC could occur during different stages of processing, including slaughtering, dressing, chilling or cutting (Bacon and Sofos, 2003; Koutsoumanis and Sofos, 2004). The ability of EHEC to form biofilms on different equipment surfaces has been attributed to its distribution and persistence in meat processing plants (Carpentier and Cerf, 1993; Dewanti and Wong, 1995; Aslam et al., 2004; Rivera-Betancourt et al., 2004). Furthermore, many EHEC outbreaks associated with the consumption of fresh produce, such as cabbage, celery, radish sprouts, lettuce, spinach, and tomatoes have been linked to surface colonization and persistence by the biofilm-forming bacteria (Beuchat 2002; Brandl 2006). Hazard Analysis and Critical Control Points (HACCP) and Good Manufacturing Practices (GMP) are employed in the food industry for ensuring food safety. However, because of potential shortcomings in clean-in-place regimens, bacteria can form biofilms on equipment surfaces in the production line, especially in the presence of moisture and nutrients (Ostrov et al., 2016). This is particularly significant since commonly used sanitizers such as hypochlorite and quaternary ammonium compounds are not fully effective in inactivating bacterial biofilms (Ostrov et al., 2016). Therefore, there is a need for controlling EHEC biofilms in food processing equipment and environments for reducing food contamination.

Historically, a variety of metals has been used as antimicrobial agents. Research has focused on heavy metal interactions on bacterial biofilms for bioremediation of soil and sediment, and wastewater management (Harrison et al., 2004). Additionally, several studies have

investigated the antibiofilm effect of metals such as zinc, copper, gallium, silver, chromium, and tellurium on various bacterial species, including *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Harrison et al., 2004, 2005a, 2005b; Kaneko et al., 2007). Selenium (Se) is a naturally occurring essential microelement recommended for daily intake by the United States Food and Drug Administration (US-FDA, 2003). Owing to its pro-oxidant property, Se generates superoxide radicals, which interact with many bacterial components, including membranes and nucleic acids, thereby exerting antimicrobial action (Tran, 2008; Tran et al., 2014; Seko and Imura, 1997). Moreover, organoselenium compounds are reported to covalently attach to solid surfaces and catalyze oxygen radical generation, thus reducing bacterial colonization and biofilm formation on surfaces (Tran et al., 2012). Sodium selenite (Na_2SeO_3) is a commonly used dietary supplement containing Se, with proven antifungal and antibacterial properties (Soriano-Garcia, 2004; Kumar et al., 2010). In this study, the efficacy Se for inhibiting EHEC biofilm synthesis and inactivating mature biofilms was investigated. Further, the effect of Se on the transcription of critical biofilm-associated genes in EHEC was determined.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions:

All bacteriological media were purchased from Difco (Becton Dickinson, Sparks, MD). Two strains of EHEC (EDL 933, E163) were used in this study. Each strain of EHEC was cultured separately in 10 ml of sterile tryptic soy broth (TSB) and incubated at 37°C for 18 h. Following incubation, the bacteria from each culture were pelleted by centrifugation (3600 ×g for 15 min), washed twice and suspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.0). The bacterial count in each of these cultures was determined by plating 100 µl of ten-fold serial

dilutions on duplicate tryptic soy agar (TSA) and Sorbitol MacConkey agar (SMA) plates, followed by incubation at 37°C for 24 h to 48 hours.

Determining the SIC, MIC, and MBC of Se against EHEC:

The SIC (sub-inhibitory concentration), MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) of Se against EHEC were determined, as described earlier (Amalaradjou and Venkitanarayanan, 2011). Sterile 24-well polystyrene tissue culture plates (Costar, Corning Incorporated, Corning, NY) containing TSB (1 ml/well) were inoculated separately with ~ 6.0 log CFU of each EHEC separately, followed by the addition of 0 to 5 µl of 50% stock solutions of Se (sodium selenite, Sigma-Aldrich, St. Louis, MO) with an increment of 0.5 µl. The plates were incubated at 37ºC for 24 h, and bacterial growth was determined by culturing on duplicate TSA and SMA plates. The highest concentration of Se that did not inhibit bacterial growth after 24 h of incubation as compared to control samples was selected as its SIC for this study, whereas the lowest concentration of Se that inhibited bacterial growth after incubation was taken as the MIC of the treatment. The lowest concentration of Se that reduced EHEC population by ~ 5.0 log CFU/ml after incubation at 37ºC for 24 h was taken as the MBC.

Biofilm Inhibition Assay on Polystyrene Surfaces:

The efficacy of SIC of Se in inhibiting EHEC biofilm formation on polystyrene was investigated using a published protocol (Amalaradjou et al., 2010). Sterile 96-well polystyrene tissue culture plates (Costar) were inoculated with 200 µl (~ 6.0 log CFU) of overnight, PBS-washed EHEC cultures, followed by the addition of SIC of Se. The plates were incubated at 25°C or 4°C for 96 h. At 24, 48, 72, and 96 h of incubation, biofilm-associated EHEC populations were be enumerated by scraping and plating 100 µl volume on duplicate TSA and SMA plates and incubation at 37°C for 24 hours.

Effect of Se on EHEC Exopolysaccharide (EPS) Production:

The efficacy of Se in reducing EPS production in EHEC biofilms was determined by ruthenium red staining assay (Borucki et al., 2003). EHEC biofilms were allowed to develop in the presence of SIC of Se at 25°C or 4°C for 96 hours, described previously. After 24, 48 and 96 hours of incubation, the biofilms developed were scraped and washed with PBS thrice, followed by the addition of 200 µl of aqueous 0.01% ruthenium red (Sigma-Aldrich) to each well and incubation at 25ºC for 60 minutes. The optical density (450 nm) of the residual stain was then measured using synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The amount of dye bound to the biofilm was determined by the formula $OD_{BF} = OD_B - OD_S$ (OD_{BF}: optical density (450 nm) of biofilms; $OD_{B:}$ optical density (450 nm) of blanks; OD_S: optical density (450 nm) of the residual stain collected from sample wells). Wells without biofilms added with 200 µl of ruthenium red stain acted as blanks (Zameer et al., 2010).

Solid Surface Attachment Assay:

The effect of Se on the initial attachment of EHEC cells to a solid surface was determined using a published method (Hay et al., 2009). Briefly, sterile 96-well polystyrene tissue culture plates were inoculated with 200 µl (~ 6.0 log CFU) of overnight grown EHEC cultures with or without SIC of Se and incubated at 37°C for 8 h. At 0, 2, 4, 6 and 8 hours of incubation, non-adherent EHEC were washed off with PBS and 125 µl of 0.1% crystal violet (Sigma-Aldrich) was added to each well and incubated at 25ºC for 10 min. Subsequently, crystal violet bound to the adherent bacterial cells was solubilized by the addition of 200 µl of 100% dimethyl sulfoxide (Sigma-Aldrich) and absorbance was measured at 595 nm.

Efficacy of Se in combination with hot water for rapid inactivation of EHEC mature biofilm.

Sterile 96-well polystyrene tissue culture plates were inoculated with 200 µl of each EHEC culture (~ 6.0 log CFU) and incubated at 37°C for 96 hours without agitation. After 96 hours of incubation, EHEC biofilms were treated with bactericidal concentrations (MBC and 2 X MBC) of Se in hot water (80°C) for 0, 30 seconds, 2 minutes and 5 minutes. The biofilm-associated EHEC populations were enumerated as described before. When EHEC was not detected by direct plating, samples were tested for surviving bacterial cells by enrichment at 37°C for 24 hours, followed by streaking on SMA plates.

Confocal Microscopy:

In situ confocal laser scanning microscopy was used to visualize the three-dimensional structure of EHEC biofilms treated with and without Se (Amalaradjou and Venkitanarayanan, 2011; Narayanan et al., 2016). EHEC biofilms were formed on Lab-Tech four-chamber no. 1 borosilicate glass coverslips (Lab-Tek, Nalge Nunc International, Rochester, NY), and treated with the MBC and 2 X MBC of Se in hot water (80°C) (as described in the inactivation assay). Live and dead cells were imaged after staining with 2.5 µM SYTO (Molecular Probes Inc., Eugene, OR) and 5 µM propidium iodide (Molecular probes). Samples were examined under a Leica true confocal scanner SP2 microscope using a water immersion lens. A krypton-argon mixed-gas laser with PMT2 filter served as the excitation source.

Effect of Se on the expression of EHEC biofilm-associated genes:

The effect of Se on the transcription of EHEC biofilm-associated genes (*motB*, *fhD*, initial attachment, and motility; *lsrA*, quorum sensing; *csgD*, master transcription regulator) was investigated by using real-time quantitative PCR (RT-qPCR) (Lee et al., 2013). Each EHEC strain was grown separately with or without the SIC of Se at 37°C in TSB to mid-log phase in sterile 12 well polystyrene plates. Total RNA was extracted using RNeasy RNA isolation kit (Qiagen,

Valencia, CA). Complementary DNA (cDNA) was synthesized using the I script cDNA synthesis kit (Biorad, CA) and used as the template for RT-qPCR. The amplification product was detected using SYBR Green reagents (Biorad, Hercules, CA). Relative gene expression was determined by the comparative critical threshold ($2^{-}\Delta\Delta Ct$) value method using a 7500 Step one Real Time PCR system (Applied Biosystems, Carlsbad, CA), and expressed as fold change in expression relative to controls. Data were normalized to the endogenous control (16S rRNA) and the level of candidate gene expression between control samples (not exposed to Se) and treated samples (exposed to SICs of Se) was compared to study the effect of Se on the expression of each biofilm-associated gene. Gene specific primers were used based on published literature (Lee at al., 2013), and 16S rRNA gene was used as a housekeeping gene or endogenous control. (Table 1).

EHEC Biofilm Inhibition on Stainless Steel Coated with Se Nanoparticles:

Synthesis and Substrate Preparation of Selenium Nanoparticles (SeNp):

Selenium nanoparticles were synthesized from sodium selenite (Na_2SeO3), where selenium in its highest oxidation state (+4) gets reduced by a mild reducing agent (L-Glutathione reduced) (Tran and Webster, 2013). For the synthesis, sodium selenite (≥ 98.0%, Sigma-Aldrich, MW = 172.94 gmol-1), reduced L-Glutathione (≥ 98.0%, Sigma-Aldrich, MW = 307.32 gmol-1) and sodium hydroxide pellets (ACS reagent, ≥ 97.0%, Sigma-Aldrich, MW = 40 gmol-1) were used. A volume of 10 ml of sterile deionized water and 10 ml of 100 mM reduced L-glutathione were added to a beaker followed by 10 ml of 25 mM of sodium selenite with continuous stirring. After gentle mixing, 2 ml of 0.5M sodium hydroxide was slowly added until the pH of the resultant reaction mixture reached ~11. Immediately following the addition of sodium hydroxide, Se nanoparticles synthesis started, which was visualized by a color change from a clear

white/transparent solution to a clear red solution indicating the formation of nanoparticles (Fig. 8A). After the solution color turned red, the beaker was left undisturbed for another 10 minutes.

Prior to SeNP coating, stainless steel coupons (type 304; diameter 1 cm) were degreased by soaking in acetone for 10 minutes, washed thoroughly with distilled water, and cleaned with ethanol. This was followed by rigorous rinsing of the coupons with distilled water and subsequent boiling in distilled water for 10 min. Finally, the coupons were autoclaved for 15 min at 121°C (Amalradjou and Venkitanarayanan, 2011). The pre-cleaned stainless steel coupon substrates were then rinsed with sterile deionized water and immersed in a beaker containing SeNP overnight. After SeNPs were adsorbed to the steel surface, individual coupons were taken out from the nanoparticle solution and washed several times with sterile deionized water to get rid of any excess unbound Se nanoparticles. Subsequently, the steel coupons coated with SeNP were kept in a heating oven (~ 100°C) for drying before using in biofilm studies.

Biofilm Inhibition:

Stainless steel coupons coated with or without SeNP were inoculated with ~ 6.0 log CFU of EHEC and biofilms were allowed to develop on the coupons for 96 hours at 25°C. At 24, 48, 72 and 96 hours, coupons were rinsed in sterile distilled water for 15–20 seconds, and placed in 50-ml centrifugation tubes with ~ 3 g of glass beads and 30 ml of sterile PBS, and subjected to sonication (Ultrasonicator, Branson Digital Sonifier, Danbury, CT) for 5 min at 50 W. The EHEC population was enumerated by serially diluting and plating the PBS suspension onto duplicate SMA agar plates with incubation at 37°C for 24 hours.

Statistical analysis. The entire study was repeated three times with duplicate samples with a completely randomized design. Data were analyzed using the PROC GLM SAS and differences

between the means were compared using least significant difference) test. Differences were considered significant when the P value was < 0.05.

RESULTS:

The SIC and MBC of Se against EHEC were 30 mM and 173 mM, respectively. Since Se was found to be equally effective in inhibiting and inactivating EHEC biofilms irrespective of the strain (P > 0.05), the results obtained with EHEC EDL 933 only are discussed in the manuscript.

Effect of SIC of Se on EHEC Biofilm Formation on Polystyrene Plates:

Selenium inhibited EHEC biofilm formation on polystyrene surfaces at both 25 and 4^{o}C (P < 0.05). As observed in Figure 1, the SIC of Se decreased EHEC counts in biofilm by ~ 4.0 log CFU/ml after 96 hours of incubation at 25°C (Figure 1A) and ~ 2.5 log CFU/ml at 4°C (Figure 1B) compared to that in control wells, which yielded a biofilm-associated bacterial count of ~ 8.0 log CFU/ml at 25°C and ~ 5.0 log CFU/ml at 4°C at the end of 96 hours.

Effect of Se on EPS Production:

Figure 2 shows the efficacy of Se in inhibiting EPS production in EHEC biofilms. With a linear positive relationship between the absorbance and EPS synthesis taken into account, higher absorbance values indicated greater EPS content. The EPS production associated with EHEC biofilms was found to be greater at 4°C (Figure 2B) than 25^{o}C (Figure 2A) (P > 0.05). However, regardless of the incubation temperature, Se decreased EPS production throughout 96 hours of incubation (P < 0.05).

Effect of Se on Initial Surface Attachment of EHEC:

Since the initial attachment of planktonic cells to a surface is one of the critical steps in biofilm development, EHEC populations that attached to polystyrene surfaces over the first 8 hours of incubation in the presence and absence of Se was determined. Results revealed that adherent

EHEC in the presence of Se at 8 hours was ~ 84% lesser compared to that in control samples devoid of Se (Figure 3). This indicates that initial surface attachment of EHEC cells to polystyrene was significantly interfered with in the presence of Se ($P < 0.05$).

Effect of Se on Pre-Formed EHEC Biofilms:

For the biofilm inactivation assay, the efficacy of MBC (173 mM) and 2 x MBC (346 mM) of Se for rapidly inactivating fully formed EHEC biofilms in combination with hot water at 80^{o}C was determined. As observed in Figure 4, EHEC counts in preformed biofilms were decreased to undetectable levels (direct plating and enrichment negative) after 5 minutes of treatment with 346 mM Se in hot water at 80°C ($P < 0.05$). In the presence of 173 mM of Se, a decrease of 3.0 log CFU/ml in biofilm-associated bacterial population was observed at 5 minutes ($P < 0.05$) (Fig 4). However, there was no significant reduction in biofilm-associated EHEC population treated with hot water alone ($P > 0.05$).

Confocal Microscopy:

The confocal images concurred with the results from the biofilm inactivation assay. In control biofilms, a dense layer of live bacterial cells stained green by SYTO dye was observed (Figure 5A), whereas biofilms exposed to Se revealed patchy breaks with disrupted organization and dead cells stained red by propidium iodide dye. The degree of biofilm disruption was found to increase with Se concentration, with only dead cells (stained red) in biofilms treated with 346 mM (Figure 5D) as against a mosaic of living and dead cells (stained green and red) in images from 173 Se treatment (Figure 5C). Similarly, a mixture of dead and living cells was observed in images from hot water treatment alone (Figure 5B).

Effect of Se on Genes Critical For Biofilm Formation in EHEC:

Real-time quantitative PCR results revealed that the SIC of Se significantly down-regulated the expression of the genes critical to EHEC biofilm synthesis except for *motB* ($P > 0.05$). Among the three genes down-regulated, the transcription of *lsr A* involved in quorum sensing in EHEC was most down-expressed.

Anti-biofilm Efficacy of Selenium Nanoparticles:

As revealed by TEM, the SeNPs synthesized were in nanometer dimensional scale and found to be $\leq$ 100 nm (Figure 7B). Results indicated that SeNP coated stainless steel coupons exerted an inhibitory effect on EHEC biofilm formation compared to that on uncoated steel surfaces (Figure 7C). Although an initial population of ~ 6.0 log CFU/ml of EHEC was inoculated onto control and SeNP-treated coupons, ~ 5.5 log CFU/ml of EHEC was recovered from uncoated coupons as against 2.0 log CFU/ml from Se-treated steel surface after 96 hours ($P < 0.05$).

DISCUSSION

Microbial biofilms are sessile bacterial aggregates with distinct architecture enclosed in a slimy layer of polysaccharides. This protective layer helps bacteria associated with the biofilm to remain resistant to the external sanitizers and disinfectants. Therefore, in this study, we evaluated the efficacy of Se for inhibiting biofilm formation from planktonic cells and inactivating pre-formed EHEC biofilms on polystyrene. This was based on the assumption that the SIC of Se may inhibit biofilm synthesis from planktonic cells by down-regulating critical biofilm-associated genes, whereas bactericidal concentrations of Se will be effective in killing mature biofilms. Since biofilm starts when bacterial cells sense the surrounding environmental conditions to form a monolayer, the initial attachment of planktonic cells to a surface is critical for successful biofilm formation (Beloin et al., 2008; Crouzel et al., 2014). Initial bacterial adhesion to abiotic surfaces depends on physicochemical and electrostatic interactions between the bacterial envelope and the

substrate (Dunne, 2002). Furthermore, exopolysaccharides are the functional and structural units of a biofilm matrix, with biofilm maturation beginning with the production of EPS. Exopolysaccharides give a three dimensional structure to the biofilm and protect the bacterial cells from disinfectants (Ghafoor et al., 2011; Lembre et al., 2012; Flemming and Wingender, 2010). Therefore, the effect of Se on the initial attachment of EHEC cells on polystyrene and EPS production was determined. The results indicated that EHEC attachment onto polystyrene at 8 hours of incubation and EPS production were significantly interfered in the presence of Se ($P < 0.05$) (Figure 2 and 3).

In the experiments evaluating the inhibitory effect of Se on EHEC biofilm formation, it was observed that the SIC of the metal (30 mM) reduced biofilm-associated bacteria by ~ 4.0 log and ~ 2.5 log CFU/ml at 25 and 4°C, respectively, at the end of 96 hours of exposure (Figure 1 A and B). The decreased inhibitory effect of Se on biofilm at 4^{o}C could be attributed to the reduced metabolic rate of the bacterium at this temperature compared to 25^{o}C (Amalaradjou and Venkitanarayanan, 2011).

Since it is known that metals such as zinc and manganese exert diminished antibacterial effect on mature biofilms due to their decreased diffusion through the biofilm architecture (Hu et al. 2007; Harrison et al., 2007), we used MBC and 2 X MBC of Se for killing pre-formed EHEC biofilms. Further, since clean in place (CIP) regimens used in food processing plant sanitation involve sanitizer treatments along with hot water at 80°C for 5 to 60 min (Gibson et al., 1999), we determined the efficacy of sodium selenite (highly soluble in water) in hot water as a sanitizer treatment for rapidly inactivating EHEC biofilms. The results from the inactivation studies indicated that Se combined with hot water at 80^{o}C was effective in killing EHEC biofilms in a

treatment time of 5 min, with 2 X MBC of the metal reducing the bacterial load to undetectable levels (> 6 log CFU/ml reduction) (Figure 4).

Since the SIC of Se was used in the experiments investigating its inhibitory effect on EHEC biofilm, the decreased biofilm-associated EHEC populations recovered from Se-treated samples were not due to EHEC killing, but rather due to an inhibitory effect of Se on the transcription of genes associated with biofilm synthesis in the bacterium. Therefore, RT-qPCR was performed to determine the effect of SIC of Se on genes involved in the various steps of biofilm formation. It was found that Se significantly decreased the expression of genes involved in the initial attachment (*flhD*) and quorum sensing (*lsr*) in EHEC (Figure 6). Moreover, the expression of *CsgD*, the master regulator of biofilm formation in *E. coli* was found to be down-regulated by Se ($P < 0.05$).

Since surface energy and composition of the immediate environment are critical external factors that influence bacterial cell attachment on a substrate (Renner and Weibel, 2011), manipulation of these external factors could be used to reduce bacterial surface attachment. In this regard, re-structuring of surfaces has been investigated to alter hydrophobicity for reducing bacterial cell surface attachment and inhibiting biofilm formation (Renner and Weibel, 2011). For example, surface coating with polymers and metal nanoparticles prevented bacterial adsorption and biofilm formation (Renner and Weibel, 2011; Lewis and Klibanov, 2005; Tran and Webster, 2013). Nanoparticles, known for their high surface to volume ratios allow greater interaction with biological molecules. Previously, Tran and Webster (2013) and Wang and Webster (2012), investigated the efficacy of Se nanoparticles for preventing biofilm formation on different medical device substrates. However, the use of nanostructured Se coating to prevent biofilm formation of food-borne pathogens, including EHEC on food processing surfaces has not been determined. Results from biofilm experiments on SeNP-coated stainless steel indicated that EHEC biofilm

formation was significantly reduced (~ 3.0 log CFU/ml) on the Se-treated surface as against control steel coupons (Figure 7C), thereby justifying follow up studies on the potential application of Se coating on food contact surfaces as a promising anti-biofilm treatment. Although the exact mechanism behind the antimicrobial property of SeNP is not known, it is hypothesized to be due to their ability to deplete thiol levels in bacteria. Since thiol is required for bacterial cell function, Se nanoparticles serve as a catalyst in oxidizing the thiol group, which in turn reduces oxygen to superoxide (Spallholz et al, 2001; Wang and Webster, 2012). In addition, a coating of stainless steel with Se nanoparticles may alter the surface hydrophobicity, resulting in decreased bacterial cell surface attachment.

In conclusion, Se was effective in inhibiting biofilm synthesis and inactivating mature biofilms in EHEC. Further, a coating of stainless steel with Se nanoparticles exerted significant anti-biofilm treatment. However, further studies are needed to determine the safety and long-term efficacy of Se coating, especially the attachment strength and stability of Se nanoparticles on surfaces.

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Figure 1. Selenium (Se) inhibited EHEC biofilm formation on polystyrene plates at 25° C and 4° C. Data represent the mean of three replicates and three experiment repetitions. Biofilm associated bacterial population was found decreased by 3-4 log CFU/ml in Se treated samples compared to control. Error bars represent standard error of the mean. *All the treatments were significantly different from control at P< 0.05.

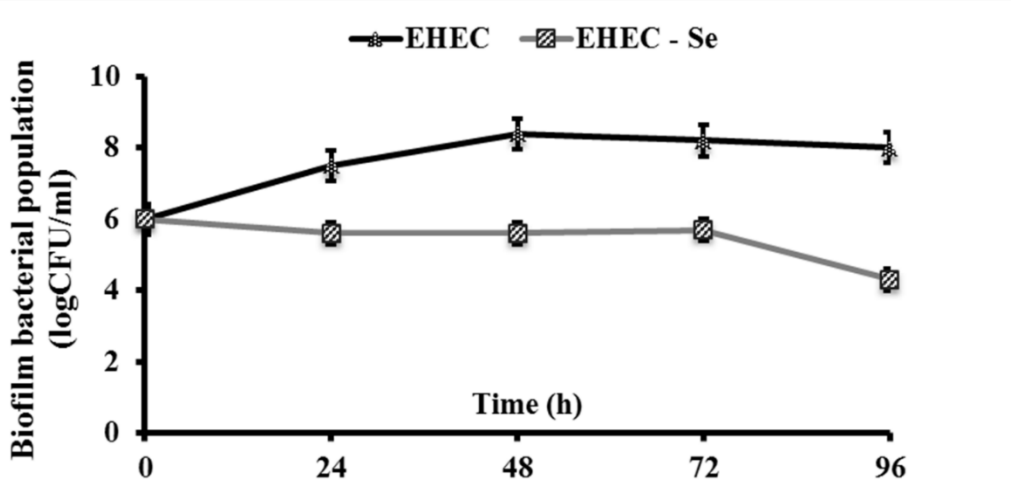

Figure 1A: Inhibition of EHEC biofilm formation at 25° C.

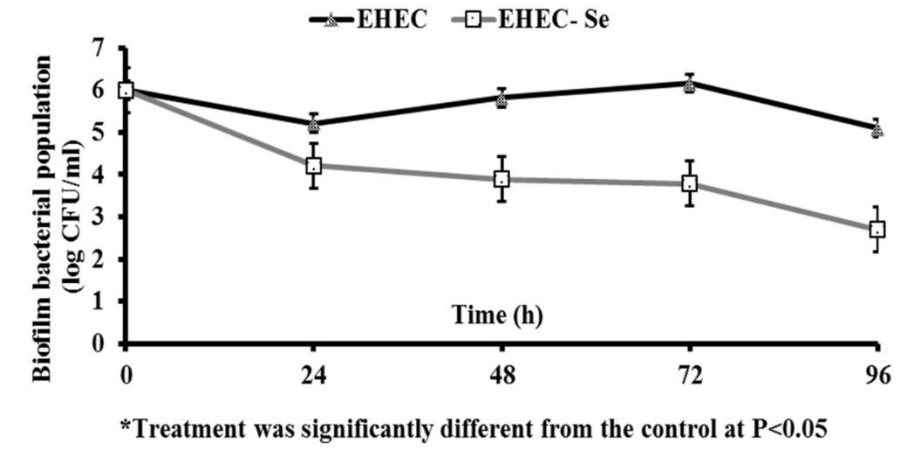

Figure 1B: Inhibition of EHEC biofilm formation at 4° C.

Figure 2.Selenium (Se)decreased EHEC EPS production at 25° C and 4° C. Throughout the 96 h of incubation, EPS production significantly decreased in Se treated samples compared to control at both the temperature tested. Data represent the mean of three replicates and three experiment repetitions. Error bars represent standard error of the mean. *All the treatments are significantly different from control at P< 0.05.

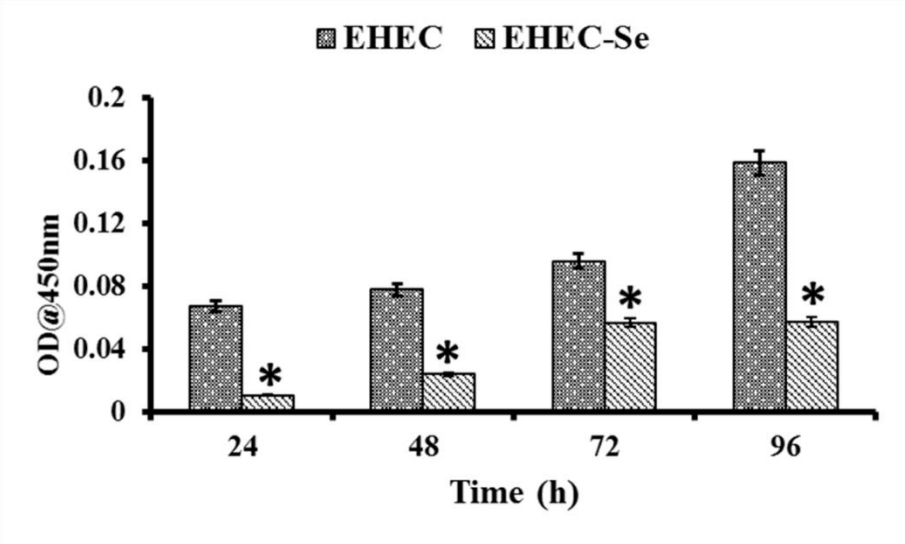

Figure 2A: Effect of Se on EHEC exopolysaccharide (EPS) production at 25° C

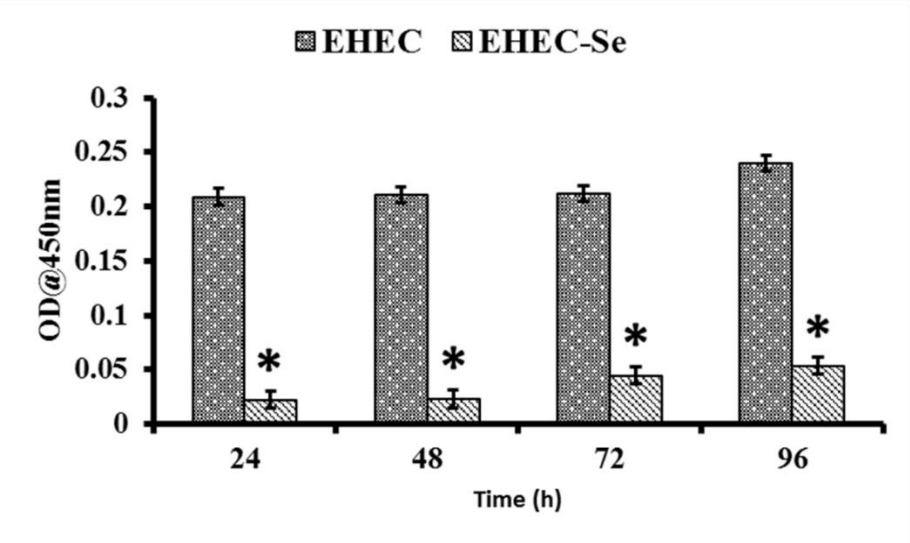

Figure 2B: Effect of Se on EHEC exopolysaccharide (EPS) production at 4° C

Figure 3: Selenium (Se) inhibited initial solid surface attachment of EHEC. Data represent the mean of three replicates and three experiment repetitions. The extent of bacterial attachment to the solid surface was 84% less in Se-treated samples compared to control. Error bars represent standard error of the mean. *All the treatments are significantly different from control at $P < 0.05$.

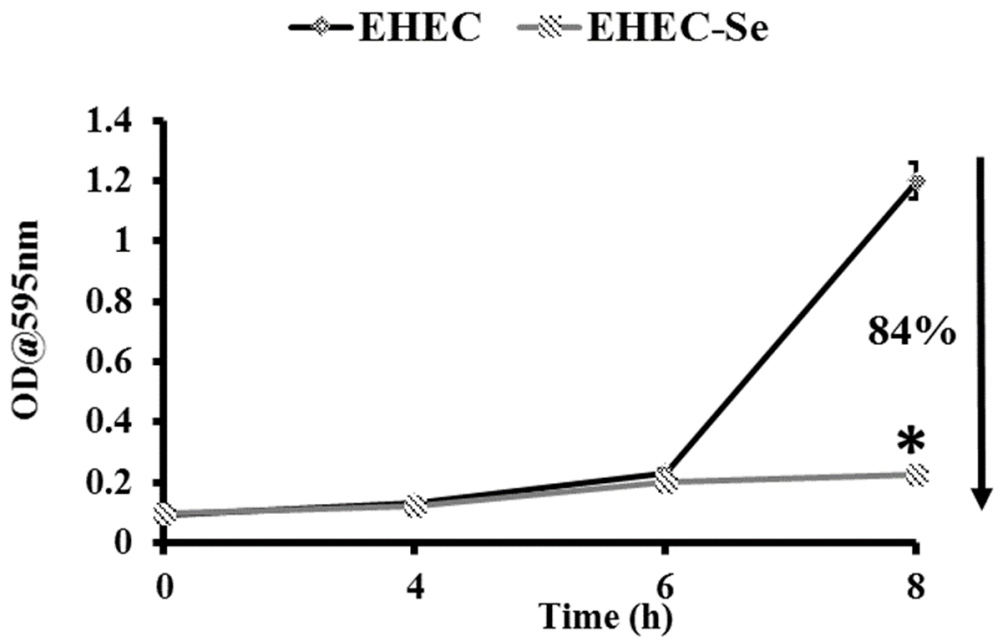

Figure 4: Selenium (Se) inactivated pre-formed EHEC biofilm on polystyrene microtiter plates. Data represent the mean of three replicates and three experiment repetitions. Error bars represent standard error of the mean. Selenium combined with hot water at 80°C was effective in killing EHEC biofilms, with 2 X MBC (346 mM Se) of the metal reducing the bacterial load to undetectable levels (> 6 log CFU/ml reduction) *All the treatments are significantly different from control at P< 0.05 except hot water treatment.

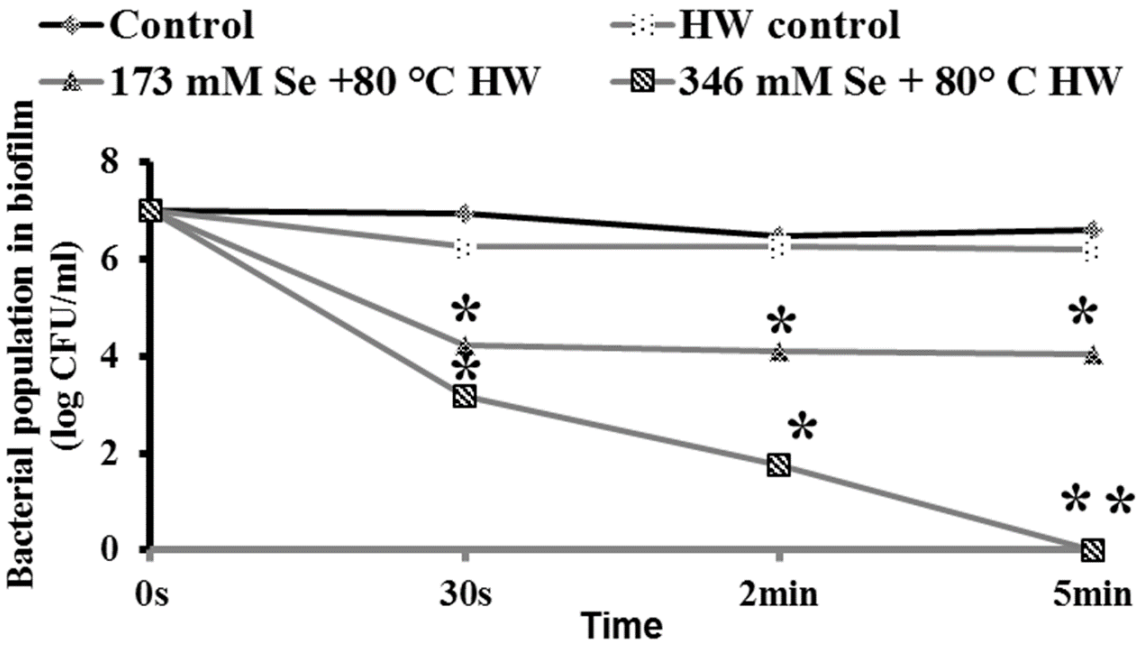

Figure 5: Scanning confocal micrographs of EHEC biofilm inactivation.

In control biofilms, a dense layer of live bacterial cells stained green by SYTO dye was observed (Figure 5A), whereas biofilms exposed to Se revealed patchy breaks with disrupted organization and dead cells stained red by propidium iodide dye. The degree of biofilm disruption was found to increase with Se concentration, with only dead cells (stained red) in biofilms treated with 346 mM (Figure 5D) as against a mosaic of living and dead cells (stained green and red) in images from 173 Se treatment (Figure 5C). Similarly, a mixture of dead and living cells was observed in images from hot water treatment alone (Figure 5B).

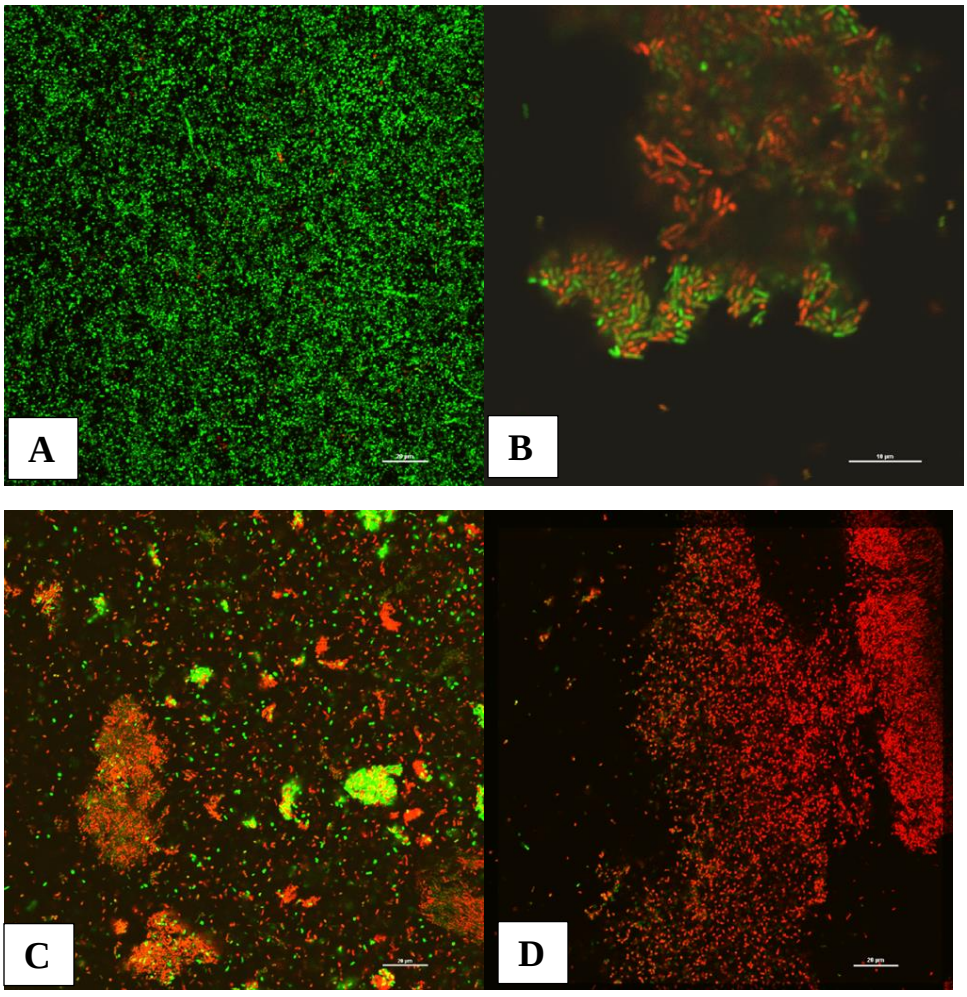

Figure 6: Effect of Se on expression of biofilm associated genes in EHEC. Data represent the mean of three replicates and two experiment repetitions. RT-qPCR results revealed that Se significantly decreased the expression of genes involved in the initial attachment (*flhD*) and quorum sensing (*lsr*) in EHEC. In addition, the expression of *csgD*, the master regulator of biofilm formation in E. coli was found to be down-regulated by Se ($P < 0.05$). *All the treatments are significantly different from control at $P< 0.05$.[a]Fold change in gene expression relative to control.

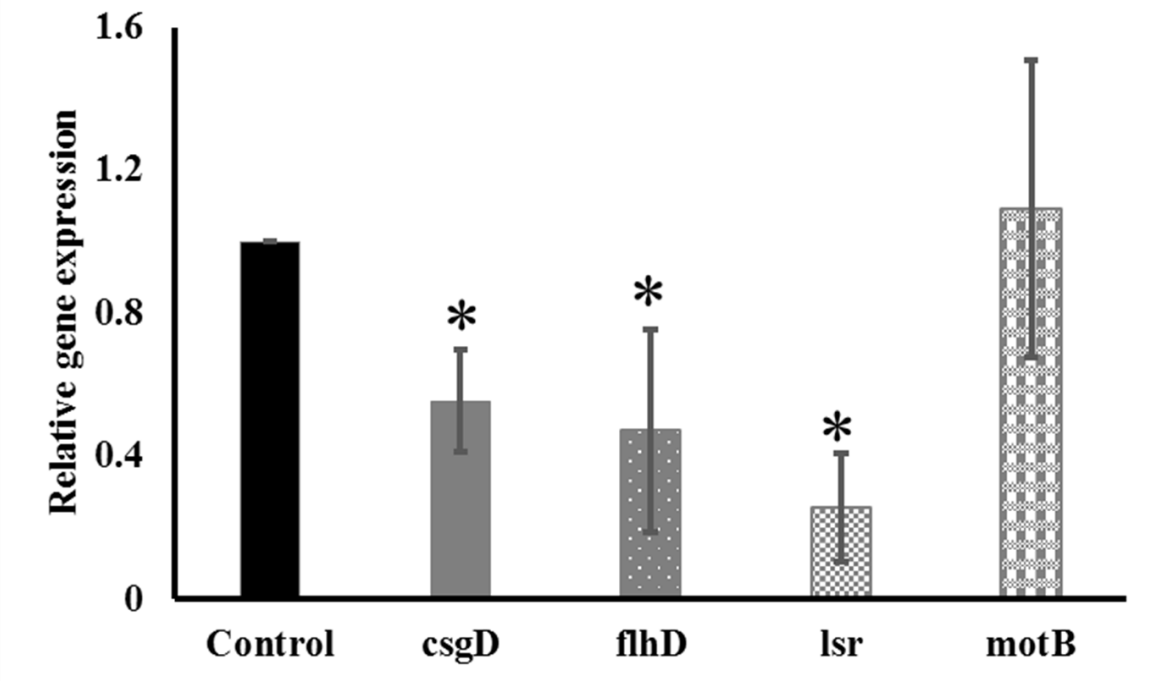

Figure 7: Anti-biofilm efficacy of Selenium nanoparticles. The dimension of the synthesized SeNps was illustrated in the TEM images (Fig 7B). Results from biofilm experiments on SeNP-coated stainless steel indicated that EHEC biofilm formation was significantly reduced (~ 3.0 log CFU/ml) on the Se-treated surface as against control steel coupons (Figure 7C). Data represent the mean of three replicates and three experiment repetitions. Error bars represent standard error of the mean. *All the treatments are significantly different from control at P< 0.05.

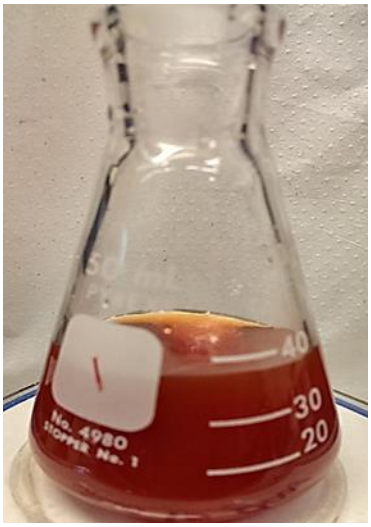

Figure 7A: Se nanoparticles in solution

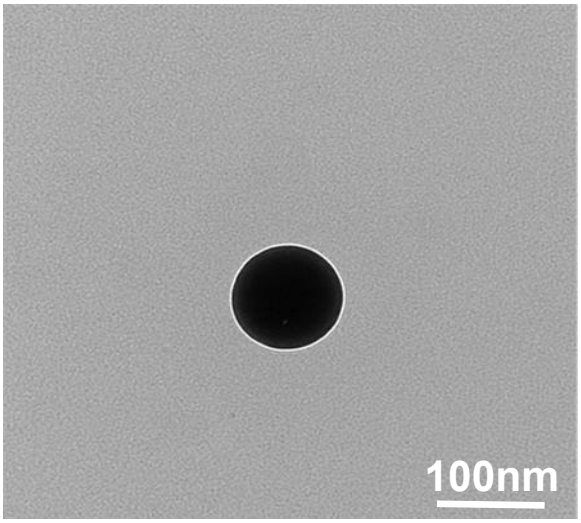

Figure 7B: TEM image of a nanoparticle

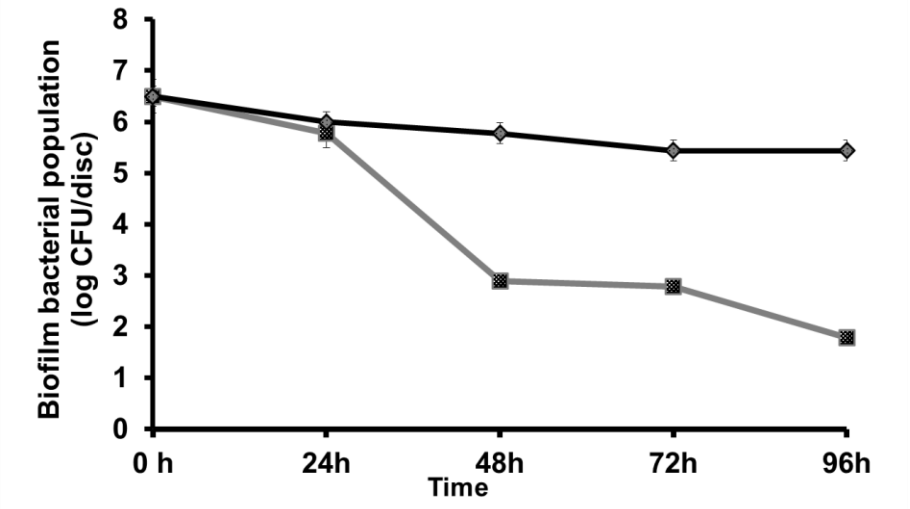

Figure 7C: Biofilm inhibition on stainless steel coated with Se nanoparticles

Table 1: List of primers used in this study.

Gene	Primer
motB	Forward 5′-CAG GGG GAA GTG AAT AAG CA-3’
	Reverse 5′-TTC TAA ACA TCG GGC GAT TC-3′
flhD	Forward 5′-TGC ATA CCT CCG AGT TGC TG-3′
	Reverse 5′-GCG TGT TGA GAG CAT GAT GC-3′
lsrA	Forward 5′-AAC ATC CTG TTT GGG CTG GCA A-3′
	Reverse 5′-AAA CAA GCG TTC GGT TTC CGC A-3′
csgD	Forward 5′-CCG CTT GTG TCC GGT TTT-3′
	Reverse 5′-GAG ATC GCT CGT TCG TTG TTC-3’
rrsG (EHEC 16s)	Forward 5′-TAT TGC ACA ATG GGC GCA AG-3′
	Reverse 5′-ACT TAA CAA ACC GCC TGC GT-3’

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CHAPTER IV

Potentiating the heat inactivation of *Escherichia coli* O157: H7 in ground beef patties by rutin

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ABSTRACT

Escherichia coli O157: H7 (EHEC) is a major foodborne pathogen largely transmitted to humans through the consumption of undercooked ground beef. This study investigated the efficacy of the food-grade, plant-derived antimicrobial, rutin (RT) with or without chitosan (CH) in enhancing EHEC inactivation in undercooked hamburger patties. Further, the effect of aforementioned treatments on beef color and lipid oxidation was analyzed. Ground beef was inoculated with a five-strain mixture of EHEC (7.0 log CFU/g), followed by the addition of RT (0.05%, 0.1% w/w) with or without CH (0.01% w/w). The meat was formed into patties (25 g) and stored at 4°C for 5 days. On days 1, 3, and 5, the patties were cooked (65°C, medium rare) and surviving EHEC was enumerated. The effect of these treatments on meat color and lipid oxidation during storage was also determined as per American Meat Science Association guidelines. The study was repeated three times with duplicate samples of each treatment. RT enhanced the thermal destruction of EHEC, and reduced the pathogen load by at least 3.0 log CFU/g compared to control ($P < 0.05$). The combination of RT with CH was found to be more effective, and reduced EHEC by 5.0 log CFU/g ($P < 0.05$). EHEC counts in treated-uncooked patties did not decline during storage for 5 days ($P > 0.05$). Moreover, patties treated with RT plus CH were more color stable with higher a^* values ($P < 0.05$). Results suggest that the aforementioned antimicrobials could be used for enhancing the thermal inactivation of EHEC in undercooked patties; however, detailed sensory studies are warranted.

Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC) is one of the major food-borne pathogens in the United States, causing an estimated 73,000 illnesses annually (Rangel et al., 2005). EHEC infection results in hemolytic uremic syndrome (HUS), characterized by hemolytic anemia, thrombocytopenia, and renal injury (Bell et al., 1997). Cattle are the principal reservoir of *E. coli* O157: H7, with fecal shedding being an important source of food and environmental contamination (Low et al., 2005). The majority of EHEC food-borne outbreaks in the US have been linked to the consumption of undercooked ground beef patties (Rhee et al., 2003; WHO, 2011), which is attributed to the thorough mixing of bacteria throughout the meat during grinding (Ferens and Hovde, 2011).

The United States Department of Agriculture (USDA) has established a zero tolerance policy for EHEC in ground beef, and recommended that beef patties be cooked to an internal temperature of 71.1°C (160°F) to ensure complete pathogen inactivation (USDA, 1998). Based on the degree of doneness, cooked patties can be classified as rare (60°C/140°F), medium-rare (65°C/149°F), medium (71.1°C/160°F) or well done (77°C/170.6°F) (Marksberry et al., 1993). A USDA survey on hamburger cooking practices in the US indicated that 20% of the participants cooked patties rare or medium rare (Ralston, 2002) which could result in EHEC survival in the meat. In order to ascertain that the required internal temperature (71.1°C) is attained during cooking of ground beef patties, the USDA advised consumers to use a meat thermometer. However, because of the lack of homogeneity in patty composition, and temperature monitoring difficulties, there is the possibility that the recommended internal temperature may not be uniformly attained (D'Sa et al., 2000). Further, the use of thermometers for cooking beef patties by consumers is limited (NCBA, 1999; McCurdy et al., 2005) due to the inconvenience of the

procedure, consumer uncertainty, and a lack of consumer confidence in thermometer's ability to ensure food safety (USDA, 1998; Research Triangle Institute [RTI], 2001; McCarty, 2008).

According to a survey conducted by the USDA-ERS, approximately 20% of the US population preferred eating rare or medium-rare patties at home, restaurants and cafeteria (Ralston, 2002). The same study revealed that at the internal cooking temperatures specified for rare and medium rare patties, EHEC numbers did not decline significantly compared to those cooked at 71.1°C. Moreover, most consumers determine the doneness of beef patties by observing the color and texture of cooked meat. However, color is not a good indicator of doneness because ground beef is prone to a non-typical color change associated with cooking called premature browning (PMB) (Claus, 2007), where the meat appears fully cooked in spite of not having achieved a safe internal temperature. Thus, PMB in ground beef can lead to inadequate cooking by consumers, who are misled by the cooked color (Warren et al., 1996a, b), potentially allowing EHEC survival. Killinger et al. (2000) reported that PMB incidence averaged about 47% in ground beef purchased from local retail stores. When compared to steaks and roasts, ground beef is more susceptible to PMB because of accelerated oxidation of the meat pigment, myoglobin that occurs as a result of grinding. The infectious dose of EHEC in humans is low (2–2,000 cells), emphasizing that undercooking of beef patties should be avoided (Buchanan, 1997; Strachan et al., 2005). Therefore, it is important to include an antimicrobial hurdle to ensure inactivation of EHEC in ground beef patties unintentionally cooked to inadequate temperatures.

Rutin (RT) is an antibacterial flavonoid obtained from buckwheat, rhubarb and berries (Kreft et al., 2006; Lupaşcu et al., 2008) with well-known antioxidant, anti-inflammatory and antimicrobial properties (Paulo et al., 2001; Sales and Resurreccion, 2014). This plant derived antimicrobial (PDA) is generally recognized as safe (GRAS) and approved for use in foods (FDA,

2007, 2010). Chitosan (CH) is a GRAS-status polymer derived from the deacetylation of chitin, a natural polysaccharide present as the main component of exoskeletons of crustaceans (Kumar, 2000). CH possesses antimicrobial properties against a wide range of Gram-positive and Gram-negative bacteria (No et al., 2002; Sagoo et al., 2002). In addition, CH is used as an antimicrobial carrier coating or film on foods due to its emulsification and gelation properties (Knorr, 1984; Jiang and Li, 2001; No et al., 2007).

The primary objective of this study was to determine the efficacy of low concentrations of RT for enhancing the inactivation of EHEC in undercooked ground beef patties (heated to 65°C, medium rare) in the presence or absence of CH. In addition, the effect of aforementioned treatments on meat color and lipid oxidation during refrigerated storage was determined.

MATERIALS AND METHODS

Bacterial Strains. Five EHEC strains (E6, E8, E10, E16, and E22) all originated from cattle were used for the study. Strains E8, E10, and E16 were isolated from meat, strain E6 from milk and E22 was a calf feces isolate. All the five strains were induced for resistance to nalidixic acid (NA, 50 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for selective enumeration (Blackburn and Davies, 1994). Each strain was grown separately in 10 ml tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with NA (Fischer Scientific, Pittsburgh, PA, USA) at 37°C for 24 h. After three consecutive transfers, all the three cultures were washed separately with phosphate buffered saline (PBS, pH 7.0) and suspended in PBS. Equal volumes of the five cultures were then combined and pelleted by centrifugation at 3600 × g for 15 min at 4°C. The pellet was suspended in 10 ml of PBS and the bacterial suspension was used as the inoculum. The EHEC count in the inoculum was determined by serially dilution (1:10 in PBS) and plating

on tryptic soy agar (TSA; Difco) plates containing NA (50 μg/ml) and incubating the plates at 37°C for 24 h.

Preparation of Ground Beef Patties. Fresh fine ground beef (80% lean and 20% fat) was purchased from a local meat processor. The ground meat was separated into portions of 25 g each, and randomly assigned to different treatments. Each meat portion was inoculated with the five-strain mixture of EHEC (~7.0 log CFU/g), followed by the addition of RT (0.05%, 0.1%w/w, Sigma–Aldrich, MW: 610.52, soluble in DMSO and Ethanol), with or without CH (0.01%w/w, Sigma–Aldrich, MW: ~1.5 KDa). CH (1%, w/v) stock solution was prepared in 1% acetic acid solution to dissolve RT. The ground beef was mixed thoroughly and formed into patties (25 g). The patties were then placed on foam trays and wrapped with oxygen-permeable fresh meat film (Koch Supplies, Kansas City, MO, USA) and stored at 4°C for 5 days.

Cooking. On days 0, 1, 3, and 5 of storage, the inoculated patties were cooked separately in a double-sided George Foreman Lean Mean Grilling Machine (Salton Inc., Columbia, MO, USA) until an internal temperature of 65°C (medium rare) was reached. A standard meat thermometer (Acutuff Model 34 Atkins 2 mm probe meat thermometer, Koch Supplies) was used to monitor the internal temperature continuously. In order to ensure uniform cooking on either sides, the patties were turned thrice during cooking (at 30°C, at 40°C, and at 50°C). The entire cooking procedure was done based on a published protocol (Suman et al., 2005; Amalaradjou et al., 2010). In addition, a set of uncooked, refrigerated patties containing each treatment was included to study the effect of antimicrobials on EHEC without applying the heat treatment

Bacterial Enumeration. Each cooked patty was immediately transferred to a sterile Whirlpak bag (Nasco, Fort Atkinson, WI, USA) containing 30 ml of sterile ice-cold neutralizing broth (Dey–Engley neutralizing broth, Sigma–Aldrich), and homogenized in a stomacher for 2

min at high speed. The surviving EHEC population was enumerated by spread plating 100 µl of serially diluted homogenate on duplicate TSA plates supplemented with NA (50 µg/ml). In addition, 1 ml of the meat homogenate was added to 9 ml of TSB containing NA and incubated at 37°C for 24 h. Following incubation, the culture was streak plated onto TSA plates supplemented with NA (50 µg/ml) and Sorbitol MacConkey agar supplemented with 4-Methylumbelliferyl-β-D-glucuronide (Oxoid Ltd, Lenexa, KS, USA).

Meat Color. Instrumental color measurements were done to determine the effect of the antimicrobial treatments on meat color (Hunt et al., 1991, 2012; Amalaradjou et al., 2010; Suman et al., 2010). Using HunterLab MiniScan XE Plus colorimeter (HunterLab Associates, Reston, VA, USA) with illuminant A, 2.54-cm diameter aperture, and 10° standard observer, a∗ values and reflectance spectra from 400 to 700 nm (at 10 nm increments) were measured on patties on 0, 1, 3, and 5 days of storage.

Lipid Oxidation. The effect of RT with or without CH on lipid oxidation in patties was determined using a procedure previously described (Sinnhuber and Yu, 1958; Hunt et al., 1991, 2012). Briefly, 0.5 g of minced portion of each ground beef patty was added with 2.5 ml of thiobarbituric acid (TBA; Sigma–Aldrich) stock solution (thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCl) to get a dilution factor of 6. After proper mixing, the samples were heated in a boiling water bath for 10 min. As soon as the samples from the control patty (without treatments) turned pink, the samples were immediately washed in tap water to cool down. The absorbance spectra of the clear supernatant obtained by centrifuging the cooled samples at 5,000 × g for 10 min was measured at 532 nm against a blank containing reagents alone (without meat). The thiobarbituric acid reactive substances (TBARS) value in ppm for each sample was determined separately using the equation: TBARS value (ppm) = sample A532 × 2.77.

Statistical analysis. The inactivation study was a split plot with completely randomized design. Each batch of ground beef was divided into 120 patties of 25 g each. The patties were randomly assigned to 1 of 20 of the treatments∗storage time combination effects. Each patty served as an experimental unit and the entire study repeated three times with duplicate samples. The data were analyzed using GLIMMIX procedure of SAS 9.4 and significance was tested at $P < 0.05$. Similar analysis was done for the TBARS estimation. The a∗ values were analyzed as repeated measures using proc mixed procedure, and the experimental design was a randomized complete block design. The significance was tested at $P < 0.05$.

RESULTS

The effect of RT with or without CH in potentiating the thermal inactivation of EHEC in undercooked ground beef patties is depicted in Figure 1. There was no decline in EHEC counts irrespective of the treatments throughout the refrigerated storage in uncooked patties, and ∼6.5 log CFU/g of the pathogen was recovered from patties at the end of storage period ($P > 0.05$) (Figure 1A). However, when the patties were cooked to 65°C, the EHEC population was decreased by ∼1.5 to 2.0 log CFU/g in untreated control patties. On the other hand, both tested concentrations of RT in combination with CH decreased EHEC counts by 2–3.0 log CFU/g on days 0 and 1, with >5.0 log CFU/g reduction in bacterial counts on days 3 and 5 of storage (enrichment negative; $P < 0.05$) (Figure 1B).

The instrumental color measurements for redness of meat (a∗ values) are shown in Table 1. As expected, the red color of control and treated patties gradually decreased over time during storage. The patties treated with RT (0.1 and 0.05%) plus CH displayed greater a∗ values on day 3 of storage compared to control patties ($P < 0.05$). The formation of TBARS in beef patties was determined on days 0, 1, 3, and 5 of storage. Although TBARS increased progressively in patties

during storage ($P < 0.05$), no significant difference in lipid oxidation was observed between the treatments on any day of storage ($P > 0.05$) (Figure 2).

DISCUSSION

This study investigated the effect of the food-grade plant-derived antimicrobial, RT in enhancing the thermal inactivation of EHEC in undercooked ground beef patties in the presence and absence of CH. CH, a known emulsifier, was primarily used for the uniform distribution of the antimicrobial in meat. It was observed that RT alone or in combination with CH did not exert any antimicrobial effect on EHEC in uncooked refrigerated ground beef patties (Figure 1A). However, when the patties were heated to an internal temperature of 65°C, RT in combination with CH brought about significant reductions in EHEC counts throughout the storage period ($P < 0.05$) when compared to control and patties containing RT or CH alone. These results indicated that the combination of RT with CH and heat had a greater lethality on EHEC in beef patties than RT or heat alone. The increased heat destruction of EHEC in patties containing the antimicrobial could be attributed to their pronounced deleterious effects on bacterial cell membrane leading to the loss of intracellular contents and destruction of the cells. In addition, heat-induced damage of bacterial plasma membrane may have potentially allowed rapid accumulation of the antimicrobial within the cells, thereby resulting in an enhanced bactericidal effect. For example, Shibasaki and Kato (1978) observed that heating causes bacterial plasma membrane to become more fluid in nature, thereby increasing the antimicrobial activity of lipid-soluble small molecules. Antimicrobial flavonoids, including rutin have been reported to act on lipid bilayers of bacteria and believed to modify the physicochemical properties of the cell membranes (Tsuchiya, 2015). They are also known to interact with functional protein systems of the cell (Koval'skii et al., 2014; Tsuchiya, 2015). Similarly, several studies showed the deleterious effect of RT on bacterial

membrane, which includes oxidative damage to the cell membrane, loss in membrane integrity and morphology (Bajpai et al., 2014; Subramanian et al., 2014; Taylor et al., 2014).

It was also observed in the study that CH by itself did not result in a significant reduction in EHEC counts ($P > 0.05$), except in patties that were cooked after 5 days of refrigerated storage (Figures 1B,). This was expected since the concentration of CH used in the study (0.01% w/w) was much below the antimicrobial concentration (0.5–1%) reported in the literature (Darmadji and Izumimoto, 1994a, b). However, the combination of RT with CH increased the thermal destruction of EHEC, which could be attributed to the improved solubility and distribution of the RT in the presence of the CH, which is a potent emulsifier (Schulz et al., 1998; Klinkesorn, 2013) and solubilizer (Zerrouk et al., 2004; Shete et al., 2015). In addition, synergistic antimicrobial effects between CH and a variety of antimicrobials, including essential oils (Wang et al., 2011) have been observed, and this could have contributed to the enhanced bacterial killing in cooked patties containing RT with CH. Previously, two plant-derived compounds, namely carvacrol and *trans*-cinnamaldehyde were found to enhance the thermal destruction of EHEC in ground beef (Juneja and Friedman, 2008), where addition of both compounds in beef sensitized the pathogen to the lethal effect of heat, as evident from the significantly lower thermal destruction times of the pathogen in the treated samples. Similarly Amalaradjou et al. (2010) reported that addition of *trans*-cinnamaldehyde (0.15, and 0.3%) significantly reduced EHEC counts in ground beef cooked to an internal temperature of 60 or 65°C compared to untreated patties cooked to the same temperatures. Yet another study found that incorporation of apple skin and tea leaf powders at 3% level in ground beef decreased the heat resistance of EHEC in Sous-wide cooked ground beef (Juneja et al., 2009).

Since the attractive red color of meat is the primary yardstick by which consumer purchasing decisions of beef and beef products are made (Mancini and Hunt, 2005), we determined the effect of RT and CH on the color of uncooked beef patties during refrigerated storage. The results from meat color analysis revealed that none of the treatments adversely affected meat color compared to control patties. Further, patties containing RT and CH demonstrated a greater redness on days 3 and 5 of storage compared to control patties ($P < 0.05$). Previous studies have revealed that CH at 1% increased the redness and color stability of frozen beef patties and pork sausages (Lin and Chao, 2001; Georgantelis et al., 2007a). However, in this study a much lower concentration of 0.01% (w/w) was used and the color of patties containing only CH was not significantly greater than that of control samples. Moreover, the aforementioned researchers observed an elevated pH in CH added meat in their studies, and the increased meat pH may have minimized myoglobin oxidation and surface discoloration, thereby maintaining the meat color stability (Suman et al., 2010). However, we did not observe any significant change in the pH of meat irrespective of the treatments throughout the study. Therefore the mechanism behind the increased redness in patties with RT in combination with CH needs to be investigated.

A primary cause for quality deterioration of meat during storage is lipid oxidation, which adversely affects color, flavor, texture and nutritional value of meat. Previous studies have shown that plant antimicrobials such as *trans*-cinnamaldehyde and rosemary oil decreased lipid oxidation in beef patties and pork sausages (Georgantelis et al., 2007b; Amalaradjou et al., 2010). In addition, CH at doses ranging from 0.2 to 1% has been documented for its ability to minimize lipid oxidation in meat. However, we did not observe any protective effect of RT or CH in reducing lipid oxidation in refrigerated beef patties, and the amount of TBARS was not different among patties throughout the storage period ($P > 0.05$).

Results of this study indicated that RT in the presence of CH significantly increased the heat inactivation of EHEC in undercooked ground beef patties without adversely affecting meat color and lipid oxidation. Therefore, this natural antimicrobial could be added to ground beef for improving the microbiological safety of undercooked beef. However, organoleptic studies are warranted for ascertaining the consumer acceptability of patties supplemented with the aforementioned additives.

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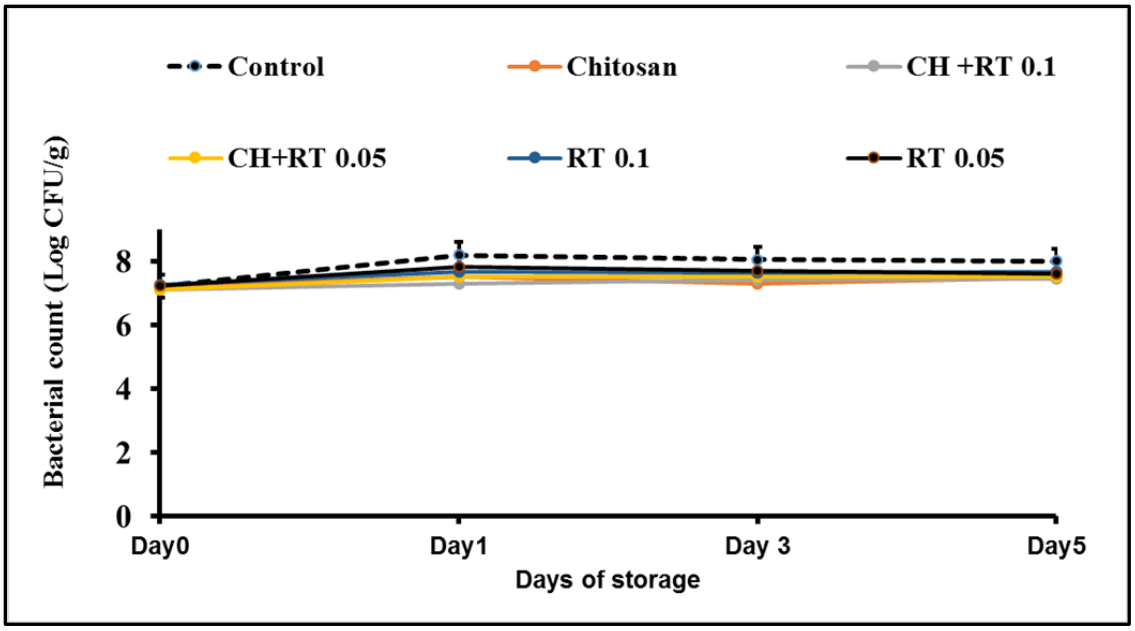
Fig 1.A. Effect of Rutin (RT) with or without Chitosan (CH) on EHEC in uncooked ground beef patties stored at 4°C for 5 days. Fig 1.B. Effect of RT with or without CH on EHEC in ground beef patties stored at 4°C for 5 days and cooked to an internal temperature of 65°C. # - Negative by Enrichment. * Treatments were significantly different from the control at P<0.05.



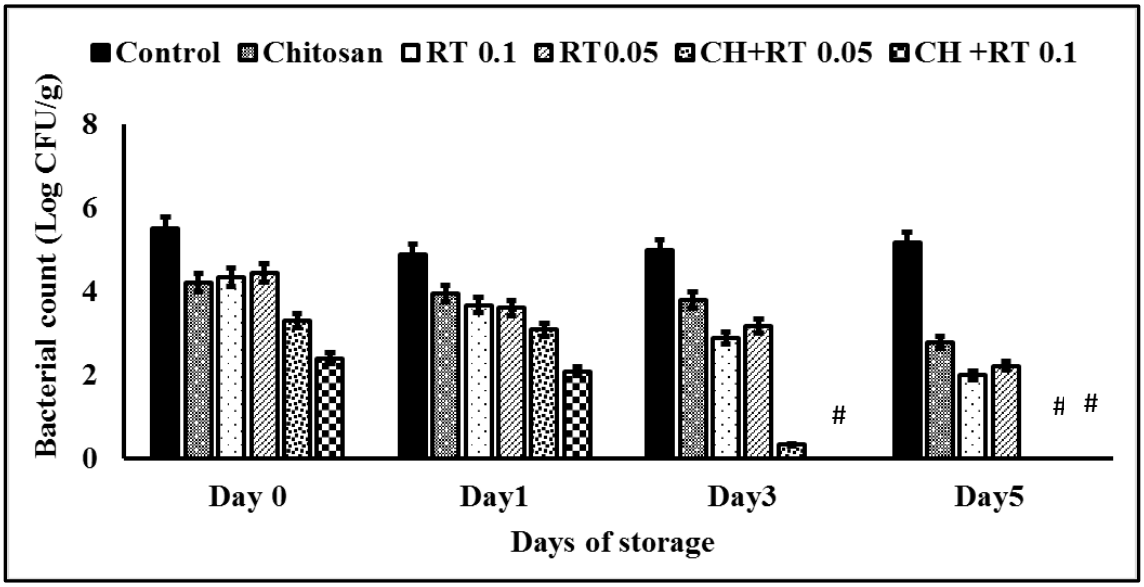


Fig 2. Effect of Rutin (RT) with or without Chitosan (CH) on lipid oxidation in ground beef patties stored at 4°C for 5 days. Higher values of TBARS denote more lipid oxidation. # - Negative by Enrichment. * Treatments were significantly different from the control at $P<0.05$.

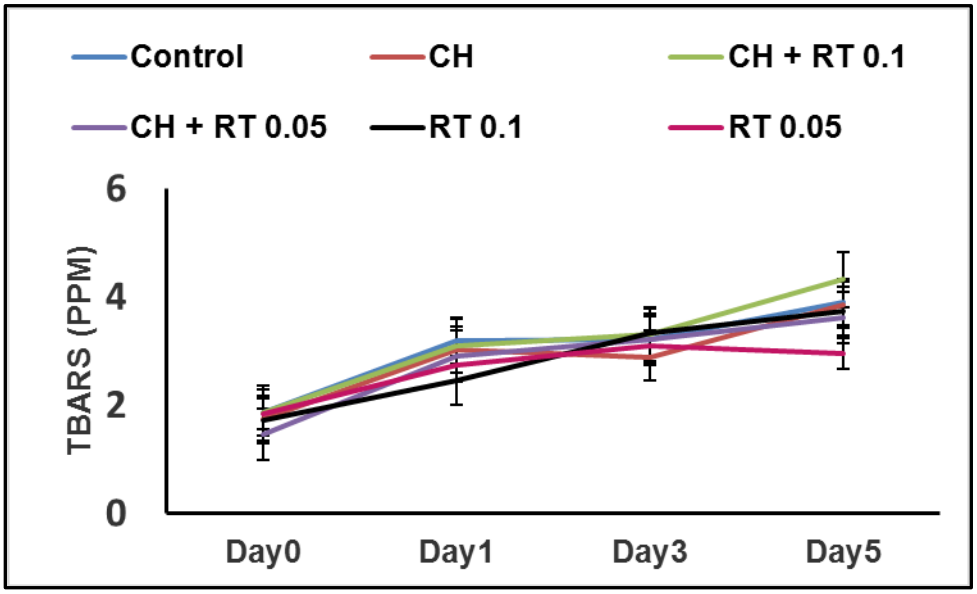


Table 1. Effect of RT with or without CH on meat color in ground beef patties stored at 4°C for 5 days. Increased a* value indicated increased redness of meat and means with same letters are not significant down each column.

Treatment	**a* value ± SE**			
	Day 0	Day 1	Day 3	Day 5
Control	29.81 ± 0.84^{ab}	25.23 ± 0.71^{bc}	17.32 ± 0.62^{f}	12.08 ± 0.95^{bc}
CH	30.26 ± 0.94^{ab}	26.67 ± 0.59^{ab}	21.72 ± 0.54^{abc}	12.35 ± 0.92^{bc}
CH + RT 0.1	29.79 ± 0.94^{ab}	26.13 ± 0.41^{b}	21.54 ± 0.37^{abcd}	12.04 ± 0.84^{bc}
CH + RT 0.05	29.87 ± 1.27^{ab}	26.06 ± 0.44^{b}	20.95 ± 0.67^{bcde}	13.57 ± 0.95^{bc}
RT 0.1	29.84 ± 0.82^{ab}	28.24 + 0.26^{bc}	20.01 ± 0.96^{cde}	15.23 ± 0.93^{ab}
RT 0.05	28.66 ± 0.71^{b}	27.85 ± 0.76^{c}	18.62 ± 0.88^{ef}	14.08 ± 0.98^{abc}

CHAPTER V

Selenium reduces Enterohemorrhagic *Escherichia coli* O157: H7 verotoxin production and Gb3 receptor expression on host cells

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Abstract

Aim: This study investigated the efficacy of selenium (Se) in reducing *Escherichia coli* O157: H7 verotoxin production and toxin gene expression. Additionally, the effect of Se on globotriaosylceramide (Gb3) receptor in human lymphoma cells was determined.

Methods: The effect of Se on verotoxin synthesis was determined by standard ELISA, whereas its effect on Gb3 receptor was determined by flow cytometry and real-time quantitative PCR. Results & conclusions: Se reduced extracellular and intracellular verotoxin concentration by 40–60% and 80–90%, respectively ($p < 0.05$), and downregulated verotoxin genes ($p < 0.05$). Se reduced Gb3 receptor synthesis in lymphoma cells, and real-time quantitative PCR data revealed a significant downregulation of *LacCer* synthase gene (*GalT2*) involved in Gb3 synthesis. Further studies are warranted to validate these results in an appropriate animal model.

Keywords: *E. coli* O157: H7, selenium, verotoxin, Gb3 receptor

1. Introduction

Shiga toxin producing (STEC) or verotoxin producing *Escherichia coli* (VTEC) poses a significant public health concern owing to its ability to produce fatal food-borne infections with a low infectious dose. Among the various VTEC serotypes, Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC) is a major food-borne pathogen in the United States, and is estimated to cost the US economy ~ one billion dollars annually [1]. Cattle are the principal reservoir of EHEC, with fecal shedding of the pathogen being an important source of food and environmental contamination [2]. The pathogenesis of EHEC infection in humans is chiefly mediated by verotoxins, and is characterized by life threatening conditions such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [3]. EHEC produces verotoxin 1 and/or verotoxin 2, which are encoded by *stx1* and *stx2*, respectively. Verotoxins belong to AB5 toxin family with A and B subunits. The pentameric B subunit binds to the host cell surface receptor, globotriaosylceramide (Gb3), whereas the A subunit enters the cells by endocytosis, arrests protein synthesis and causes apoptosis of cells [4, 5]. The total verotoxin produced by EHEC includes two components, namely extracellular and intracellular. The extracellular verotoxin component is released into the environment, whereas the intracellular portion is accumulated in the bacterial cells, and is released when bacterial cells are lysed [6, 7, 8].

The use of antibiotics for treating EHEC infection is contraindicated since they induce the phage lytic cycle thereby augmenting the expression of *stx* genes encoded within the prophage genome [9, 10]. Neely and Friedman (11) showed that the activation of this phage lytic cycle resulted in increased Stx production in EHEC.

Thus, there is a need for alternate strategies for controlling EHEC infection in humans. Potential novel approaches for treating EHEC infections in humans include those targeted against

the verotoxins, toxin receptor and their interaction, thereby reducing the cellular uptake of toxins and downstream pathology [12, 13, 14**]**.

The usage of metals as antimicrobial agents can be traced back to historical periods, with their application in human medicine and agriculture gradually decreasing after the discovery of antibiotics. However, due to the emergence of multidrug resistant pathogens and shortage of new antibiotics, the use of antimicrobial metals is undergoing a period of resurgence [15]. Many transition metals and metalloids such as copper, silver and gallium have been reported as effective antimicrobials and anti-biofilm agents against *E. coli, Salmonella, Pseudomonas aeruginosa* and *Staphylococcus aureus* [15, 16, 17, 18]. In addition, the efficacy of zinc in attenuating the virulence of EHEC both *in vitro* and *in vivo* was reported [19]. Similarly, manganese was found to block intracellular trafficking of shiga toxin, and protect mice when challenged with the toxin [20].

Selenium (Se) is a naturally occurring essential microelement recommended for daily intake by the United States Food and Drug Administration. Selenium is present in a wide variety of foods, and is available as a dietary supplement. In humans, Se is an essential cofactor in selenoproteins, which play structural and enzymatic functions in many biological processes in the body, including reproduction, DNA synthesis, immunity and antioxidant defense [21, 22]. The daily average intake of Se in the United States ranges from 70 to 100 µg [23], with a recommended dietary allowance of 55 µg and tolerable upper intake level of 400 µg in adults [24]. Selenium and its compounds have been used in anti-cancer treatment, and as a potential orthopedic implant [25, 26]. Sodium selenite (Na_2SeO_3) is a commonly used dietary supplement containing Se, with proven antifungal and antibacterial properties [27, 28].

This study investigated the efficacy of sub-inhibitory concentration (SIC; highest concentration that is neither bactericidal nor bacteriostatic) of Se in reducing EHEC verotoxin

production and toxin gene expression. In addition, the effect of Se on verotoxin binding to Gb3, and the expression of Gb3 receptor in human lymphoma cells was determined.

2. Materials and methods

2.1.Bacterial strains and culture conditions

All bacteriological media used in this study were purchased from Difco (Becton Dickinson, Sparks, MD). Three strains of EHEC, namely ATCC 43895 (EDL 933), E163 (meat isolate) and E137 (salami isolate) were used in this study. Each strain was cultured separately in 10 ml of sterile tryptic soy broth (TSB, Sigma Aldrich, St. Louis, MO) in 15 ml screw-cap tubes, and incubated at 37°C for 18 h. Following incubation, bacteria from each culture were pelleted by centrifugation (3,600 ×*g* for 15 min), washed twice and suspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.0). The bacterial count in each of the three cultures was determined by plating 100 µl of ten-fold serial dilutions of the culture on duplicate tryptic soy agar (TSA, Sigma Aldrich) and Sorbitol MacConkey agar (SMA, Sigma Aldrich) plates, followed by incubation at 37°C for 24 h.

2.2.Sub-inhibitory concentration of selenium

The SIC of Se was determined as previously described [29]. Sterile 24-well polystyrene tissue culture plates (Costar, Corning Incorporated, Corning, NY) containing 2 ml of TSB per well were inoculated separately with ~ 6 $\log_{10}$ CFU/ml of each EHEC culture, and 5–50 µl of a 50% (w/v) stock solution of sodium selenite (99% purity, Sigma-Aldrich) with an increment of 5 µl was added to each well. The plates were incubated at 37°C for 24 h, and bacterial growth was monitored by plating on TSA and SMA plates. The highest concentration of Se that did not inhibit bacterial growth after 24 h of incubation was selected as the SIC for the study.

2.3.Determination of verotoxin concentration

The verotoxin produced by each EHEC strain was *quantified* by enzyme-linked immunosorbent assay (ELISA) using the *Premier EHEC* test (Meridian Bioscience, Cincinnati, OH), as described previously [6, 30]. Briefly, TSB containing the SIC of Se was separately inoculated with each EHEC strain (~6.0 log CFU/ml), and incubated at 37ºC for 24 h. EHEC cultured in the absence of Se was included as control. After incubation, the bacterial cells were pelleted by centrifugation at 3,800× *g* for 15 min, and the culture supernatant and cell pellets were separated. The cell pellets were washed three times with PBS. The final washed pellet was resuspended in Tris-EDTA lysis buffer, and the cell membranes were disrupted using an ultrasonicator (Branson Digital Sonifier, Danbury, CT) at 50 W for 1 min. The extracellular and intracellular verotoxin concentrations were estimated in cell-free culture supernatant and cell lysate at both 0 and 24 h of incubation, respectively using the aforementioned ELISA kit. A standard curve of optical density at 450 nm versus known standard verotoxin (BEI resources, Manassas, VA) concentration was used to determine the unknown concentration of verotoxin in control and Se treated samples [6].

2.4.Toxin gene expression using real-time quantitative PCR (RT-qPCR)

The effect of Se on the expression of genes encoding verotoxins (*stx1* and *stx2*) was investigated using RT-qPCR, as described previously [31]. Each EHEC strain was grown overnight in TSB at 37°C with or without the SIC of Se, and total RNA was extracted using an RNeasy RNA isolation kit (Qiagen, Valencia, CA). The complementary DNA (cDNA) synthesized using the Iscript cDNA synthesis kit (Biorad, Hercules, CA) was used as the template for RT-qPCR. SYBR green reagents (Applied Biosystems, Inc., CA) were used to detect the amplification products. RT-qPCR analysis of the verotoxin genes was performed using published primers [31], and normalized against 16S rRNA gene expression. Twenty-five microliter reactions were

performed in duplicate using iTaq SYBR RT-PCR (Bio-Rad) and repeated three times. The relative fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [32].

2.5.Flow cytometry

The effect of Se on the surface expression of Gb3 receptor protein in the Ramos cell line was studied using flow cytometry [33, 34, 35, 36]. Ramos cells (human Burkitt's lymphoma; ATCC CRL-1596) were maintained in Roswell Park Memorial Institute medium (RPMI, Gibco, Invitrogen) containing 20% FBS at 37°C under 5% CO_2. The cells (~1 x 10^4 per well) were seeded onto a 6-well plate, and allowed to grow overnight at 37°C in the presence of 5% CO_2. The cells were then treated with the SIC of Se for 1 h, sedimented by centrifugation at 300 x g for 10 min and washed with fluorescence activated cell sorting (FACS) buffer (PBS containing 0.1% fetal bovine serum and 0.01% sodium azide). The cells were subsequently resuspended in FACS buffer and labeled with FITC-conjugated mouse anti-human CD77/Gb3 antibody (BD Bioscience, San Jose, CA) for 30 min on ice. Unlabeled control wells (cells neither treated with Se nor incubated with antibody), labeled control wells (cells not treated with Se, but labeled with antibody) and isotype control wells (cells not treated with SIC, but labeled with FITC mouse IgM κ (BD Bioscience) were included. Isotype controls were used to confirm the primary antibody specificity and determine any non-specific reactivity of antibody with the cells [37]. After labeling, cells were washed twice with FACS buffer and analyzed by flow cytometry using a BD FACS-Calibur flow cytometer. The mean fluorescence intensity was calculated for all the samples [37, 38].

In addition, the recovery of Gb3 expression on the Se-treated cells after removing the Se pressure was determined. As previously mentioned, the cells (~1 x 10^4 per well) were seeded onto a 6-well plate, and allowed to grow overnight at 37°C in the presence of 5% CO_2. The cells were then treated with the SIC of Se for 1 h, pelleted by centrifugation at 300 x g, and washed 5 times

with FACS buffer. The cells were seeded onto 6 well plates, allowed to grow overnight, and analyzed for Gb3 expression by flow cytometry.

2.6.Effect of selenium on the expression of Gb3 synthesis genes

Ramos cells were seeded onto a 6-well plate, and allowed to grow overnight, as described previously. The cells were treated with the SIC of Se for 1 h, following which total RNA was extracted using TRIzol reagent (Ambion, NY) –Chloroform (Sigma Aldrich) followed by RNeasy RNA isolation kit (Qiagen). RT-qPCR was performed to analyze the expression of genes involved in the synthesis of Gb3. The primers used for the amplification of Gb3 synthesis genes are listed in Table 1. The gene *actB* was used as endogenous control, and the level of gene expression in Se-treated samples was compared to untreated control samples.

2.7.Effect of Se on verotoxin binding to Gb3 receptor

2.8.1. Isolation and quantification of Gb3

The Gb3 receptor was isolated from Vero cells as described previously [39]. Vero cells (CCL-81, ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen) containing 20% FBS in six well polystyrene plates, and the growth was arrested at confluence by transferring the cells to serum deficit media. Vero cells were stimulated with the inflammatory factor, TNFά (100U/ml; Sigma Aldrich) for 24 h followed by the extraction of total cellular lipids. The cells were washed with ice cold PBS (pH 7), scraped off the plates and centrifuged at 500 x *g* for 10 min. The cell pellet was then lysed using an ultrasonicator at 50 W for 5 min. Subsequently, cells were extracted in 1ml of chloroform: methanol: water solvent (5:10:3) three times, and dried under nitrogen gas. The total dried lipid was suspended in 2:1 chloroform: methanol, and separated on silica gel thin layer chromatography plates (10 x 20 cm, Sigma-Aldrich) by ascending chromatography in chloroform: methanol: water (65:35:8) solvent

system. The lipid content on silica plates was then visualized with iodine vapor. The Gb3 standard (Ceramide Trihexosides Gb3, Matreya LLC, PA) was run at different concentrations (10 to 40 µg) on TLC plates to identify the glycolipid of interest, and determine the approximate amount of Gb3 extracted from the cells. Additionally, a standard curve of optical density at 490 nm versus Gb3 concentration (µg/ml) was used to determine the unknown concentration of the Gb3 extracted from the cells.

The effect of Se on the binding of verotoxin to the Gb3 receptor was determined according to a published protocol [40]. The extracted Gb3 was retrieved from the TLC plate and dissolved in 2:1 chloroform: methanol solvent. Polystyrene microdilution plates (Fisher Scientific, Pittsburg, PA) were inoculated with Gb3 (36 µg/ml), left uncovered for 4 h at room temperature for the evaporation of the diluent, and subsequently washed with Tween 20-PBS buffer. Verotoxin extracted from an overnight culture of EHEC EDL 933 was added to the plates (50 ng/well), and allowed to bind to the receptor with or without the SIC of Se at 37°C for 1 h. Following this, mouse anti-human CD77/Gb3 antibody (BD Bioscience) was added to the wells at 1:1000 dilution, and allowed to bind to Gb3 for 1 h at 37°C. Subsequently after washing with Tween-PBS, peroxidase conjugated rabbit anti-mouse immunoglobulin G (Promega, Madison, WI) was added and incubated at 37°C for another hour. The substrate orthophenyldiamine (0.4 mg/ml, Sigma-Aldrich)-0.1% H_2O_2 in citrate-phosphate buffer (25 mM citrate, 50 mM phosphate, pH 5) was then allowed to react with the available enzyme peroxidase in the well for 15 min at room temperature following the washing step. The supernatants from the wells were transferred to new plates after incubation and the absorbance was measured at 490 nm. Wells with 0.1% BSA in 0.05% Tween 20-PBS (diluent in which samples were diluted) instead of the sample served as negative controls.

3. Statistical analysis

All the experiments had duplicate samples for each treatment and control, and were replicated three times. The data were analyzed using a generalized linear model of proc genmod procedure of SAS 9.3 version (SAS institute, Cary, NC). The differences between the means were compared using least significant difference (LSD) test and were found to be significant compared to control when $P<0.05$.

4. Results

4.1.Determination of SIC of selenium

The highest concentration of Se that did not inhibit EHEC growth when compared to control was 30 mM (0.5% w/v). The average initial EHEC count in Se-treated and control samples was ~ 5 log CFU/ml. After 24 h of incubation at 37^{o}C, ~ 8.5 log CFU/ml of EHEC was recovered from control and treated samples, thereby confirming that the aforementioned Se concentration was not inhibitory to bacterial growth (data not shown).

4.2.Effect of selenium on verotoxin production and toxin gene expression

Selenium reduced both extracellular and intracellular verotoxin concentrations in all three EHEC strains (P<0.05) (Fig. 1 and 2). At 0 hour of incubation, the extracellular verotoxin concentration in both treatments and control was found to be ~ 4 to 6 ng/ml, irrespective of the strains, whereas the intracellular verotoxin concentration was under the detection limit (< 1 ng/ml). However, by the end of 24 h of incubation, compared to control (436 ng/ml) the extracellular verotoxin concentration in the presence of Se was decreased by 200 – 300 ng/ml (40-60%) (P<0.05) (Fig. 1). Similarly, the intracellular verotoxin concentration in Se-treated EHEC was reduced by 80-90% after 24 h of incubation (P<0.05) (Fig. 2). The magnitude of extracellular and intracellular verotoxin production was found to be strain specific. Among the three different strains, EDL 933 produced the highest amount of extracellular and intracellular toxins. Concurring

with the ELISA results, RT-qPCR data revealed that Se down-regulated both *stx1* and/or *stx2* expression in all the three strains of EHEC ($P<0.05$) (Fig. 3).

4.3.Effect of selenium on Gb3 receptor and Gb3 synthesis genes in Ramos cells

The cell surface expression of Gb3 was determined by measuring the mean fluorescence intensity of gated population of live cells using flow cytometry. Live cell population as determined by side scatter-forward scatter gate (confirmed with propidium iodide staining) were analyzed. As shown in Table 2, the mean fluorescence of cells treated with Se was decreased by ~ 80% (observed a mean fluorescence of 10) compared to control cells (observed mean fluorescence of 45), thereby suggesting the inhibitory effect of the Se on Gb3 receptor expression. Histograms showing Gb3 receptor fluorescence intensity of representative samples of unlabeled, labeled, Se treated cells and isotype control are shown in Fig 4. As discussed above, there was approximately a log shift in the fluorescence intensity in Se-treated samples compared to the labeled control. This was further supported by RT-qPCR results, which indicated that Se down-regulated the expression of LacCer synthase gene (*GALT2*) involved in Gb3 synthesis ($P < 0.05$) (Fig. 5). In addition, Se-treated cells were found to regain 75% of their original Gb3 expression following the removal of the treatment pressure (data not shown).

4.4.Effect of Se on verotoxin binding to Gb3 receptor

Gb3 receptor was extracted and the concentration was estimated from a standard curve generated using various concentrations of pure Gb3 standard. The average yield of Gb3 was found to be ~ 36 µg/ml (Fig 6A). The extracted Gb3 was then used to determine the effect of Se on toxin binding to the receptor. As shown in Fig. 6B, when verotoxin alone was added to the wells coated with Gb3, the absorbance decreased to 56% compared to control, indicating a decreased free Gb3 availability due to active binding of toxin to the receptor. However, the toxin binding to the

receptor was found to be interfered to some extent in the presence of Se, where a Gb3 availability (83%) was observed compared to wells added with only verotoxins. This suggested that a greater concentration of free Gb3 was available for the antibody to bind when toxin was added with Se. However, the difference in Gb3 availability observed between wells containing toxin and those with toxin and Se was not significant ($P > 0.05$). Further, selenium by itself did not affect the antibody binding to the receptor compared to control wells ($P > 0.05$).

5. Discussion

Despite being a bacterial infection, the current treatment strategies against EHEC in humans are limited, and do not include the use of antibiotics. As mentioned previously, this is because of the fact that antibiotic usage has been linked to enhanced risk of systemic complications such as acute renal failure [10] and elevated mortality in patients [9], due to increased verotoxin release from EHEC lysis [9, 10]. In addition, antibiotics can inhibit the growth of normal gut flora, leading to EHEC overgrowth [9]. Moreover, no effective vaccine is available against EHEC in humans [41, 42, 43]. Thus, there is a critical need for identifying alternate interventions for controlling EHEC infection in humans.

A relatively new strategy which is being increasingly investigated for combating infectious diseases involves targeting microbial virulence rather than growth, where a pathogen's specific mechanisms critical for causing infection or disease symptoms in hosts are inhibited [44, 45]. Since anti-virulence agents are neither bacteriostatic nor bactericidal, they exert a reduced selection pressure for the development of bacterial drug resistance [46, 47, 48], and are minimally deleterious on the host gut microflora.

We observed that the SIC of Se neither inhibited EHEC growth nor killed the bacterium, but significantly inhibited both extracellular and intracellular verotoxin. The RT-qPCR results also

concurred with the aforementioned findings, where Se was found to down-regulate the expression of *stx1* and *stx2* in EHEC.

As verotoxins are the key virulence factors that cause HUS, therapeutic interventions which can impede toxin binding, uptake, or receptor function are potential approaches for the treatment of EHEC infections [12, 13]. Moreover, Gb3 is considered as the only functional receptor for verotoxins in most mammals, with the cell surface expression of Gb3 being a prerequisite for toxin sensitivity [49, 50, 51, 52, 53, 54], and toxin-induced pathology in the host. Further, inhibitors of Gb3 synthesis enzymes have been shown to reduce host cell sensitivity to verotoxin [55, 56]. Several studies have been conducted to identify compounds that could reduce Gb3 synthesis or binding to the toxin in order to prevent toxin receptor interaction. One such compound is C-9, which was found to inhibit the conversion of ceramide to glucosylceramide during Gb3 synthesis in renal tubular epithelial cells [57]. The renal tubular epithelial cells pretreated with C-9 expressed reduced levels of Gb3, thereby demonstrating decreased sensitivity to verotoxin type 2.

In this study, to determine the effect of Se on the receptor expression, we used Ramos cells, which are known to over express Gb3 receptor. Ramos cells have been extensively used for studying physico-chemical characteristics of Gb3 receptor [34, 35, 36]. The level of Gb3 expression has been reported to be high in these tumor cells which were found to be much more sensitive to verotoxins compared to normal renal and intestinal cells [54, 56].

Flow cytometry is a fluorescence-based technique commonly used to analyze cells for extracellular and intracellular protein expression, and it provides information on the proportion of cells expressing a targeted receptor and the relative number of receptors expressed by a single or population of cells [56]. Using fluorochromes conjugated to a specific antibody that can recognize the protein of interest, the surface expression of receptors can be determined [58, 59]. Therefore,

flow cytometry was used for the quantitative analysis of the cell surface expression of Gb3 on Ramos cells. As shown in the results (Table 2 and Fig. 4), the mean fluorescence data indicated that Gb3 expression on Se-treated cells was significantly reduced in comparison to that in the controls ($P < 0.05$). These results are supported by RT-qPCR data, which revealed a significant down-regulation in the expression of GALT2 gene involved in Gb3 synthesis [60].

Furthermore, we investigated the effect of Se on reducing verotoxin binding to Gb3 receptor using an *ex vivo* Gb3 binding assay. Although the results showed a relative decrease in toxin binding to the receptor in the presence of Se, the reduction was not significantly different from that of the toxin control ($P > 0.05$). These results indicate that the potential therapeutic effect of Se is mainly mediated by decreasing EHEC verotoxin production and host receptor expression, rather than interference with toxin binding to the receptor.

6. Conclusion

In summary, results of this study indicated that Se could potentially be used to attenuate EHEC infection by reducing verotoxin-mediated pathogenesis. The SIC of Se at which the down-regulation of Gb3 expression observed in this study is approximately at the recommended upper tolerable level (400 µg), and half of the no-observed-adverse-effect level (NOAEL) of selenium (800 µg/day) [61, 62]. Therefore, the down-expression of Gb3 on host cells is not expected to occur at the much lower daily intake levels of Se. Further studies are needed to unveil the molecular mechanisms behind the anti-verotoxin effect of Se on EHEC and its down-regulation of Gb3 on host cells. Moreover, the effective blood concentration of Se required for the anti-verotoxin property and its pharmacokinetics in a living system needs to be determined. Our follow up *in vivo* studies will validate the safety and efficacy of Se-based approach for EHEC control.

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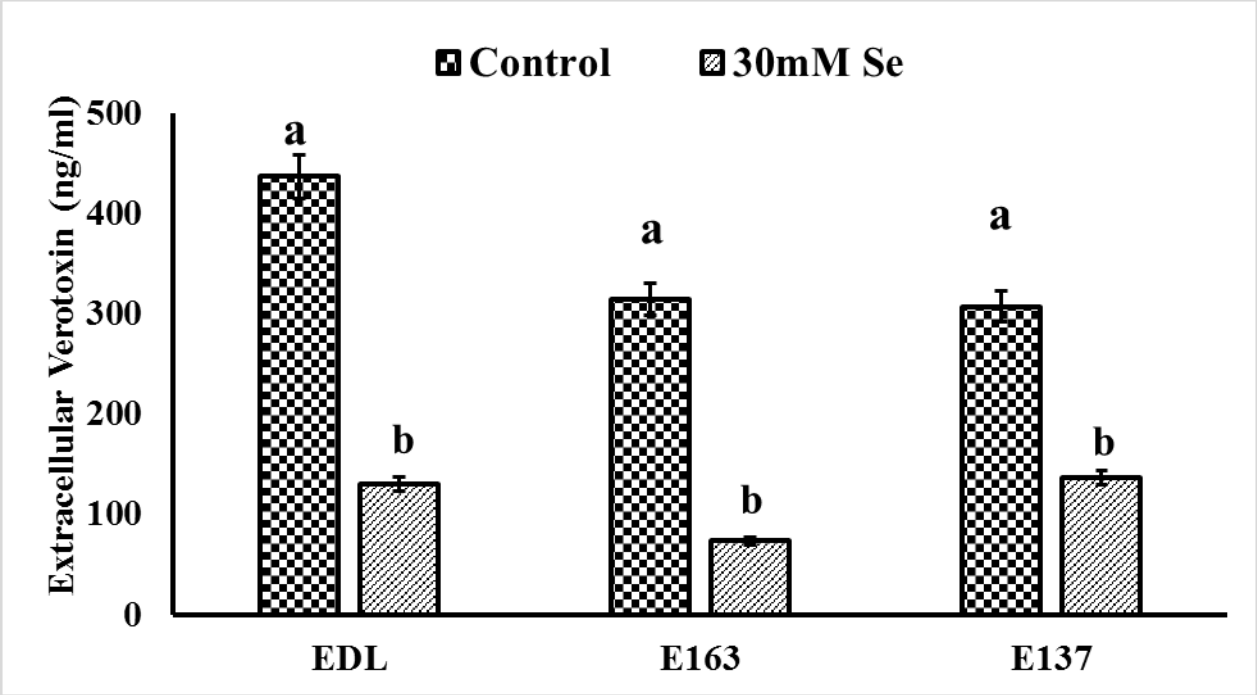
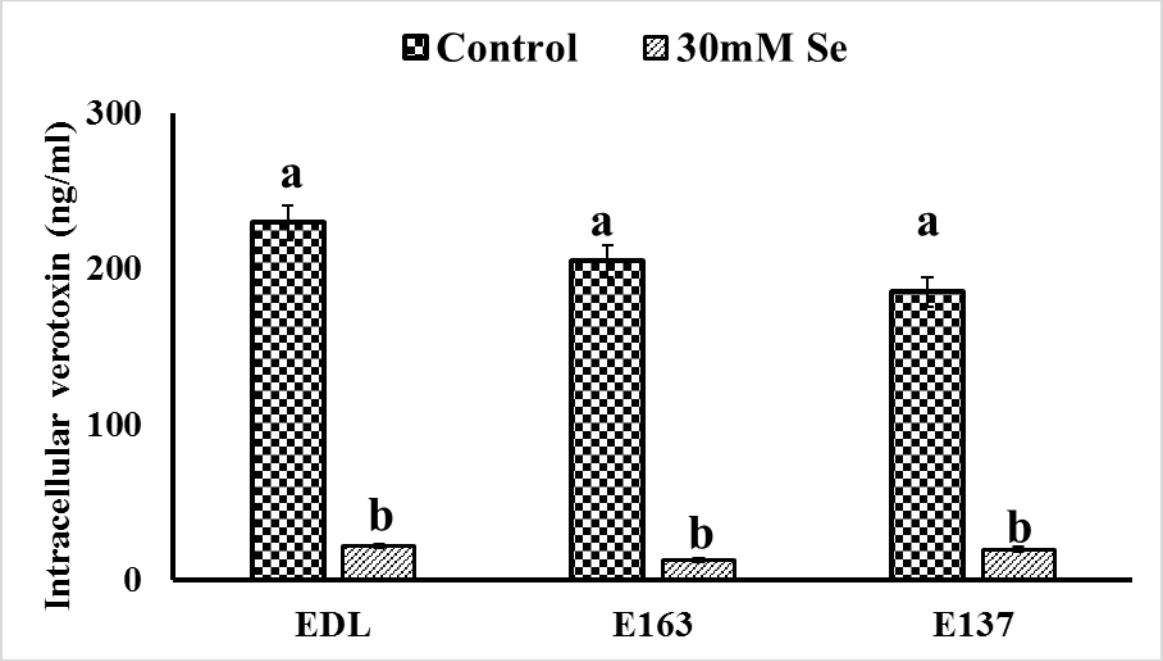
Figure 1: Effect of Se on extracellular verotoxin concentration.



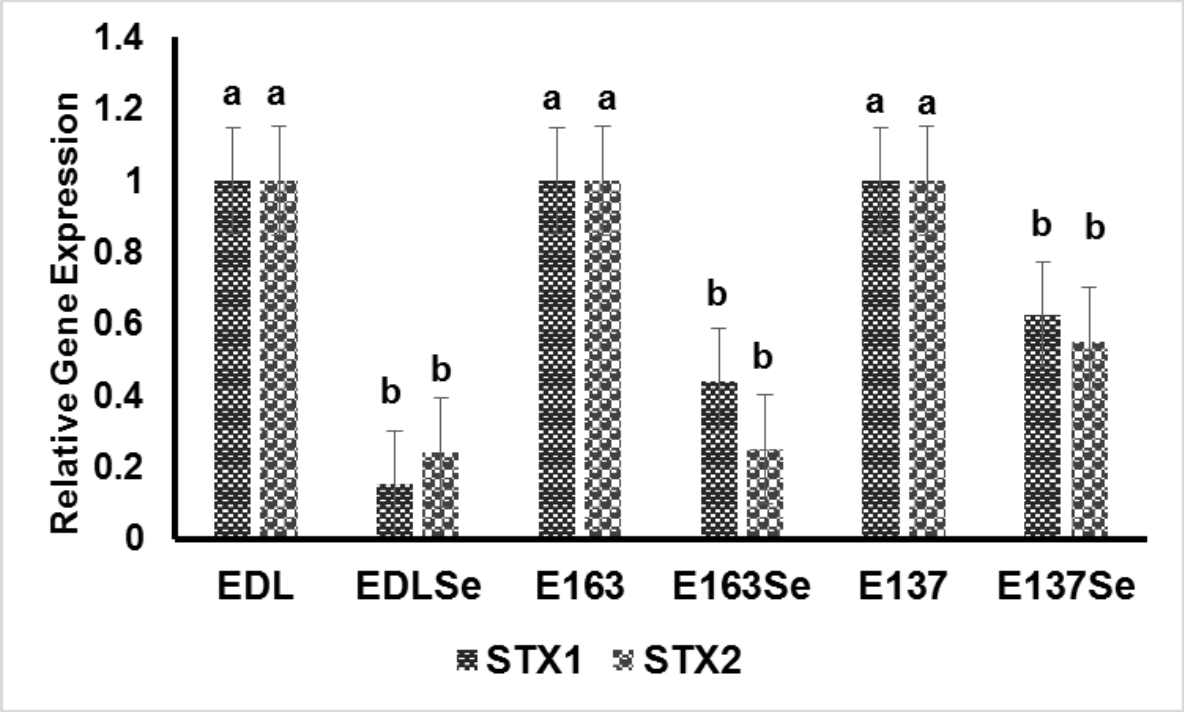
Three strains of EHEC (EDL 933, E163, and E137) were separately cultured with or without the SIC of Se (30 mM) at 37°C for 24 h, and extracellular verotoxin in cell-free culture supernatant was determined by ELISA. Bars with different letters (a and b) denote significant difference between the treatments and respective control ($P < 0.05$).

Figure 2: Effect of Se on intracellular verotoxin concentration.



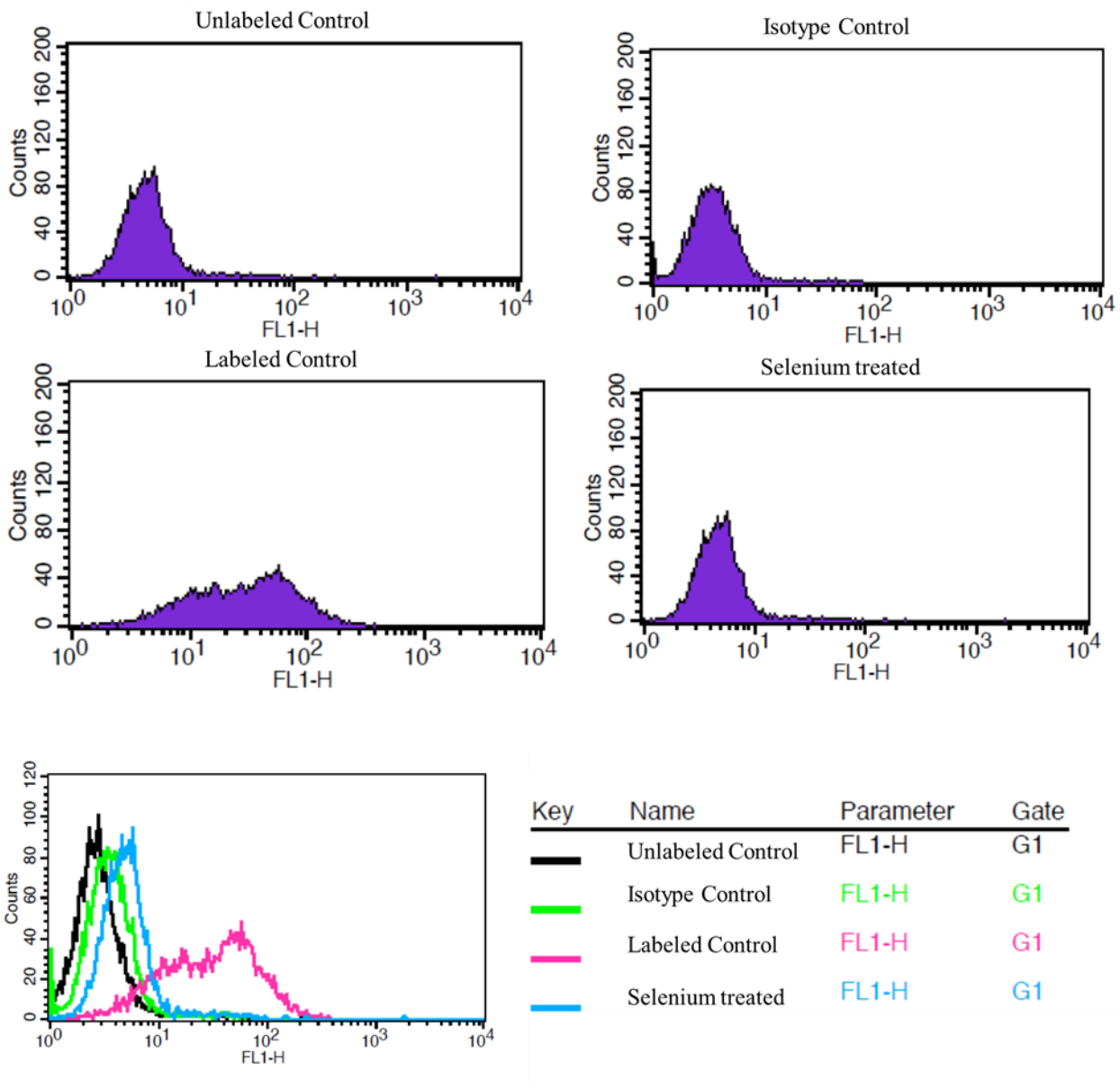
Three strains of EHEC (EDL 933, E163, and E137) were separately cultured with or without the SIC of Se (30 mM) at 37°C for 24 h, and intracellular verotoxin concentration in cell lysate was determined by ELISA. Bars with different letters (a, and b) denote significant difference between the treatments and respective control ($P < 0.05$).

Figure 3. Effect of Se on verotoxin gene expression.



RNA extracted from EHEC grown with and without SIC of Se was converted to cDNA, and subjected to RT-qPCR using published primers specific for *stx1* and *stx2*. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Bars with different letters (a, b) denote significant difference between the treatment and control for respective genes ($P < 0.05$).

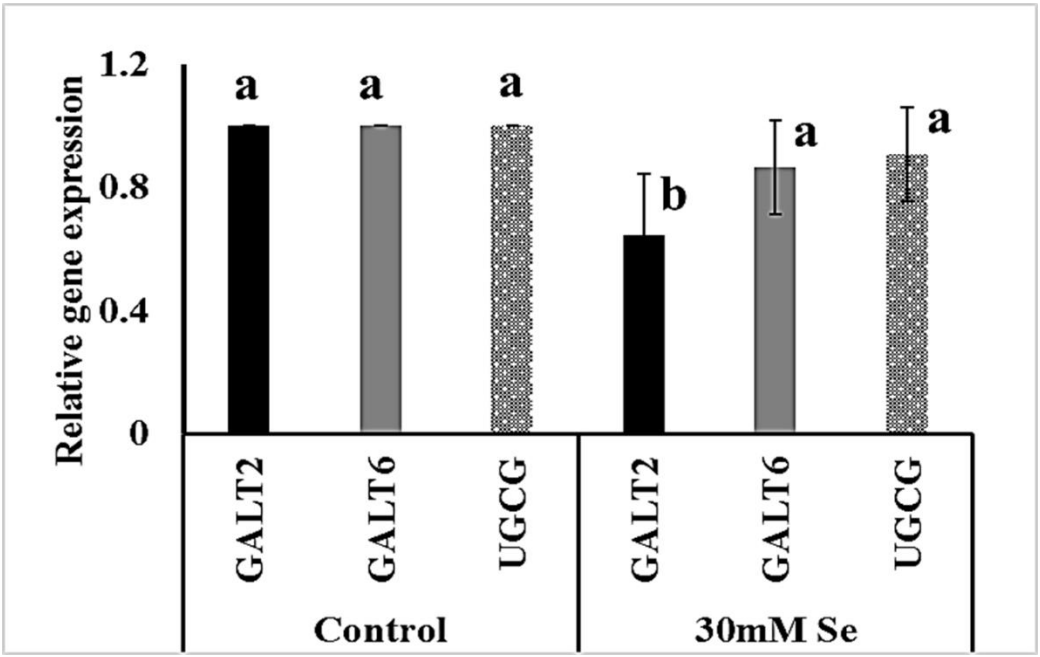
Figure 4. Effect of Se on Gb3 receptor expression on Ramos cells.



The cells were treated with the SIC of sodium selenite for 1 h, sedimented by centrifugation at 300 x g for 10 min, and washed with fluorescence activated cell sorting (FACS) buffer (PBS containing 0.1% fetal bovine serum and 0.01% sodium azide). The cells were subsequently labeled with FITC mouse anti-human CD77/Gb3 antibody and incubated for 30 min on ice. Appropriate unlabeled, labeled and isotype controls were included. Histograms showing Gb3 receptor fluorescence

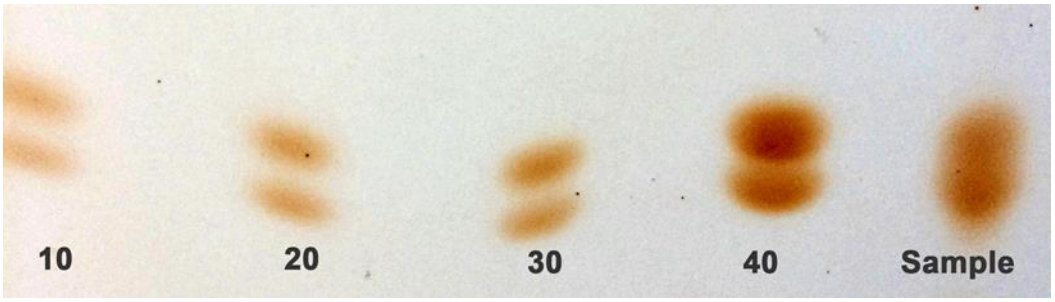
intensity of gated Ramos cells. FACS sorted Ramos cells after treating with selenium compared to unlabeled, labeled Ramos cells and isotype control.

Figure 5. Effect of Se on Gb3 gene expression in Ramos cells.



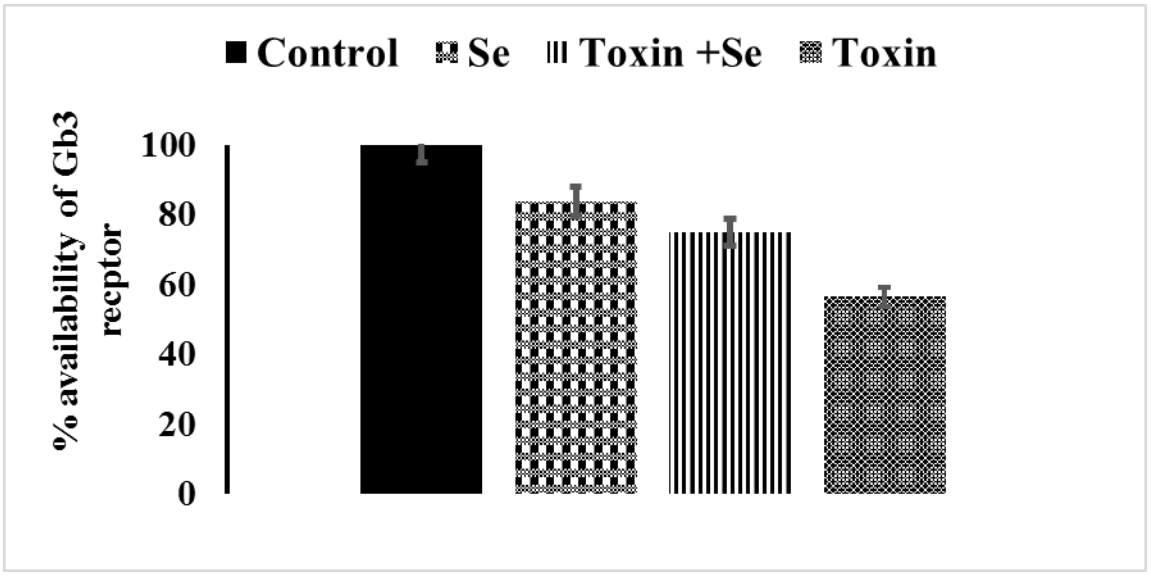
RNA extracted from Ramos cells grown with and without SIC of Se was converted to cDNA, and subjected to RT-qPCR using published primers specific for genes involved in the synthesis of Gb3. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Bars with different letters (a, b) denote significant difference between the treatment and control for respective genes ($P < 0.05$).

Figure 6A. Globotriaosylceramide (Gb3) content in Vero cells.



Each experimental lane represents different concentrations (µg/ml) of Gb3 standard and the extracted sample.

Figure 6B: Gb3 binding.



Gb3 was extracted from the Vero cells using thin layer chromatography and coated on to microdilution plates. Elisa was performed to detect the percentage availability of Gb3 receptor for binding with the antibody after incubating with the verotoxin in presence or absence of Se. Even though, there was decreased binding of receptor with the toxin in the presence of Se, the percentage availability of Gb3 receptor was not significantly different between toxin treatment and toxin plus Se treatment (P=0.11).

Table 1: List of primers used in the study

Gene	Primer	Sequence (5' – 3')
stx1	Forward	GTGGCATTAATACTGAATTGTCATCA
	Reverse	GCGTAATCCCACGGACTCTTC
stx2	Forward	GATGTTTATGGCGGTTTTATTTGCTGG
	Reverse	AAAACTCAATTTTACCTTTAGCA
actB	Forward	TGGCGCTTTTGACTCAGGAT
	Reverse	GGGATGTTTGCTCCAACCAA
GALT2	Forward	AACGGTACAGATTATCCCGAAGG
	Reverse	TGGAGCTAACTCTGGCATGAGG
GALT6	Forward	GATCTGGGGATACCATGTCCAAG
	Reverse	CAGTAGCGGGCATGCAGCTGG
UGCG	Forward	GCTGTGGCTGATGCATTTCATGG
	Reverse	CAGTTCTCCAGCTTATAGTTGGG

CHAPTER VI

Inhibitory Effect of Rutin on *Escherichia coli* O157: H7 virulence

ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) is a pathogen causing severe bloody diarrhea, hemorrhagic colitis, and a potentially fatal renal disease. Due to bacterial lysis and release of bacterial cytotoxins called verotoxins (VT) in patients, the use of antibiotics for treating EHEC infection is contraindicated. Thus, there is a need for alternate strategies for controlling EHEC infection in humans. This study investigated the efficacy of rutin (RT), a GRAS-status (Generally recognized as safe) antimicrobial flavonoid, in attenuating EHEC virulence *in vitro.* The effect of sub-inhibitory concentration (SIC, highest concentration neither bacteriostatic nor bactericidal) of RT on EHEC motility, attachment to intestinal epithelium (Caco2 cells,) VT production, VT-induced cytotoxicity, and VT binding to cell surface receptor, Gb3, was studied. Moreover, the anti-verotoxin effect of RT was investigated by determining the binding ability of RT to VT by molecular docking and protein-ligand interactions.

Rutin decreased EHEC motility and attachment to Caco2 cells ($P<0.05$). Rutin inhibited both extracellular and intracellular VT production by ~ 40 to 60% in EHEC, compared to untreated controls ($P < 0.05$). RT-qPCR results revealed that RT down-regulated the transcription of genes mediating EHEC attachment (*eae*) and VT synthesis (*stx*1 *and stx*2) ($P < 0.05$). Verotoxin binding to Gb3 receptor was decreased in the presence of RT ($P < 0.05$). Further experiments revealed that RT can competitively bind VT at the receptor binding sites, thereby possibly inhibiting toxin-induced pathology. Results justify follow-up animal studies for validating the anti-verotoxin effect of RT in EHEC.

Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC) is a subset of Shiga toxin producing *E. coli*, which causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. EHEC poses a significant public health concern owing to its ability to produce fatal food-borne infections with a low infectious dose (2–2,000 cells) (Buchanan, 1997; Strachan et al., 2005). EHEC is responsible for causing over 63,000 estimated cases of foodborne illnesses annually in the United States, costing the U.S. economy an estimated $1 billion per year (Schraff, 2012). Cattle serve as the principal reservoir of EHEC (Laegreid et al 1999, Shere et al., 1998), with fecal contamination of food products as an important source of human infections. A wide range of foods have been implicated as vehicles of EHEC infection, with a majority of outbreaks linked to the ingestion of undercooked ground beef products (Croxen et al., 2013; Shere et al., 1998; Naylor et al., 2003).

EHEC colonization of the human gut mucosa is mediated by bacterial motility, which enables the pathogen to traverse through the intestine to reach a favorable niche (Tobe et al., 2011). In addition, several adherence factors, including intimin, fimbriae and pili contribute to EHEC adherence and colonization of host mucosal surface (Nataro and Kaper, 1995; Wang et al., 2006; Serna and Boedeker, 2008). After attaching to intestinal epithelial cells, EHEC elaborates verotoxins (VT), the key virulence factor that brings about EHEC pathogenesis and causes HUS in humans (Sakagami et al., 2001). Verotoxins are of two types, verotoxin 1 and verotoxin 2, and they can be extracellular and intracellular. The extracellular VT is secreted to the growth environment, whereas the intracellular portion gets accumulated in bacterial cells and is released when cells are lysed (Yuk and Marshall, 2003, 2004, 2006).

Verotoxins belong to the AB5 toxin family with A and B subunits. The pentameric B subunit of VT binds to the target host cell glycolipid receptor, globotriaosylceramide (Gb3), and

gets internalized by receptor-mediated endocytosis. After internalization, the subunit A of VT interacts with 60S ribosomal subunit and inhibits protein synthesis, thereby leading to cell death or apoptosis (Weeratna and Doyle, 1991; Tam and Lingwood, 2007). Thus, an adequate expression of Gb3 receptor and effective VT binding are pre-requisites for toxin-mediated pathogenesis.

Verotoxins are encoded by *stx1* and *stx2* genes, which are located within Stx-encoding phages present in the bacterial chromosome (Shaikh and Tarr, 2003; Mauro and Koudelka, 2011). Antibiotics can induce phage lytic cycle through SOS response (global response to DNA damage in which the cell cycle is arrested), thereby augmenting the expression of *stx* genes encoded within the prophage. Since this results in increased VT production (Su and Brandt, 1995; Neely and Friedman, 1998; Suwalak and Voravuthikunchai, 2009), the use of antibiotics for treating EHEC infections is contraindicated (Croxen et al., 2013). Antibiotic usage in patients has been linked to enhanced risk of systemic complications such as acute renal failure (Zhang et al., 2000) and elevated mortality in patients (Matsushiro et al., 1999). Moreover, antibiotics can negatively affect the normal gut flora, which in turn can lead to EHEC overgrowth (Su and Brandt, 1995). Thus the current treatment options against EHEC infections are mainly supportive (Ho et al., 2013), underscoring the critical need for alternate approaches for controlling EHEC in humans. Prospective strategies for treating EHEC infections target VT, its toxin receptor and their interaction, with the aim of decreasing cellular uptake of toxin and downstream pathology (Goldwater and Bettelheim, 2012; Pacheco and Sperandio, 2012).

In light of microbial antibiotic resistance, an emerging strategy progressively explored for controlling infectious diseases is called antivirulence therapy, which primarily targets microbial virulence rather than growth, where bacterial factors critical for causing infection or disease in

hosts are attenuated (Defoirdt, 2016; Rasko and Sperandio, 2010; Khodaverdian et al., 2013; Clatworthy et al., 2007; Escaich, 2008). Antivirulence agents being neither bacteriostatic nor bactericidal, potentially lead to a reduced selection pressure for resistance development (Rasko and Sperandio, 2010; Hung et al., 2005; Cegelski et al., 2008; Mellbye and Schuster, 2011) compared to conventional antimicrobials, and are minimally deleterious on the host gut microflora.

Phytochemicals constitute an important ingredient of traditional and herbal medicine. Several studies have documented a wide range of beneficial properties with phytochemicals, ranging from antiseptic, anti-inflammatory, antioxidant and antimicrobial effects (Deans and Ritchie; 1987; Baratta et al., 1998a, b; Alma et al., 2003). Rutin (RT, quercetin-3-rhamnosyl glucoside) is a natural flavone derivative that was first extracted from buckwheat. Other sources of RT include asparagus, rhubarb, and berries (Kreft et al., 2006). Various pharmacological effects of RT, including anti-allergic, anti-inflammatory, antitumor, antibacterial, antiviral and antiprotozoal properties have been reported (Yang et al., 2008). Based on the hypothesis that RT attenuates EHEC virulence, disengages VT with its biochemical interactions, and impedes toxin-mediated pathogenesis, this study investigated the potential of RT as a next generation drug candidate to treat EHEC infections effectively.

Materials and Methods

Bacterial strains and culture conditions

Three strains of EHEC (EDL 933, E163, and E137) were used in this study. Each strain of EHEC was cultured separately in 10 ml of sterile tryptic soy broth (TSB, Sigma-Aldrich, St. Louis, MO) with incubation at 37°C for 18 h. Following incubation, bacterial cultures were pelleted by centrifugation (3600 ×g for 15 min), washed twice and suspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.0). The bacterial count in each of these cultures was determined by

plating 100 µl of ten-fold serial dilutions on duplicate tryptic soy agar (TSA, Sigma-Aldrich), Sorbitol MacConkey agar (SMA, Sigma-Aldrich), followed by incubation at 37°C for 24 h.

Sub-inhibitory concentration (SIC) of RT

The SIC of RT (Sigma-Aldrich, MW: 610.52) was determined, as previously described (Surendran Nair et al., 2016). Tryptic soy broth containing RT ranging from 0 to 0.5% (v/v) in increments of 0.01% was inoculated with each strain of EHEC (5.0 log CFU/ml) and incubated at 37°C for 24 h. Control samples containing TSB without RT were also included. After 24 h of incubation, samples were serially diluted (1:10) in PBS and appropriate dilutions were surface-plated on TSA. The plates were incubated at 37°C for 24 h. The highest concentration of RT that permitted bacterial growth similar to the control after incubation at 37°C for 24 h was taken as the SIC for the study.

Bacterial motility assay

The effect of RT on EHEC motility was determined according to a previously published method (Wang et al., 2006). RT at its SIC was added to petriplates containing 20 ml of LB broth containing 0.3% agar. The plates were mixed by swirling for 15-20 sec. A volume of 10 µl of EHEC culture containing ~ 6.0 log CFU was inoculated at the center, and the plates were kept still for 1 h at 25°C, followed by incubation at 37°C for 12 h. After incubation, the zone of motility was measured.

Bacterial adherence assay

The effect of RT on EHEC adherence to human colonic epithelial cells (Caco-2) was investigated as described (Xicohtencatl-Cortes et al., 2007). Caco-2 cells were maintained in Dulbecco's high glucose Modified Eagle Medium (DMEM, Gibco, Invitrogen) containing 10% FBS at 37°C under 5% CO_2. Polystyrene 24-well plates were seeded with Caco-2 cells at a density of 1×10^5 cells per well, and allowed to form a monolayer in high glucose DMEM containing 10% FBS. The

monolayer was washed three times with PBS and inoculated separately with each EHEC strain at 6 log CFU/well (MOI 1:10), followed by treatment with RT. The cells were incubated for 2 h and washed three times with PBS to remove unattached bacteria. Subsequently, the cells were permeabilized with 0.1% triton X-100 for 15 min to enable the quantification of the bacterial population adhered to cells by serial dilution and plating on TSA and SMA.

Effect of RT on verotoxin production

Verotoxin produced by each EHEC strain was quantified by enzyme-linked immunosorbent assay (ELISA) using the Premier EHEC test (Meridian Bioscience, Cincinnati, OH), as described previously (Yuk and Marshall, 2003; Staples et al., 2012). Briefly, TSB containing the SIC of RT was separately inoculated with each EHEC strain (~ 6.0 log CFU/ml) and incubated at 37°C for 24 h. EHEC cultured in the absence of RT was included as control. After incubation, the bacterial cells were pelleted by centrifugation at 3,800× g for 15 min, and culture supernatant and cell pellets were separated. The final washed cell pellet was resuspended in Tris-EDTA lysis buffer, and bacterial cell membrane was disrupted using an ultrasonicator (Branson Digital Sonifier, Danbury, CT) at 50 W for 1 min. The extracellular and intracellular VT concentrations were be estimated in cell-free culture supernatant and cell lysate at both 0 and 24 h of incubation using the aforementioned ELISA kit. A standard curve of optical density at 450 nm versus known standard VT (BEI resources, Manassas, VA) concentration was created to determine the unknown VT concentration in control and RT-treated samples (Yuk and Marshall, 2003).

Virulence gene expression using real-time quantitative PCR (RT-qPCR)

The effect of RT on the transcription of genes encoding VT (*stx1* and *stx2*), motility (*motB*) and adherence (*eae*) was investigated using RT-qPCR (Pollard et al., 1990) using the primers listed in Table 2. Each EHEC strain was grown overnight in TSB at 37°C with or without the SIC of RT,

and total RNA extracted using an RNeasy RNA isolation kit (Qiagen, Germantown, MD). The cDNA was synthesized using the I script cDNA synthesis kit (Biorad, Hercules, CA) and used as the template. RT-qPCR analysis of the verotoxin genes was performed using published primers (Pollard et al., 1990) and normalized against 16S rRNA gene expression. Twenty-five microliter reactions were performed in duplicate using iTaq SYBR RT-PCR (Bio-Rad) and repeated three times. The relative fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

Neutral red cytotoxicity assay

The effect of RT on the cytotoxicity of EHEC culture supernatant and its efficacy in protecting host cells from VT was determined using neutral red cytotoxicity assay (Bouzari et al., 2009). Vero cells (CCL-81, ATCC, Manassas, VA) were plated in 96-well plates and grown to sub-confluence in Eagle's minimal essential medium (MEM, Gibco, Invitrogen) containing 10% fetal bovine serum. Cells were then washed and treated with the culture supernatant of EHEC grown with RT overnight. In addition, Vero cells were also treated with SIC of RT and VT extracted from an overnight culture of EHEC. Appropriate cell and solvent controls were included. Two hundred microliters of freshly diluted neutral red in MEM medium were then added to a final concentration of 50 ng/ml and cells were incubated for an additional 3 h at 37°C in 5% CO2. Cells were washed with 1% CaCl2 and 4% formaldehyde, and solubilized in 1% acetic acid and 50% ethanol. Absorption in each well was read in a plate spectrophotometer at 546 nm. Results were expressed as neutral red uptake percent, with 100% representing cells incubated under identical conditions but without toxin treatment.

Effect of RT on VT binding to Gb3 receptor

Immunofluorescent staining of vero cells.

To detect levels of available Gb3 on Vero cells for VT binding in the presence of RT, immunofluorescent staining was done (Zumbrun et al., 2010). Briefly, Vero cells were grown in MEM, containing 10% FBS on Lab-Tech four-chamber no. 1 borosilicate glass coverslips (Lab-tek, Nalge Nunc International, Rochester, NY). The cells were stimulated with the inflammatory factor, TNFά (100U/ml; Sigma Aldrich) 24 h prior to treatment. Binding of VT to cells was then be facilitated by incubating with Stx1 holotoxin (NR-857; BEI resources, Manassas, VA) (0.1 ng/µl MEM) overnight at 37°C in the presence or absence of SIC of RT. The treated wells were then gently washed with PBS and blocked with PBS containing 3% bovine serum albumin (BSA) for 45 min. FITC-conjugated mouse anti-human CD77/Gb3 antibody (BD Bioscience, San Jose, CA) (diluted 1:50 in PBS), or PBS (negative control) was added to appropriate wells and incubated for 1 h on ice. Cells were again washed with PBS and counterstained with 0.01% Hoescht blue dye. The stained cells were assessed with a Zeiss Axiovert 200M fluorescent microscope

Isolation and quantification of Gb3

Gb3 receptor was isolated from Vero cells as described previously (Hughes et al., 2000; Surendran_Nair et al., 2016). Vero cells (CCL-81, ATCC) were grown in Eagle's minimal essential medium (MEM, Gibco, Invitrogen, city, State) containing 20% FBS in six-well polystyrene plates, and growth was arrested at confluence by transferring cells to serum deficit medium. The cells were stimulated with the inflammatory factor, TNFά (100U/ml; Sigma-Aldrich) for 24 h followed by the extraction of total cellular lipids. The cells were washed with ice-cold PBS (pH 7), scraped off the plates and centrifuged at 500 x *g* for 10 min. The cell pellet was then lysed using an ultrasonicator at 50 W for 5 min. Subsequently, cells were extracted in 1ml of chloroform: methanol: water solvent (5:10:3) three times, and dried under nitrogen gas. The total dried lipid was suspended in 2:1 chloroform: methanol, and separated on silica gel thin layer

chromatography plates (10 x 20 cm, Sigma-Aldrich) by ascending chromatography in chloroform: methanol: water (65:35:8) solvent system. The lipid content on silica plates was then visualized with iodine vapor. The Gb3 standard (Ceramide Trihexosides Gb3, Matreya LLC, and PA) was run at different concentrations (10 to 40 µg) on TLC plates to identify the glycolipid of interest and determine the approximate amount of Gb3 extracted from the cells. Additionally, a standard curve of optical density at 490 nm versus Gb3 concentration (µg/ml) was used to determine the unknown concentration of Gb3 extracted from the cells.

The effect of RT on VT binding to Gb3 receptor was determined according to a published protocol (Ashkenazi and Cleary, 1989). The extracted Gb3 was retrieved from the TLC plate above and dissolved in 2:1 chloroform: methanol solvent. Polystyrene microdilution plates (Fisher Scientific, Pittsburg, PA) were inoculated with Gb3 (36 µg/ml), left uncovered for 4 h at 25ºC for the evaporation of the diluent, and subsequently washed with Tween 20-PBS buffer. Verotoxin extracted from an overnight culture of EHEC was added to the plates (50 ng/well), and allowed to bind to the receptor with or without the SIC of RT at 37°C for 1 h. Following this, mouse anti-human CD77/Gb3 antibody (BD Bioscience, Billerica, MA) was added to the wells at 1:1000 dilution, and allowed to bind to Gb3 for 1 h at 37°C. Subsequently, after washing with Tween-PBS, peroxidase conjugated rabbit anti-mouse immunoglobulin G (Promega, Madison, WI) was added and incubated at 37°C for another hour. The substrate ortho phenyl diamine (0.4 mg/ml, Sigma-Aldrich)-0.1% H_2O_2 in citrate-phosphate buffer (25 mM citrate, 50 mM phosphate, pH 5) was allowed to react with the available enzyme peroxidase in the well for 15 min at room temperature following the washing step. The supernatants from the wells were transferred to new plates after incubation and the absorbance were measured at 490 nm. Wells with 0.1% BSA in

0.05% Tween 20-PBS (diluent in which samples were diluted) instead of the sample served as negative controls.

Flow cytometry

The effect of RT on surface expression of the Gb3 receptor protein in Ramos cell line (human Burkitt's lymphoma; ATCC CRL-1596) was studied using flow cytometry (Tetaud et al., 2003; Desselle et al., 2012). Ramos cells were maintained in Roswell Park Memorial Institute medium (RPMI, Gibco, Invitrogen) containing 20% FBS at 37°C under 5% CO_2. The cells (~1 x 10^4 per well) were seeded onto a 6-well plate and will be allowed to grow overnight at 37°C in the presence of 5% CO_2. The cells were then treated with the SIC of RT for 1 h, sedimented by centrifugation at 300 x g for 10 min and washed with fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.1% fetal bovine serum and 0.01% sodium azide). The cells were subsequently resuspended in FACS buffer and labeled with FITC-conjugated mouse anti-human CD77/Gb3 antibody (BD Bioscience) for 30 min on ice. Unlabeled control wells (cells neither treated with RT nor incubated with antibody), labeled control wells (cells not treated with RT, but labeled with antibody) and isotype control wells (cells not treated with SIC of RT, but labeled with FITC mouse IgM κ (BD Bioscience) were included. Isotype controls were used to confirm the primary antibody specificity and determine any non-specific reactivity of antibody with the cells (Keeney et al., 1998). After labeling, cells were washed twice with FACS buffer and analyzed by flow cytometry using a BD FACS-Calibur flow cytometer. The mean fluorescence intensity was calculated for all the samples (Keeney et al., 1998; Herzenberg et al., 2006).

Molecular modeling and docking

In order to test the hypothesis that RT interacts with the toxin, inducing a protein modification, molecular modeling and docking were done using the Schrödinger small-molecule drug discovery

suite (version: 2017 – 2) as described by Benedik et al (2014) and. Friesner et al (2004). The details on the binding sites of VT we discussed in this study were extracted from the crystal structures PDB 4M1U and 1BOS. Owing to the better resolution for testing the plausible binding affinity of RT to toxin, docking experiments were done using the 1.53 Å resolution crystal structure of Stx2 (PDB ID 4M1U). Prior to the docking experiments the crystal structure was prepared (e.g. H atoms added, protonation and tautomeric states assigned, and H-bond donor/acceptor groups reoriented) with the Protein Preparation module in Maestro version 10.6.014 (Sastry et al., 2013).

The grid-based ligand docking with energetics (Glide) methodology in standard precision mode was used to dock rutin into the five Site 1 and five Site 2 carbohydrate-binding pockets identified on the B5 subunit of Stx (Halgren et al., 2004; Friesner et al., 2004). For binding pockets shown to be occupied by a co-crystallized disaccharide analogue of Gb3, or methyl α-D-GalNAcp-(1→4)-β-D-Galp-(1-O), the receptor grid was centered at the location of the bound molecule. For unoccupied sites in the crystal structure, the centroid of the residues defining the pocket was used to position the docking grid. The top ten poses from each of the 10 Site 1 and Site 2 binding sites were analyzed, and the best-ranked poses for Site 1 and Site 2 pockets were retained.

Statistical analysis

All experiments included duplicate samples for each treatment and control, and were replicated three times. The data were analyzed using a generalized linear model of PROC GENMODE procedure of SAS 9.3 version (SAS institute, Cary, NC). The differences between the means were compared using least significant difference (LSD) test and the significance was tested at $P<0.05$.

Results

The highest concentration of RT which was neither bacteriostatic nor bactericidal was first determined. RT was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of

DMSO used in the treatment solution was 1%, which did not affect EHEC viability, as previously reported by Markarian et al (2002). The average initial EHEC count in RT-treated and control samples was ~ 5.0 log CFU/ml. After 24 h of incubation at 37°C, ~ 8.5 log CFU/ml of EHEC was recovered from control and 1.7 mM RT-treated samples, thereby confirming that the aforementioned RT concentration was the SIC. Further studies were conducted using this concentration unlessnoted otherwise.

EHEC motility was affected in the presence of RT *in vitro*

Bacterial motility is essential for host-microbial interactions, colonization, and virulence in the host (Josehans and Suerbaum, 2002). Therefore, the effect of RT on the motility of EHEC was determined. The extent of bacterial spread over a constant period of time on semi-solid LB agar plates was estimated by measuring the zone of motility, as shown in Figure 1. In the presence of RT, the zone of motility was significantly reduced in all three strains of EHEC (EDL 933, E163 and E137), indicating an anti-motility effect of the treatment ($P<0.05$). The downstream protein involved in the chemotaxis of EHEC, the *motB* mRNA expression, was also downregulated in the presence of RT (Figure 2).

Decreased EHEC attachment to human intestinal cells in the presence of RT

Being an extracellular pathogen, EHEC requires firm adhesion to host intestinal epithelial cells in order to colonize, persist and establish disease in the host. It also contributes to tissue tropism during the process of infection (McWilliams and Torres, 2014; Shoaf-Sweeney and Hutkins, 2008). Cell culture studies revealed that RT decreased EHEC adhesion to human colon epithelial carcinoma cells ($P<0.05$) (Figure 3). When co-incubated with RT, the number of EHEC adhered to the intestinal cells were lesser than those infected with EHEC alone ($P<0.05$). Since *eae* gene and its protein product intimin play a significant role in the initial EHEC attachment and

colonization of the intestine, RT-qPCR studies revealed that RT down-expressed the respective genes which contributed to the phenotype observed (Figure 4).

Rutin reduced extracellular and intracellular verotoxin production

Verotoxins are the major virulence factors elaborated by EHEC. They are pentameric cytotoxins causing apoptosis following entry into the host cells. Since antibiotics can cause bacterial lysis and can release increased amount of toxins to host cells, they are not commonly used for treating EHEC infections in humans. Therefore, EHEC cultures (EDL 933, E163 and E137) were treated with the SIC of RT, which is neither bacteriostatic nor bactericidal. The basal level of extracellular VT concentration in RT treatment and control at 0 h was ~ 5 $\pm$ 1 ng/ml, irrespective of the strains, whereas the intracellular VT concentration was under the detection limit (<1 ng/ml). After 24 h of incubation, the extracellular VT concentration in the presence of RT was decreased to $\leq$ 100 ng/ml compared to control (350-400 ng/ml) ($P < 0.05$) (Figure 5). Similarly, the intracellular VT level in RT-treated EHEC was reduced by 80–90% after 24 h of incubation ($P < 0.05$) (Figure 6). However, the magnitude of extracellular and intracellular VT production was found to be strain specific. Among the three different strains, EDL 933 produced the highest amount of extracellular and intracellular toxins. Additionally, in accord with the ELISA results, RT-qPCR data revealed that RT downregulated both *stx1* and/or *stx2* expression in all the three strains of EHEC ($P < 0.05$) (Figure 7).

Rutin protected Vero cells from the cytotoxic effects of verotoxin

Cytotoxicity of the Vero cells (African green monkey kidney cells) has been identified as the gold standard assay for determining the presence of VT and its cytotoxicity. Therefore, in this study, we determined the cytotoxic activity of VT on Vero cells in the presence of RT by measuring cell viability using the neutral red (NR) uptake assay (Ma´rquez et al., 2014). Neutral Red assay is

based on the ability of viable cells to incorporate and bind the vital dye called neutral red in lysosomes. Consequently, when the cell dies, the dye cannot be retained in the cells, and thus the amount of retained dye is proportional to the number of viable cells. The linear relationship between the number of live cells and absorbance at 540 nm after NR test subsequently identifies the cell viability and/or cytotoxicity levels.

For this purpose, confluent Vero cells were incubated with SIC of RT and VT extracted from the overnight culture of type strain EDL 933 (Kavaliauskiene et al., 2016). Incubation with VT produced a significant reduction in the cell viability, whereas a diminished cytotoxic effect was observed when cells were co-incubated with RT and VT (Figure 8). In addition, as expected, the supernatant (containing VT) retrieved from EHEC culture grown in the presence of RT did not produce any cytotoxicity on Vero cells, confirming the decreased production by EHEC. However, the supernatant from cultures grown without RT caused a complete rounding of Vero cells (data not shown).

Verotoxin binding to Gb3 receptor decreased in the presence of RT

As mentioned before, the efficient binding of VT to Gb3 is a prerequisite for toxin induced cytotoxicity and further downstream pathology. Subsequent to decreased cytotoxicity observed in the presence of RT, we tested the hypothesis that RT interfered with the effective binding of toxin to the receptor, using immunofluorescent staining of Vero cells (Zumbrun et al., 2010). As seen in Figure 9, in Vero cells not exposed to VT, a high green fluorescence intensity was observed indicating the maximum availability of Gb3. However, the cells treated with VT alone showed significantly less green fluorescence indicating the increased binding of the receptor with toxin leaving less free receptors behind. Furthermore, in Vero cells treated with VT in the presence of

RT, there was significantly greater green fluorescence indicating more available Gb3 and less efficient binding to the toxin.

As a follow up for the quantitative estimation, the glycolipid receptor protein, Gb3, was isolated from Vero cells and the concentration was estimated from a standard curve generated using different concentrations of Gb3 standard. The average yield of Gb3 was found to be ~ 36 µg/ml (Figure 10A). The extracted Gb3 was then used to determine the effect of RT on toxin binding to the receptor using ELISA. As shown in Figure 10B, when VT alone was added to wells coated with Gb3, the absorbance decreased to 49% compared to control, indicating a decreased Gb3 availability due to active binding of toxin to receptor. However, the toxin binding to the receptor was found to be impeded in the presence of RT leaving more Gb3 available for the antibody to bind when VT was added with RT. Thus, RT alone affected VT binding to the receptor compared with control samples ($P < 0.05$).

Effect of RT on Gb3 receptor expression

To determine if RT exerts any inhibitory effect on Gb3 expression, we used Ramos cells, since these are known to overexpress Gb3 receptor, and have been extensively used for studying physicochemical characteristics of Gb3 receptor and its expression (Furukawa et al., 2002; Engedal et al., 2011). In order to assess the cell surface expression of Gb3 in Ramos cells (human Burkitt's lymphoma; ATCC CRL-1596), the mean fluorescence intensity of gated population of live cells was measured using flow cytometry. Live cell population as determined by side scatter-forward scatter gate (confirmed with propidium iodide staining) were analyzed. However, the mean fluorescence of cells treated with RT was not different compared to control cells ($P>0.05$), thereby suggesting that Gb3 expression in host cells was not modulated in the presence of RT (Figure 11).

On other hand, this finding along with the decreased binding of VT to the Gb3 led us to hypothesize potential structural interactions of RT with the binding sites of the VT. To test this, we investigated the binding interactions experimentally using molecular docking and protein-ligand interactions.

Molecular modeling and docking

To understand the molecular basis of RT-induced VT inhibition, we performed simulations via the grid-based ligand docking with energetics (Glide) method using the Schrödinger small-molecule drug discovery suite (Version: 2017 – 2). Since we had no information where the RT binding sites might be, we had to determine the potential binding site locations for our ligand using blind docking. Subsequently, RT was found docked into the pentameric array of receptor binding sites 1 and 2 identified in the Stx crystal structures (PDB 4M1U and 1BOS) (Figure 12A). Furthermore, to predict the plausibility of competitive binding hypotheses, the binding scores were determined in comparison to the disaccharide receptor analog (*Methyl α-D-GalNAcp-(1→4)-β-D-Galp-(1-O)*) identified by Jacobson et al. (2014).

Figure 12B shows the molecular surfaces for Site 1 (blue) and Site 2 locations (red), the co-crystalized disaccharide (green), and the best ranked docking poses for RT (yellow) in all Site 1 and Site 2 grooves. As mentioned, the docking scores (Table 1) and docking poses (Figure 13) were then compared to the co-crystalized disaccharide, methyl α-D-GalNAcp-(1→4)-β-D-Galp-(1-O), the analogue of Gb3.

As observed in Table 1, RT is predicted to bind with comparable or slightly better affinity to the receptor binding sites of StxB compared to the co-crystalized disaccharide ligand. Figure 12C demonstrates that these relatively favorable binding scores are attributable to the complementarity of RT structure for the topology of both Site 1 and Site 2 binding grooves on the

B subunit. Rutin better utilizes an array of H-bonding and π-π stacking interactions, which are not accessible to the disaccharide Gb3 analogue. Interestingly, the best docking pose for RT in Site 1 binding grooves coincided with the disaccharide ligand, whereas different Site 2 binding pockets were preferred by the disaccharide and RT. This may reflect different binding site conformations in the crystal structure of the protein. Collectively, these docking results suggest that RT is a promising lead for structure-activity investigations on the anti-verotoxin effect of phytochemicals. Although the docking results suggest the plausibility of competitive binding hypothesis, these calculations do not predict which of the possible inhibitory mechanisms is operative or more relevant.

Discussion

In this study, we investigated the phytochemical, RT, as a potential antivirulence agent to attenuate EHEC's determinants contributing to pathogenesis in humans. To this end, we demonstrated that RT at its highest SIC level did not disrupt bacterial growth or viability, but rather disarmed the pathogen using multiple mechanisms.

Motility and host tissue attachment are critical processes required for EHEC intestinal colonization and pathogenesis. Rutin was effective in reducing both these virulence factors in all three EHEC strains. Intimin is an adhesin in EHEC critical for intimate attachment of the pathogen to host tissue (Frankel et al., 1998; Hicks et al., 1998). It is documented that EHEC mutants lacking intimin displayed diminished intestinal colonization and virulence in calves and gnotobiotic piglets (Dean-Nystrom et al., 1998; Tzipori et al., 1995). RT-qPCR data revealed that RT significantly decreased the transcription of intimin-encoding gene, *eae* in EHEC.

As discussed before, verotoxins are the most significant virulence factors in EHEC. Therefore interventions inhibiting VT synthesis, toxin binding, uptake, or function represent a

promising avenue for the development of antivirulence therapy against the pathogen. In this study, we identified the efficacy of RT to impede both extracellular and intracellular VT production in all three EHEC isolates, which concurred with RT-qPCR data revealing RT-mediated downregulation in the expression of *stx1* and *stx2* genes. Previously, green tea catechins, epigallocatechin gallate and gallocatechin gallate were documented to exert an inhibitory effect on extracellular VT secretion in EHEC (Sugita-Konishi et al., 1999). Similarly, Takemasa et al. (34) reported a concentration-dependent reduction in EHEC extracellular and intracellular VT expression by the essential oil, eugenol. Likewise, Baskaran et al. (2016) observed an inhibitory effect of several essential oils, including trans-cinnamaldehyde, carvacrol, and thymol on EHEC motility, adhesion to human intestinal cells and VT synthesis.

Gb3 is the only functional receptor for VT in mammals and cell surface expression of this receptor and effective VT binding to the receptor are prerequisites for toxin sensitivity and toxin-induced pathology in the host (Okuda et al., 2006; Sandvig et al., 1992, 1994; 2001; Jacewiz et al., 1994; Lingwood 1994). Consequently, we investigated the effect of RT on reducing VT binding to Gb3 receptor using an *in vitro* Gb3 binding assay. The results showed a significant decrease in toxin binding to Gb3 in the presence of RT ($P< 0.05$). These results were confirmed by immunofluorescence staining of Vero cells, where a greater proportion of available Gb3 and decreased binding to the toxin were observed in the presence of RT compared to control. However, the flow cytometry data revealed no effect of RT on Gb3 expression. These results indicate that the potential therapeutic effect of RT is mainly mediated by decreasing VT production and toxin-receptor interaction rather any direct effect on Gb3 receptor expression. Consequently, we investigated the probability of structural interactions of RT with VT. Several flavonoids have been identified to possess potential binding sites on enterotoxins, including the staphylococcal

enterotoxins (Benedik et al., 2014). Our molecular docking results suggested that RT can bind to two of the three receptor binding sites on each monomer subunit, which is likely to influence receptor binding affinity of the toxin. The crystal structure of radially symmetric pentameric B subunit (B_5) possess three binding sites for Gb3, numbered 1–3, for a total of 15 binding sites per pentamer (Figure 12A). The results thus imply that RT could inhibit VT toxicity by effectively competing with the natural cell-surface Gb3 receptor for the carbohydrate binding sites of the holotoxin. In a study conducted by Dong et al., (2015), a structurally related phytochemical, baicalin inhibited Stx2 activity by directly interacting with toxin inducing protein oligomerization. Given the fact that the activities of RT and baicalin are similar and they share a high-level resemblance in their structures (Figure 13), we attempted to identify residues on Stx potentially involved in binding baicalin and RT. Although weak associations were identified in similar sites identified with baicalin, the hypothesis of protein oligomerization has to be further elucidated using techniques such as analytical centrifugation or crystallization of Stx with RT. Therefore further biochemical studies are warranted in this direction to the fully understand the anti-VT effect of RT.

In conclusion, the potential efficacy of RT as an antivirulence agent to control EHEC pathogenesis has been identified and discussed in this manuscript. However, follow up *in vivo* studies would validate the safety and efficacy of an RT-based approach for control of EHEC .

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Figure 1 (Left panel). Effect of rutin on EHEC motility. In the presence of RT, the zone of motility was significantly reduced in all three strains of EHEC (EDL 933, E163 and E137), indicating an anti-motility effect of the treatment ($P<0.05$). Figure 2 (Right panel). Effect of rutin on EHEC *motB* mRNA expression. The protein involved in the chemotaxis of EHEC, the *motB* mRNA expression, was downregulated in the presence of RT ($P<0.05$).

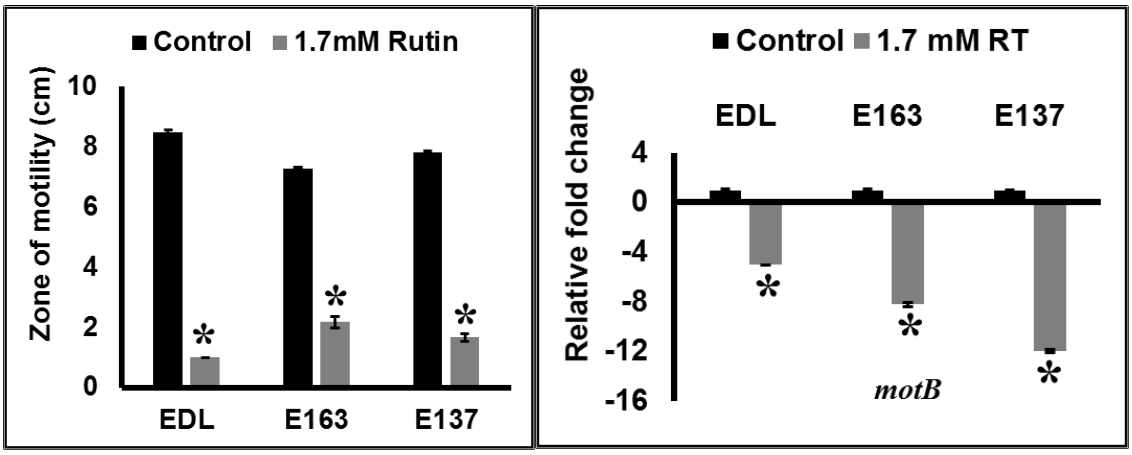


Figure 3 (Left panel). Effect of rutin on EHEC adhesion to human intestinal cells. RT decreased all three strains of EHEC (EDL 933, E163 and E137) adhesion to human colon epithelial carcinoma cells ($P<0.05$). When co-incubated with RT, the number of EHEC adhered to the intestinal cells were lesser than those infected with EHEC alone ($P<0.05$).

Figure 4 (Right panel). Effect of rutin on EHEC *eae* mRNA expression. Since *eae* gene and its protein product intimin play a significant role in the initial EHEC attachment and colonization of the intestine, RT-qPCR studies revealed that RT down-expressed the respective genes which contributed to the phenotype observed (Figure 4).

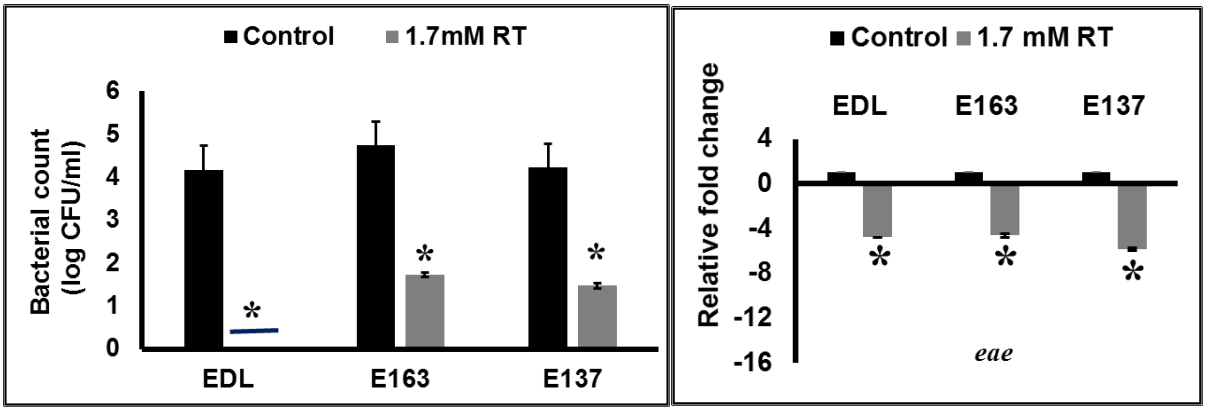


Figure 5. Effect of rutin on extracellular verotoxin production (P<0.05). Figure 6. Effect of rutin on intracellular verotoxin production (P<0.05). After 24 h of incubation, the extracellular VT concentration in the presence of RT was decreased to < 100 ng/ml compared to control (350-400 ng/ml) (P < 0.05). Similarly, the intracellular VT level in RT-treated EHEC was reduced by 80–90% after 24 h of incubation (P < 0.05).

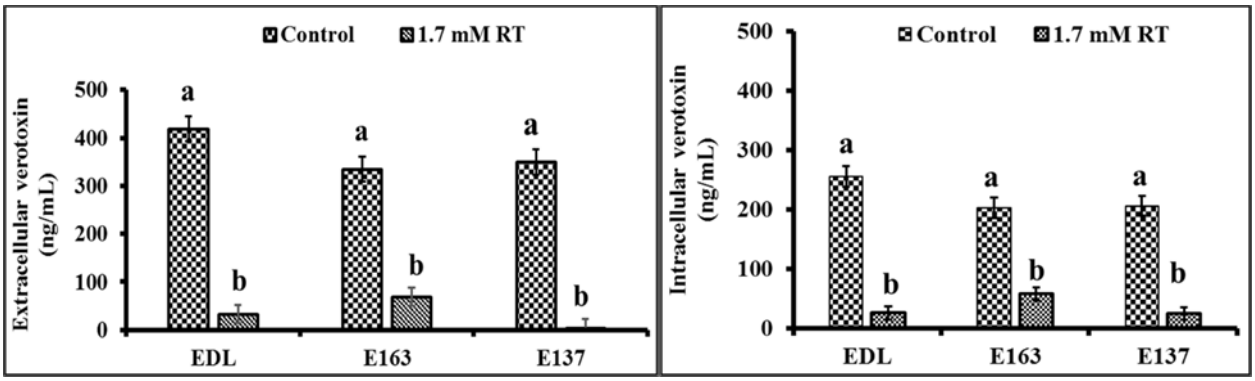


Figure 7. Effect of rutin on verotoxin gene transcription. RT-qPCR data revealed that RT downregulated both stx1 and/or stx2 expression in all the three strains of EHEC (P < 0.05)

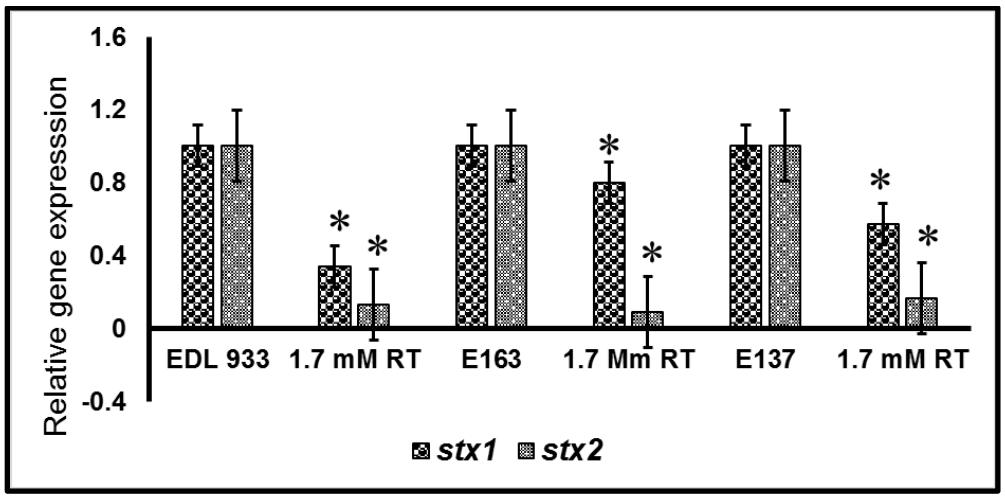
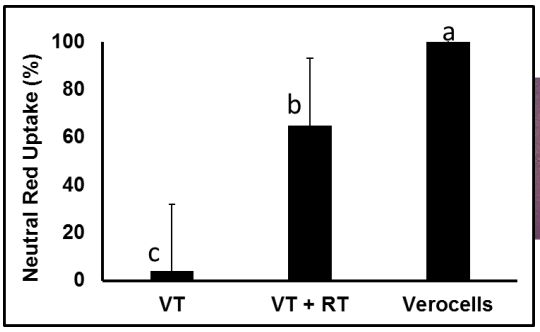
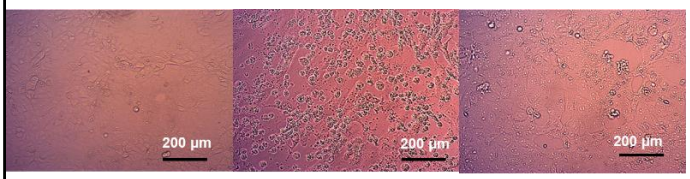


Figure 8. Effect of rutin on Vero cell cytotoxicity using neutral red uptake assay ($P<0.05$). Confluent Vero cells were incubated with SIC of RT and VT extracted from the overnight culture of type strain EDL 933. Incubation with VT produced a significant reduction in the cell viability, whereas a diminished cytotoxic effect was observed when cells were co-incubated with RT and VT. Clear rounding was observed in cells incubated with verotoxin as observed in the light microscopy images.



Light microscopy images of Vero cells before neutral red assay



Vero cells | **Vero cells + verotoxin** | **Vero cells + verotoxin + 1.7mM RT**

Figure 9: Effect of rutin on verotoxin binding to Gb3. Immunofluorescence staining using FITC labelled anti-Gb3 antibody (green) and the Hoechst staining for the nucleus (blue). In Vero cells not exposed to VT, a high green fluorescence intensity was observed indicating the maximum availability of Gb3 whereas the cells treated with VT alone showed significantly less green fluorescence indicating the increased binding of the receptor with toxin leaving less free receptors behind. Furthermore, in Vero cells treated with VT in the presence of RT, there was significantly greater green fluorescence indicating more available Gb3 and less efficient binding to the toxin.

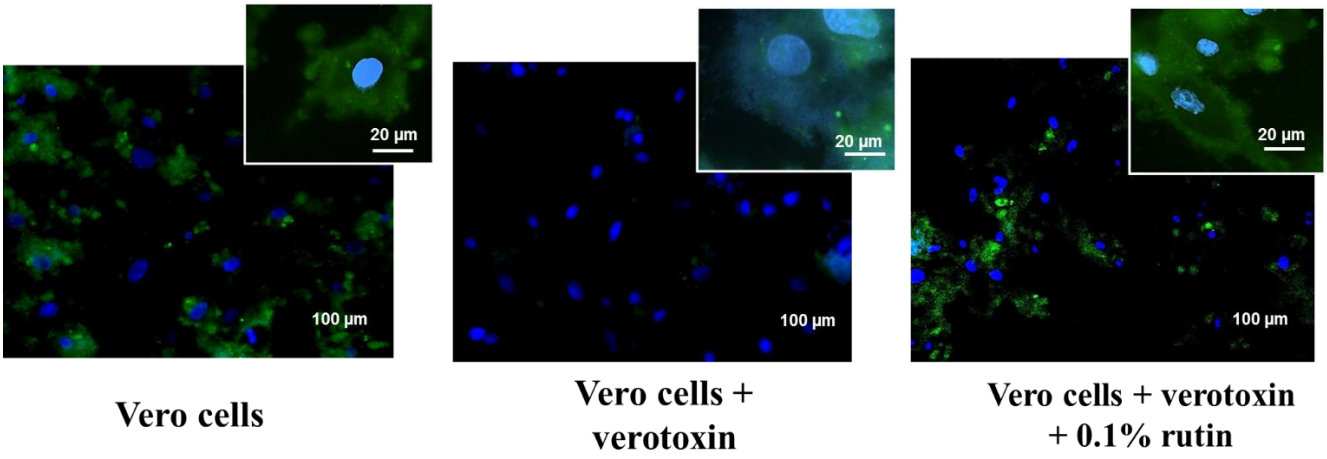
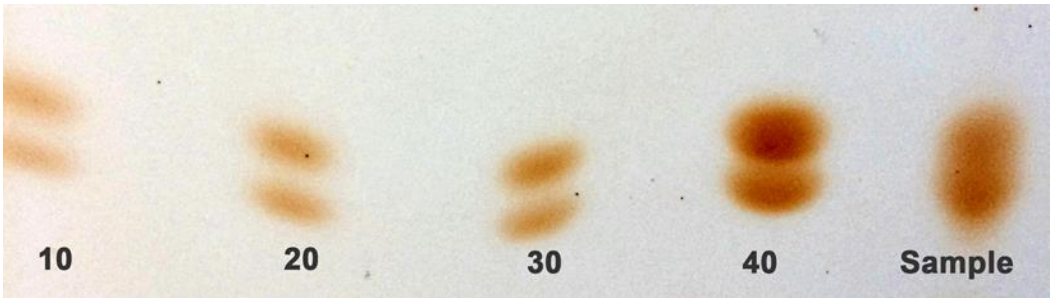


Figure 10A. Globotriaosylceramide (Gb3) content in Vero cells



Each experimental lane represents different concentrations (µg/ml) of Gb3 standard and the extracted sample.

Figure 10B. Gb3 binding. Gb3 was extracted from the Vero cells using thin layer chromatography and coated on to microdilution plates. ELISA was performed to detect the percentage availability of Gb3 receptor for binding with the antibody after incubating with the verotoxin in presence or absence of RT. Results indicate, there was decreased binding of receptor with the toxin in the presence of RT, the percentage availability of Gb3 receptor was significantly different between toxin treatment and toxin plus RT treatment ($P < 0.05$).

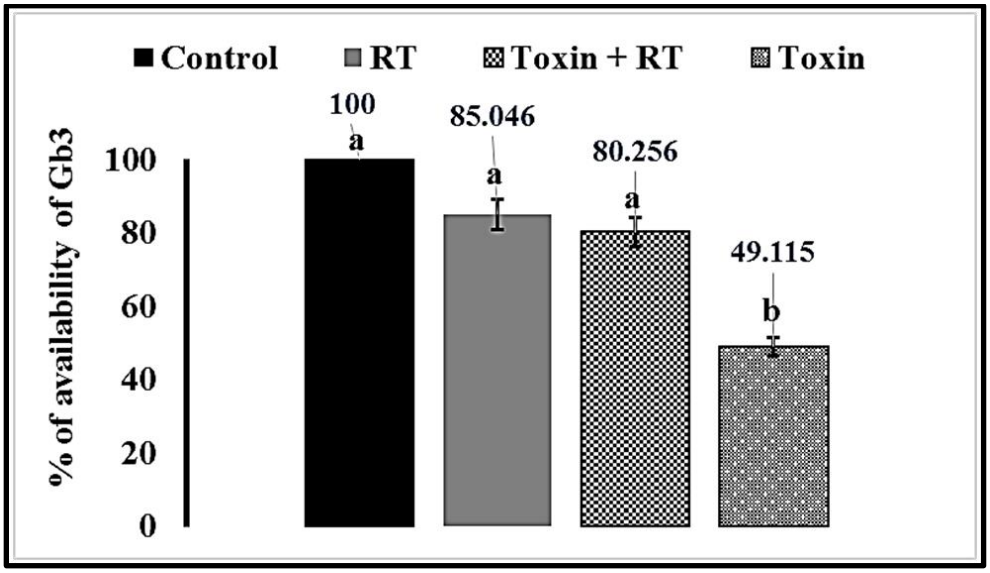
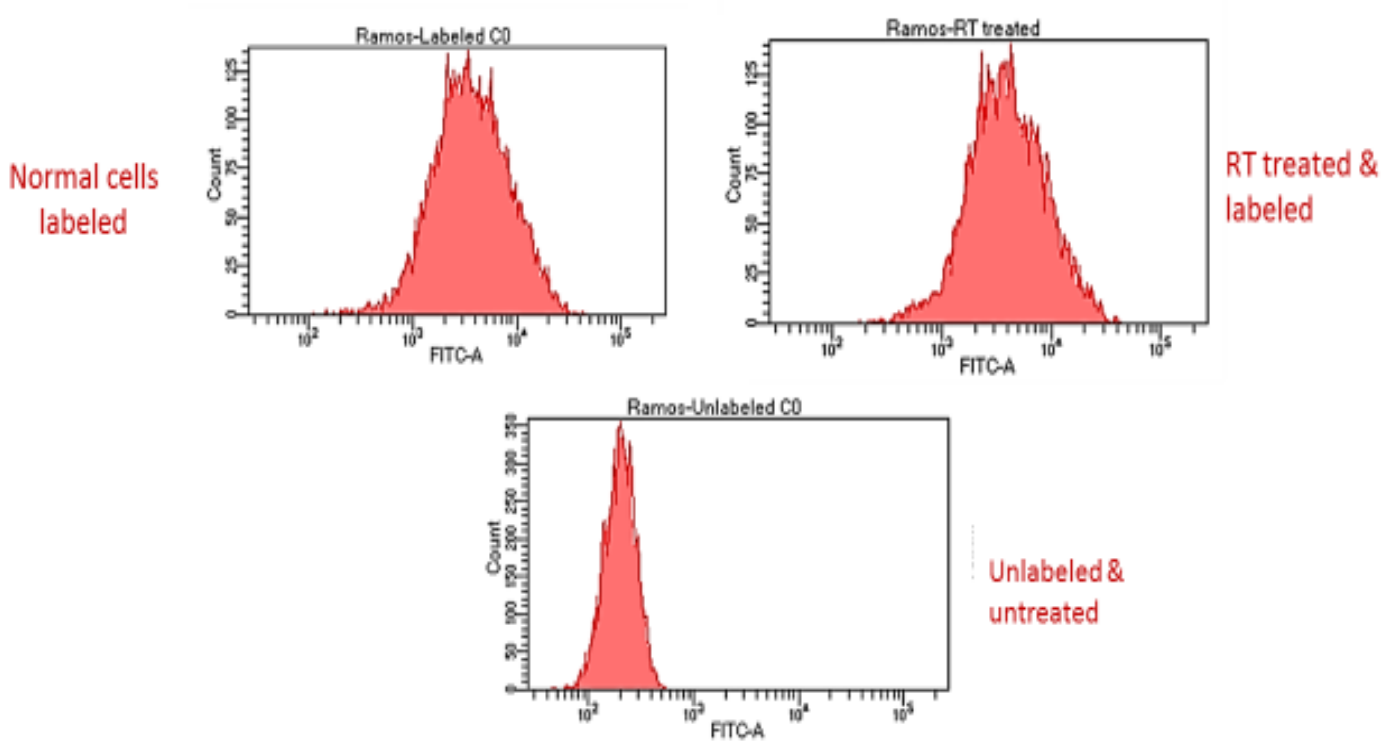


Figure 11. Effect of RT on Gb3 receptor expression on Ramos cells. The cells were treated with the SIC of RT for 1 h, sedimented by centrifugation at 300 x g for 10 min, and washed with fluorescence activated cell sorting (FACS) buffer (PBS containing 0.1% fetal bovine serum and 0.01% sodium azide). The cells were subsequently labeled with FITC mouse anti-human CD77/Gb3 antibody and incubated for 30 min on ice. Appropriate unlabeled, labeled and isotype controls were included. Histograms showing Gb3 receptor fluorescence intensity of gated Ramos cells. FACS sorted Ramos cells after treating with RT compared to unlabeled, labeled Ramos cells and isotype control.



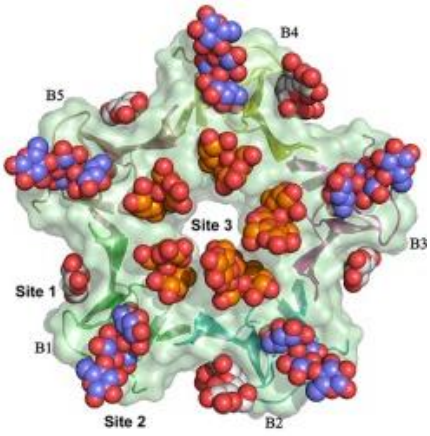


Figure 12A. (Adapted from Jacobson et al. (2014)). The Stx-Gb3 receptor analog complex. The five B subunits are named B1 through B5. The bound carbohydrates are shown as spheres with oxygen and nitrogen atoms colored red and blue, respectively; the carbon atoms of the carbohydrate ligands bound in sites 1, 2, and 3 are colored in gray, slate, and gold, respectively.

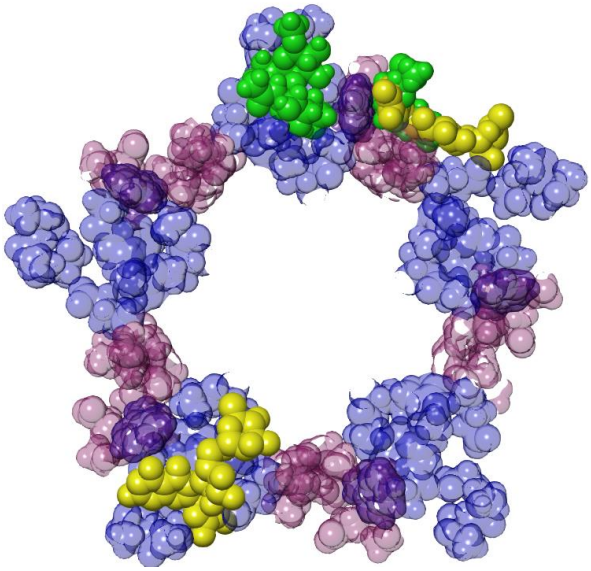


Figure 12B. The pentameric arrangement of the Site 1 (blue) and Site 2 (red) carbohydrate binding sites of Stx. Only the molecular surfaces for these binding sites are shown for the protein. The co-crystalized disaccharide and the best ranked docking poses for rutin in the two different types of binding sites are shown in green and yellow CPK view, respectively.

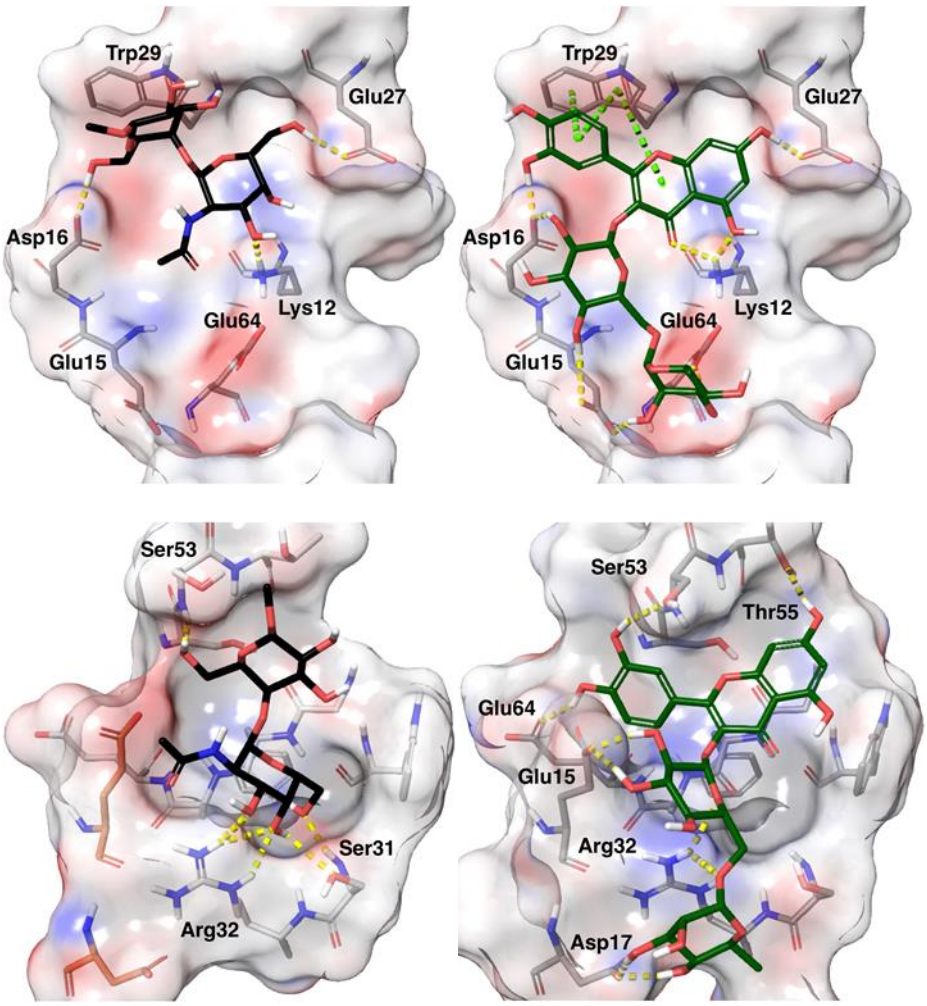


Figure 12C. Poses of the co-crystalized Gb3 disaccharide analogue (left), and the docked rutin (right) are shown for Site 1 (top panel) and Site 2 (bottom panel). H-bonding and π-π stacking interactions are indicated with yellow and green dashed lines, respectively. Residues participating in these non-covalent interactions are labeled.

Figure 13. Chemical structure of RT in the context of the previously identified Stx favonol inhibitor baicalin. The flavonol core of both structures is depicted in blue. The glucuronic acid specific to baicalin is shown in green. The aromatic diol and α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose moieties in red are specific to rutin. The black bond signifies that this glycosylated position in baicalin is a free hydroxyl in rutin.

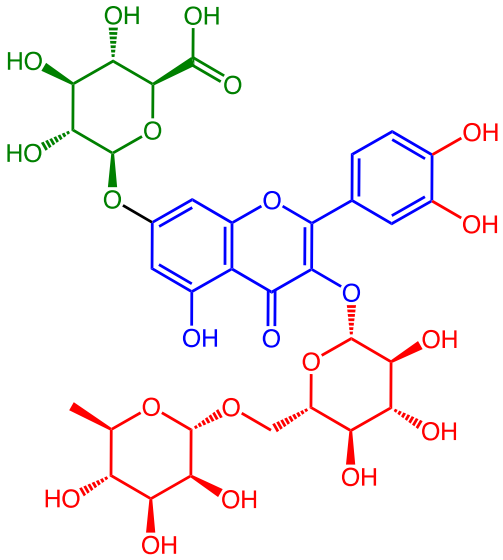


Table 1. Glide scores (kcal/mol) for the co-crystalized disaccharide and rutin in the carbohydrate binding sites of STX2.

	Site 1	Site 2
Methyl α-D-GalNAc*p*-(1→4)-β-D-Gal*p*-(1-O)	-5.07	-5.65
Rutin	-5.73	-6.92

Table 2: List of primers used in this study.

Gene	**Oligonucleotide sequences (5'-3')**	
16S rRNA	Forward	CATTGACGTTACCCGCAGAA
	Reverse	CGCTTTACGCCCAGTAATTCC
eaeA	Forward	TGGTGGCGAATACTGGCGAGA
	Reverse	TGTATGACTCATGCCAGCCGC
motB	Forward	CAGGGGGAAGTGAATAAGCA
	Reverse	TTCTAAACATCGGGCGATTC
stx1	Forward	GTGGCATTAATACTGAATTGTCATCA
	Reverse	GCGTAATCCCACGGACTCTTC
stx2	Forward	GATGTTTATGGCGGTTTTATTTGC
	Reverse	TGGAAAACTCAATTTTACCTTTAGCA

CHAPTER VII

Anti-EHEC effect of Selenium and Rutin in *Caenorhabditis elegans* infection model

ABSTRACT

Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC) is an important foodborne pathogen causing severe symptoms and mortality in humans. Verotoxins (VT) produced by EHEC are the most critical virulence factor for causing hemorrhagic colitis, hemolytic uremic syndrome, and renal failure. Antibiotics are contraindicated in the treatment against EHEC due to enhanced chances of renal failure and mortality stemming from increased bacterial release of VT in the presence of antibiotics. Therefore, there is significant interest in identifying effective treatment strategies against EHEC, especially those targeting the VT. Our previous studies indicated that rutin (RT), a phytochemical, and selenium (Se), a naturally occurring essential trace mineral, significantly inhibited VT synthesis in EHEC.

Caenorhabditis elegans is a nematode, increasingly used as an invertebrate model to study microbial pathogenesis and validate in-infective strategies against pathogens. Therefore, in this study, we used *C. elegans* as an *in vivo* model for studying the anti-EHEC activity of RT and Se. Supplementation of RT significantly increased the survival of EHEC-infected *C. elegans* compared to control worms. The survivability was increased by ~ 65% in infected worms after treating with RT for a period of 7 days ($P < 0.05$). However, RT failed to protect *C. elegans* when the worms were intoxicated directly with VT. On the other hand, since Se was found to be lethal to *C. elegans*, a significantly lower concentration than that used *in vitro* was supplemented to EHEC-infected worms. However, at the lower concentration used, Se lacked anti-EHEC activity and the percentage survival of Se-treated worms was not significantly different from the infected control group ($P > 0.05$). The aforementioned results justify further studies in mammalian models to validate the anti-virulence effect of RT and Se against EHEC.

INTRODUCTION

Diarrheal diseases are the second major threat for global mortality among children (Lanata et al., 2013; Walker et al., 2013). Enterohemorrhagic *E. coli* (EHEC) is one of the diarrheagenic pathogens causing lethal complications such as hemolytic uremic syndrome (HUS) and renal failure worldwide (Ciccarelli et al., 2013). The global burden of EHEC comprises of 2,801,000 acute illnesses, 3890 cases of HUS, 270 cases of permanent end-stage renal disease (ESRD), and 230 deaths annually (Majowicz et al., 2014). Unfortunately, the only mainstream therapy for EHEC-HUS are supportive care measures such as fluid resuscitation and dialysis, since antibiotics are contraindicated. Several studies reported increased chance of developing renal failure in children on antibiotic therapy for HUS associated with EHEC infection (Wong et al., 2000; Zimmerhackl, 2000; Safdar et al., 2002; Tarr et al., 2005). This is because conventional antibiotics usage is reported to exacerbate verotoxin-mediated cytotoxicity. Verotoxins (VT) are the key virulence factors of EHEC responsible for causing HUS in humans (Sakagami et al., 2001). EHEC produces verotoxin 1 and/or verotoxin 2, which are encoded by *stx1* and *stx2*, genes respectively. Antibiotics enhance the replication and expression of these *stx* genes encoded within a chromosomally integrated lambdoid prophage genome, resulting in increased VT production. Antibiotics also cause phage-mediated lysis of the EHEC cell membrane, allowing the release and dissemination of VT into the environment (Karch et al., 1999; Matsushiro et al., 1999; Wagner et al., 2002). Therefore, there is immense interest among clinicians and scientists to explore alternate strategies for treating EHEC infections in humans.

Since VT represents the major virulence factor in EHEC, identification of antivirulence agents such as toxin neutralizers or inhibitors will lead to the development more specific and efficacious therapeutic strategies in the treatment of EHEC associated HUS. Our previous studies

reported two natural molecules, rutin (RT) a phytochemical, and selenium (Se), an essential trace mineral, were each effective in inhibiting the synthesis of extracellular and intracellular VT synthesis in EHEC (Surendran Nair et al., 2016).

Several animal models, including piglets, mice and rats have been used to investigate EHEC pathogenesis specifically the toxicity of VT, the pathogenesis of HUS and to some extent the protective capacity of different therapeutic methodologies (Sheoran et al., 2005; Armstrong et al., 2006; Sauter et al., 2008; Tesh et al., 1993). However, no animal model reproduces the full spectrum of clinical disease induced by EHEC in humans (Ritchie et al., 2013; Mohawk and O'Brien, 2011). Recently, the nematode *Caenorhabditis elegans* has been recognized as a naturally infected and genetic tractable animal host to study EHEC infection (Ritchie et al., 2013).

Several characteristics of *C. elegans*, including its rapid life cycle, ease of conducting research on host-pathogen interactions, genetic screens, and sharing of conserved innate immune pathways with humans make it invaluable for investigating microbial pathogenesis (Irazoqui et al., 2010). Moreover, reports indicate that *C. elegans* intestinal cells share similar anatomic features with those of humans (McGhee, 2007), which makes it an ideal model for the study of intestinal pathogens, including EHEC. Studies demonstrated that EHEC can colonize and induce characteristic A/E (attaching and effacing) lesions in the intestine of *C. elegans*, and VT1 1 (Stx1) is partly required for the manifestation of EHEC pathogenicity (Chou et al., 2013). Additionally, the advantage of using *C. elegans* is that the pathogenic bacterium under study can be substituted in place of the normal feed of the nematode (*E.coli* OP50) as the nutritional source, and the primary site of the infection is the intestine. Moreover, phenotypes such as animal survival, motility, and pathogen burden can be easily and noninvasively examined in *C. elegans* (Marsh and May, 2012). Therefore, we used *C. elegans* as an *in vivo* model for studying the anti-EHEC activity of Se and

RT. Moreover, the study serves as a follow-up *in vivo* validation of the protective effect of Se and RT against EHEC *in vitro* (Surendran Nair et al., 2015, 2016).

Materials and methods

Bacterial and nematode strains.

Young wild-type *C. elegans* (N2 Bristol) was used in all experiments (Caenorhabditis Genetics Center, MN). The nematodes were maintained on nematode growth medium agar (NGM, US Biologicals, MA) plates using the standard laboratory *E. coli* strain OP50 as feed (Brenner, 1974). N2 strains were synchronized by isolating eggs from gravid adults, hatching the eggs overnight in M9 buffer, and plating L1-stage worms onto lawns of *E. coli* on NGM agar media. Worms were grown to sterile, young adults by incubating at 25°C for 48–52 h (Moy et al., 2006; Stiernagel, 2006). Two strains of EHEC, namely EDL933 (ATCC 43895) which produces VT1 and VT2, and B6914 that does not produce either VT1 or VT2 were used. For the direct intoxication study, we procured the Stx1 holotoxin (NR-857) from the BEI resources (Manassas, VA).

Effect of Se and RT on C. elegans

C. elegans strain N2 was grown on nematode growth medium (NGM) agar plates and fed with *E. coli* OP50 (Stiernagel, 2006). Synchronized adult nematodes were transferred to 48-well plates (20 worms/well), each well containing 150 µl of S medium with *E. coli* OP50 (Stiernagel, 2006). Selenium (Sigma-Aldrich, St. Loius, MO) and Rutin (Sigma-Aldrich, St. Loius, MO) at different concentrations, were added in each well from 0% to 0.5% (v/v) with 0.01% increment. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Loius, MO) was used as the diluent for RT; the final concentration of DMSO was $\leq$1%. At every 48 h of post-treatment, in order to score for worm survival, the plates were hand shaken, and the worms were considered dead if they did not

move or exhibit muscle tone (Moy et al., 2006). Living nematodes maintain a sinusoidal shape, whereas dead nematodes appear as straight rods (Moy et al., 2006) (Figure 1).

C. elegans infection

EHEC strain EDL 933 was grown overnight in Luria-Bertani (LB, Fisher Scientific) broth at 37°C. Bacterial lawns used for *C. elegans* infection assays were prepared by spreading 200 µl of the overnight EHEC culture on modified NGM (50 mM NaCl and 0.35% peptone) agar plates. The plates were incubated at 37°C for 12 h before seeding with young adult nematodes (Stiernagel, 2006). The infections were performed at 25°C overnight.

Survival assays of infected C. elegans treated with Se and RT

Nematode survival was evaluated by liquid medium assay, as described previously (Uccelletti et al, 2010). EHEC-infected adult worms were washed three times with M9 buffer and transferred to 48-well plates (20 worms/well). Each well containing 150 µl of S medium with *E. coli* OP50 along with RT (Sigma-Aldrich) and Se (sodium selenite, Sigma-Aldrich) separately (at the highest concentration non-toxic to the worms) added to the wells. Wells without Se and RT served as positive controls and uninfected worms were the negative controls. The plates were incubated at 25°C for 7 days. Worm survival was monitored every 48 h for 7 days, and survivability index was determined.

Estimation of EHEC within the nematode gut

The numbers of viable EHEC in the worm intestine were determined before and after RT treatment, as described by Uccelletti et al (2010). Briefly, for each replicate, 10 infected worms were transferred into a 1.5-ml Eppendorf tube and washed three times with M9 buffer (500 µl) containing 20 µg/ml gentamicin to remove surface *E. coli*. The nematodes were broken with 50 µl of M9 buffer-1% Triton X-100. Appropriate serial dilutions of whole worm lysate were plated on

Sorbitol MacConkey agar in order to determine bacterial colonization in the intestinal tract of the worm.

Toxicity study on C. elegans using extracellular verotoxin

Synchronized adult nematodes were transferred to 48-well plates (20 worms/well), each well containing 150 µl of S medium with *E. coli* OP50. A volume of 100 µl of the supernatant retrieved from an overnight grown EHEC EDL 933 culture (containing ~ 100 ng VT estimated using Premier EHEC ELISA Kit) and B6914 supernatant (tested negative for VT) were added to the respective wells. Worms exposed to 100 ng standard Stx1 holotoxin (BEI resources, Manassas, VA) were included as positive controls and those not exposed to any toxin served as negative controls. Worm survival was estimated every 48 h, as described above for 7 days, and the survivability index was determined.

Protective effect of RT against the toxicity effects of verotoxin

Synchronized adult nematodes were exposed to overnight culture supernatant of EDL933, B6914, and VT, as described above. RT was added to the wells at concentrations identified from the previous experiment, and wells without RT served as positive controls. Uninfected worms served as negative controls. The plates will be incubated at 25°C for 7 days. Worm survival was estimated every 48 h as described above for 7 days and survivability index determined.

Statistical analysis

A completely randomized design was used for the study. All the experiments had duplicate samples for each treatment and control, and were replicated three times. The data were analyzed using the PROC MIXED procedure of the Statistical Analysis Software (SAS 9.3 version, SAS institute, Cary, NC). The differences between means were compared and significance was tested at $P<0.05$. The log-rank test was used to analyze survival curves, with a P -value of < 0.05

indicating statistical significance. The survival analysis experiments were performed three times with triplicate samples (N =180).

Results

Effect of Se and RT on *C. elegans*

A toxicity study was first conducted to determine the *in vivo* lethal doses of Se and RT in uninfected nematodes. Accordingly, the anti-virulence concentrations determined against EHEC in our previous *in vitro* studies were found to be non-lethal in the case of RT, whereas for Se, those *in vitro* sub inhibitory concentrations were lethal. In concordance with studies conducted using other flavonoids such as catechin (Saul et al., 2009), 1.7 mM of RT was found to be non-lethal during a 7 day period of observation (Figure 1). Dimethyl sulfoxide used as the diluent for RT (final concentration 1%) neither affected worm survival in the non-infected group nor protected the worms from EHEC infection. In the case of Se, concentrations above 0.15 mM were found to be toxic, where the worms displayed sluggishness and delayed response within 24 h of exposure. Therefore in the further experiments, we used 1.7 mM RT (the *in vitro* anti-virulence concentration against EHEC) and 0.15 mM Se (~150 times lesser than the *in vitro* anti-virulence concentration) (Figure 1).

Effect of RT and Se on the survival of EHEC infected worms

The adult nematodes were infected with EHEC strain EDL 933 overnight and then transferred to S buffer containing RT or Se at the indicated concentrations. Worm survival was monitored every 48 h for 7 days and survivability index was determined. The results showed that treating EHEC infected worms with RT protected the nematode against the lethal bacterial infection (Figure 2). Rutin increased the infected worm survival by at least three-fold throughout the treatment period in liquid medium. In addition, a difference in the activity of infected worms

treated with RT compared to controls was noted. The RT-treated worms were very active and similar to uninfected controls when compared to the sluggish infected worms. However, Se did not exert any anti-EHEC activity and percentage worm survival after treatment was not significantly different from the infected control group (Figure 3). The negative control group, which was neither infected nor treated, showed 100% survivability throughout the seven days. Based on these observations, further antivirulence mechanisms of RT against EHEC in the nematode were elucidated and discussed below. However, the antivirulence mechanisms of Se needs to be investigated in mammalian models because of the sensitivity of *C. elegans* to the metal.

Effect of RT on EHEC colonization in the nematode intestinal tract

The effect of RT on the colonization of EHEC in the intestinal lumen of *C. elegans* was further examined. EHEC colony-forming units (CFU) per nematode were determined in infected and treated worms. As shown in Fig. 2B, the average number of EHEC per worm recovered from worms infected with EDL933 overnight was ~ 4.5 log. However, the pathogen count dropped significantly (~ 2 log) in RT treated worms with respect to untreated infected live worms at the end of 7 days of observation. The retrieved EHEC on the agar plates were then confirmed as O157: H7 strain by using latex agglutination assay (Thermo Fisher, Waltham, MA). These results suggest that RT interfered with the ability of EHEC to colonize the nematode gut (Figure 4).

Toxicity study on *C. elegans* using extracellular verotoxin and protective effect of RT against the toxicity effects of extracellular verotoxin

Since the EDL 933 strain elaborates Stx1 and Stx2 toxins, and previous studies showed the association of Stx1 with EHEC pathogenesis (Chou et al. 2013), the anti-toxigenic potential of RT was investigated *in vivo.* Synchronized adult nematodes were exposed to culture supernatant of strains EDL933 and B6914 (does not produce either Stx1 or Stx2), and commercially available

standard VT (Stx1) separately, as described in the methods. Interestingly, exposure to the extracellular supernatant from EDL 933 produced maximal lethality compared to pure toxin and the supernatant retrieved from the strain B6914 (Figure 5). However, treating the worms with RT after intoxication did not protect the worms from EHEC infection significantly (Figure 6).

Discussion

C. elegans has been used widely as a host system to study the pathogenesis of various human pathogens, including EHEC and EPEC (Enteropathogenic *E.coli*) (Garsin et al. 2001; Nicholas and Hodgkin 2004; Chou et al. 2013). Similarly, Chou et al (2013) observed Shiga toxin-dependent disease development and involvement of attaching and effacing lesions, both typical with EHEC virulence, in the nematode gut infection process. Therefore, this study was undertaken to validate the antivirulence effects of Se and RT observed in our previous *in vitro* studies (Surendran Nair et al., 2016).

In concordance with other studies conducted, we first observed that EHEC could infect, colonize and kill *C. elegans* in a period of seven days (Frankel et al., 1998; Anyanful et al., 2005; Chou et al., 2013). We also noted that EHEC colonized and persisted in the digestive tract of *C. elegans*, and several phenotypic changes, including sluggish activity, paler appearance and dilated body surface were observed compared to control worms. Similar to the antimicrobial effect of RT against EHEC in *C. elegans*, Lee and coworkers (2009) observed that the green tea catechin, epigallocatechin gallate, protected the nematode against EHEC by inhibiting quorum sensing. Further, Kampkötter et al (2007) while studying the effect of RT on stress resistance in *C elegans*, observed a reduced ROS accumulation and decreased extent of induced oxidative stress with RT administration. A similar modulating effect of different flavonoids on oxidative stress, redox-sensitive signaling pathways and life span in *C. elegans* was also reported by Koch et al (2014).

However, the protective effect of RT against EHEC in *C. elegans* was not observed when the worms were directly intoxicated with pure toxin or EHEC culture supernatant containing the toxin. Therefore, RT's protective effect against EHEC could be attributed to the inhibition on VT production by the pathogen in the nematode gut, as observed in our *in vitro* study. On the other hand, despite using a RT concentration that is sub-inhibitory on EHEC, we observed significant decreased bacterial load in the treated worms compared to controls. This suggests that there could be additional mechanisms involved in the RT-mediated anti-EHEC effect conferred in the worms.

Based on our *in vitro* results that showed that Se was inhibitory on VT synthesis in EHEC, we hypothesized that the dietary essential mineral will be effective in protecting *C. elegans* against the pathogen challenge. Previously, Li et al. (2009) reported that *C. elegans* infected with *P. aeruginosa* were rescued by supplementation of Se, where the protective effect was attributed to the upregulation of putative antimicrobial genes in the worm. However, in our studies, Se did not display any protective effect in the worms against EHEC challenge. This could be attributed to the significantly lesser Se concentration (0.15 mM) used in our worm study compared to that used *in vitro* (30mM), where VT synthesis by EHEC was significantly inhibited. Selenium at concentrations above 0.15 mM were found to be lethal to *C. elegans* in our study. Previously, Boehler et al. (2014) observed that Se toxicity in *C. elegans* causes an increase in ROS and stress responses, marked by increased expression of oxidoreductases and reduced expression of cuticle-associated genes. Further, an impaired growth was observed when the worms were exposed to concentrations as low as 0.2 mM, thereby concurring with our findings.

In conclusion, the results from this study confirm that *C. elegans* could be used as a model to study EHEC pathogenesis. Further, concurring with our *in vitro* studies, RT exerted significant anti-EHEC effect in the nematode. However, Se was found to be toxic to *C. elegans*, and

considering the increased sensitivity of worms to the mineral, the protective effect of Se against EHEC needs to be further expounded in higher mammalian models.

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Figure 1: Effect of Se and RT on the survival of the nematodes. Synchronized adult nematodes were fed with Se and RT to determine the non lethal concentrations in uninfected nematodes. 1.7 mM of RT and 0.15 mM Se were found to be non-lethal during a 7 day period of observation.

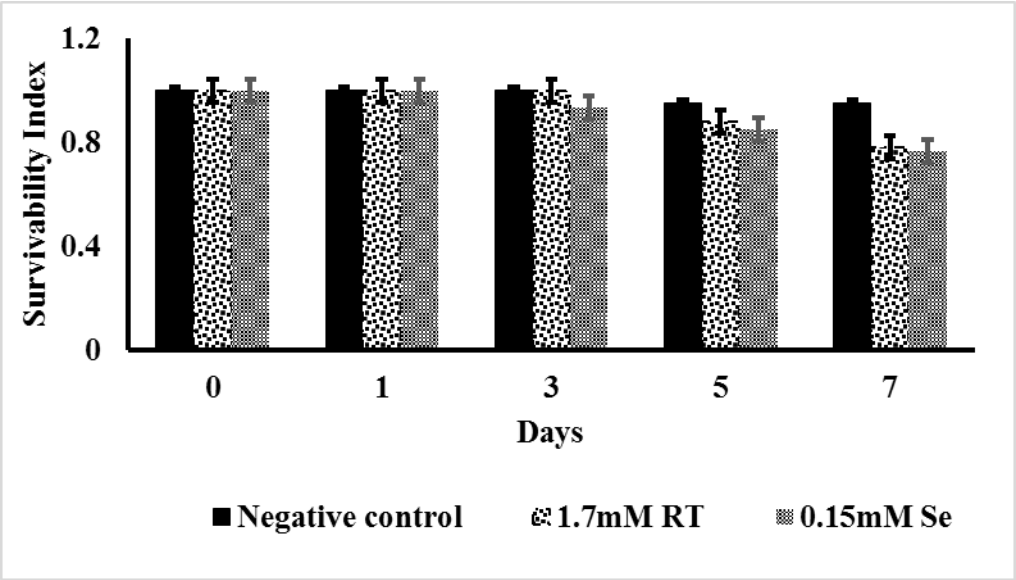


Figure 2. Effect of RT on the survival of EHEC infected worms ($P<0.05$). Synchronized adult nematodes were infected with EHEC followed by treating with 1.7 mM RT and survivability was observed. RT increased the infected worm survival by at least three-fold throughout the treatment period in liquid medium. 1% DMSO was used as drug solvent control in worm survivability assays.

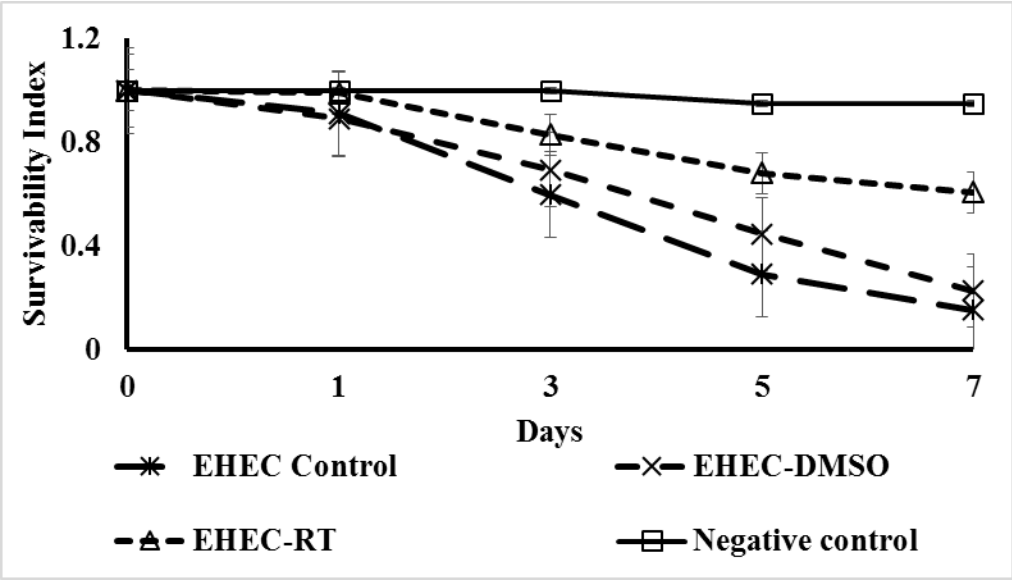


Figure 3. Effect of Se on the survival of EHEC-infected worms. Synchronized adult nematodes were infected with EHEC followed by treating with 0.15mM Se and survivability was observed. Se did not exert any anti-EHEC activity and percentage worm survival after treatment was not significantly different from the infected control group ($P > 0.05$).

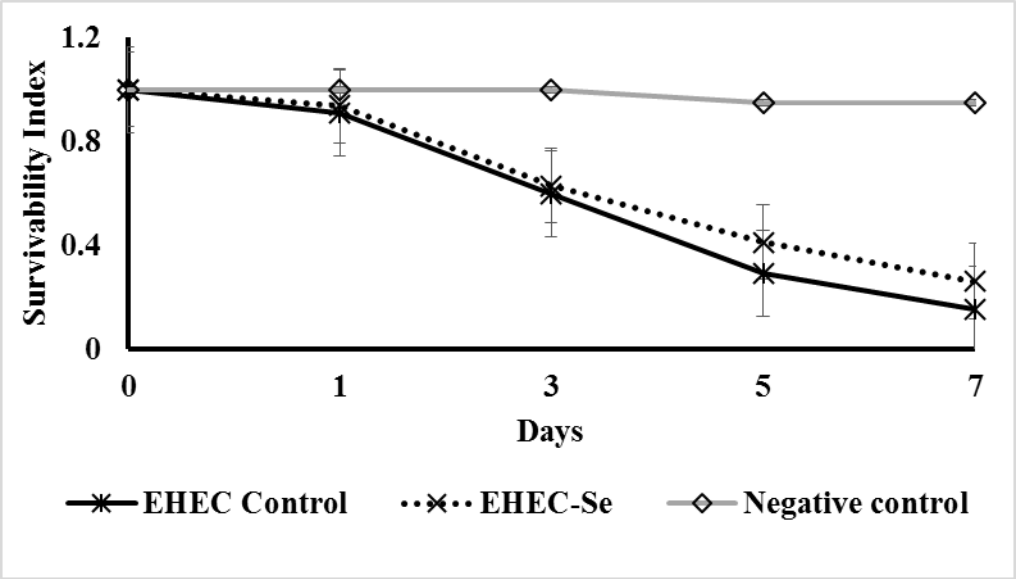


Figure 4. Effect of RT on bacterial colonization in the nematode intestinal tract ($P<0.05$). EHEC colony-forming units (CFU) per nematode were determined in infected and treated worms. The average number of EHEC per worm recovered from worms infected with EDL933 overnight was ~ 4.5 log. The pathogen count dropped significantly (~ 2 log) in RT treated worms with respect to untreated infected live worms at the end of 7 days of observation.

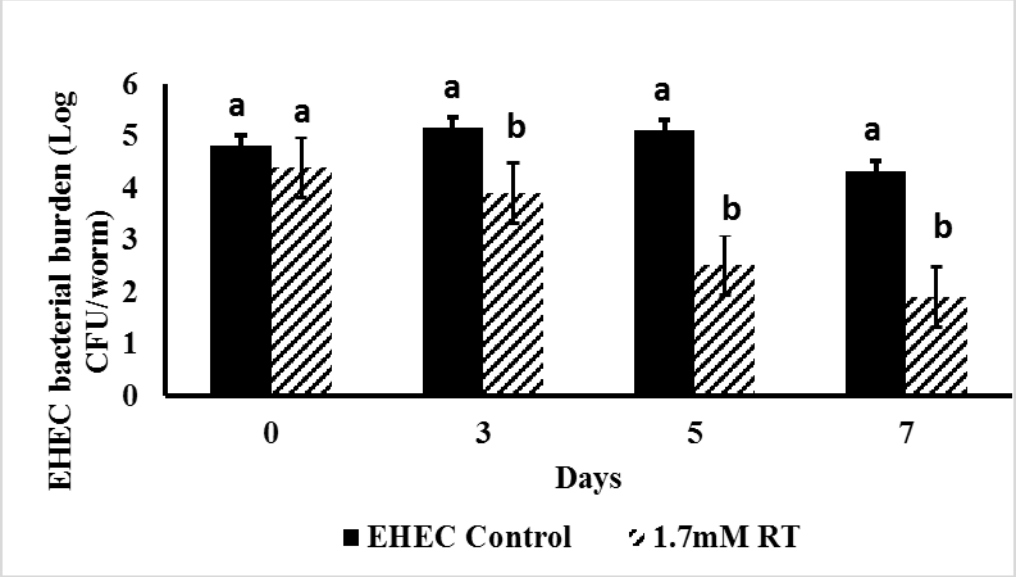


Figure 5. Toxicity of extracellular EHEC culture supernatants and Stx1 holotoxin on *C. elegans* ($P<0.05$). Synchronized adult nematodes were exposed to culture supernatant of strains EDL933 and B6914 (does not produce either Stx1 or Stx2), and commercially available standard VT (Stx1) separately. Exposure to the extracellular supernatant from EDL 933 produced maximal lethality compared to pure toxin and the supernatant retrieved from the strain B6914.

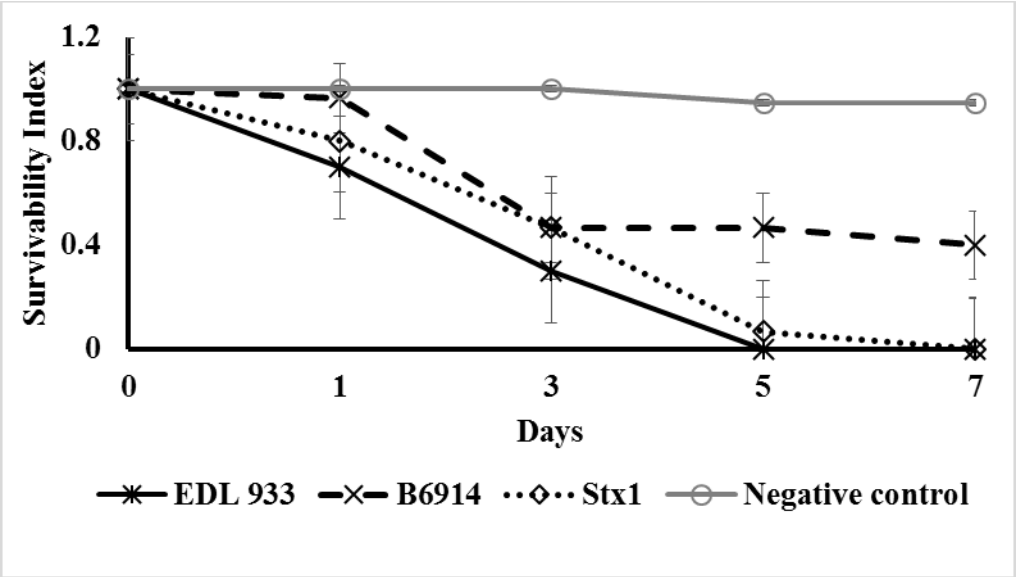


Figure 6a. Protective effect of RT against the toxicity effects of culture supernatants and Stx1 holotoxin. Synchronized adult nematodes were exposed to culture supernatant of strains EDL933 and B6914 (does not produce either Stx1 or Stx2), and commercially available standard VT (Stx1) separately followed by treating with SIC of RT. Treating the worms with RT after intoxication did not protect the worms from EHEC infection significantly. ($P < 0.05$ within the treatments between days and $P > 0.05$ between respective treatments and controls in a given day).

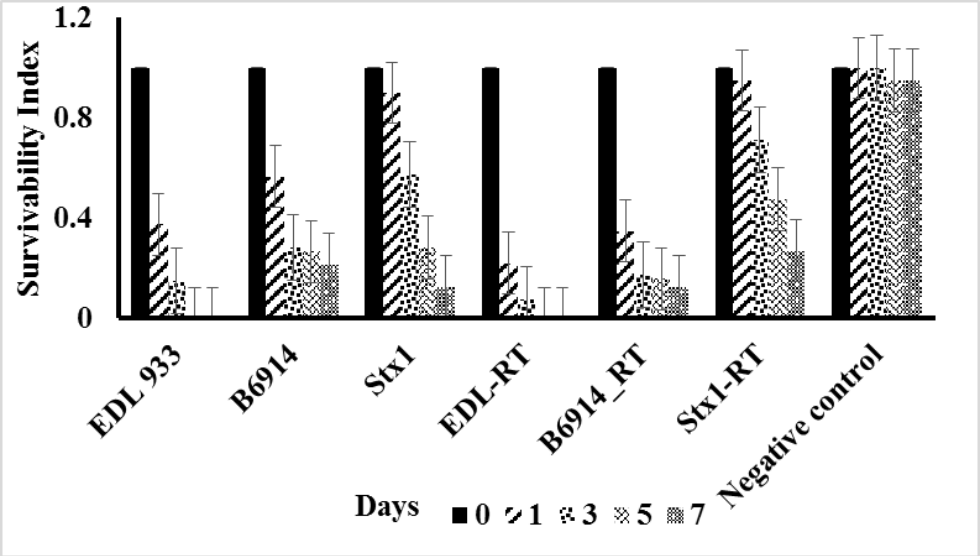
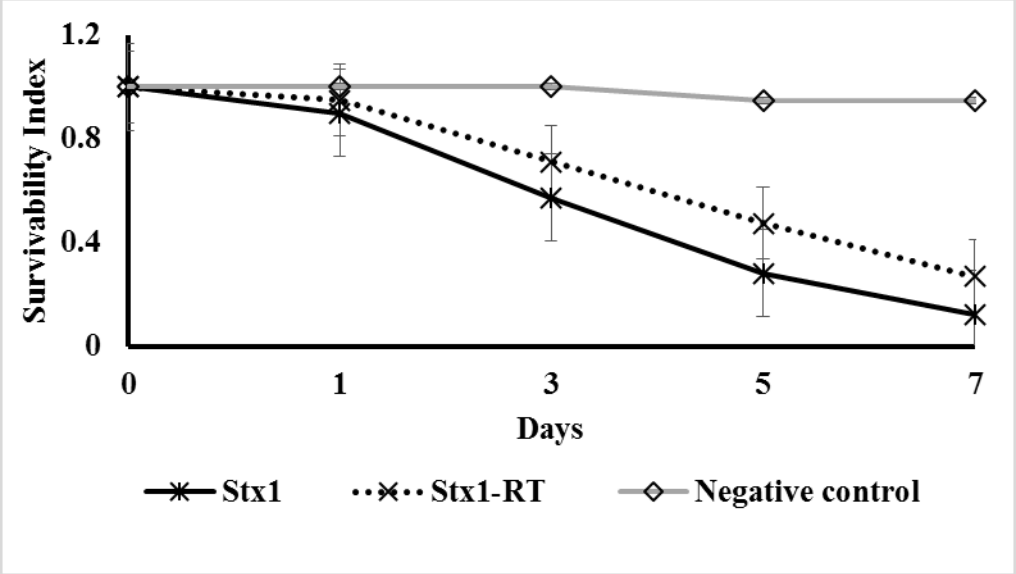


Figure 6b. Protective effect of RT against the toxicity effects of pure standard Stx1 holotoxin.



CHAPTER VIII

Summary

Escherichia coli O157:H7 (EHEC) is a major foodborne pathogen in the United States and poses a significant public health concern owing to its ability to produce fatal food-borne infections with a low infectious dose. The pathophysiology of EHEC infection is primarily mediated by verotoxins, which bind to globotriaosylceramide (Gb3) receptor on host cells, resulting in hemorrhagic diarrhea and kidney failure. Therefore, inhibition of EHEC toxin mediated pathology can significantly reduce its illness, and improve disease outcome in humans. This study investigated the efficacy of two natural antimicrobials selenium (Se) and rutin (RT), for potentially controlling EHEC outbreaks in the food continuum, and their efficacy as virulence inhibitors against EHEC infections in humans.

In objective 1, the efficacy of Se in inhibiting and inactivating EHEC biofilms was investigated. Additionally, the effect of Se on surface attachment of planktonic cells and exopolysaccharide (EPS) synthesis was studied. Moreover, the efficacy of Se in combination with hot water for rapidly inactivating fully formed EHEC biofilms was determined. For biofilm inhibition assay, polystyrene plates inoculated with EHEC (~6.0 log CFU/well) were treated with the sub-inhibitory concentration of Se, and incubated at 25°C and 4°C. The bacterial population in the biofilm was enumerated every 24 h for 96 h. Initial EHEC planktonic cell attachment and EPS production were analysed using solid surface assay and ruthenium red staining assay, respectively. The inactivation of mature EHEC biofilms was studied by treating pre-formed biofilms with the bactericidal concentration of Se in combination with hot water (80^{o}C) for 0, 2 and 5 min. Selenium reduced attachment of planktonic cells, biofilm formation and EHEC EPS synthesis ($P < 0.05$). Se in combination with hot water reduced biofilm-associated EHEC counts to undetectable levels at 5 min of exposure compared to control ($P < 0.05$). In addition, the coating of stainless steel surfaces with Se nanoparticles exerted

significant anti-biofilm effect. The results suggest that Se could potentially be incorporated on food contact surfaces in processing plants for controlling EHEC biofilms, and improve food safety.

Objective 2 investigated the efficacy of a food-grade, plant-derived flavonoid, rutin (RT), either alone or in combination with chitosan, in enhancing the thermal inactivation of EHEC in undercooked ground beef patties. Fresh, fine ground beef (80% lean) purchased locally was divided into patties (25 g) and inoculated with a 5-strain mixture of EHEC (7.0 log CFU/g), followed by addition of RT (0.05%, 0.1%w/w) with or without CH (0.01%v/w). The patties were packaged on Styrofoam trays, wrapped with oxygen-permeable PVC film and stored at 4°C for 5 days. On days 1, 3 and 5, patties were cooked to an internal temperature of 65°C and surviving EHEC were enumerated. The effect of treatments on meat color using MiniScan XE Plus colorimeter and lipid oxidation during storage was determined according to American Meat Sciene Association (AMSA) guidelines. RT enhanced thermal destruction of EHEC and reduced pathogen load by at least 3 log CFU/g compared with control ($P<0.05$). The combination of RT and CH was more effective, and reduced EHEC to undetectable levels by day 7 of storage ($P<0.05$). The EHEC counts in uncooked patties did not decline during storage ($P>0.05$). The treatments did not alter shelf-life compared with controls ($P>0.05$), whereas there was an increased redness (a* value) in patties treated with RT ($P<0.05$). Results suggested that RT could be used to kill EHEC in undercooked patties; however, sensory studies are warranted.

In objective 3, the effect of Se on EHEC motility, verotoxin production, cytotoxicity and toxin gene expression was determined. Additionally, the effect of selenium on Gb3 receptor protein production and expression of Gb3 synthesis genes in human lymphoma cells was investigated by flow cytometry and real-time quantitative PCR (RT-qPCR), respectively.

Selenium reduced extracellular verotoxin production by 40–60% and intracellular verotoxin synthesis by 80-90% in EHEC ($P<0.05$). Selenium also inhibited toxin-mediated Vero cell cytotoxicity, and down-regulated the expression of EHEC verotoxin genes ($P<0.05$). In addition, mean fluorescence data from flow cytometry revealed that selenium reduced the production of Gb3 receptor ($P<0.05$) compared to untreated control. This was further validated by RT-qPCR data, which revealed a significant down-regulation of LacCer synthase gene (GalT2) involved in Gb3 synthesis. Results justify follow up *in vivo* studies for validating the efficacy of selenium in controlling EHEC infection.

In objective 4, the efficacy of sub-inhibitory concentration (SIC, highest concentration that is neither bacteriostatic nor bactericidal) of RT on EHEC motility, attachment to cultured intestinal epithelial cells, verotoxin production and verotoxin binding to Gb3 receptor was investigated. In addition, the effect of RT on EHEC virulence gene expression was determined. Following treatment, extracellular and intracellular verotoxin concentration in each culture was determined by enzyme linked immunosorbent assay. In addition, the effect of RT on the expression of EHEC virulence genes, including the verotoxin genes (*stx1* and *stx2*) was investigated using real-time quantitative polymerase chain reaction (RT-qPCR). The effect of RT on EHEC motility and adhesion to human intestinal epithelial cells (CaCO2) was also studied using standard protocols. Rutin decreased EHEC motility and attachment to CaCO2 cells ($P<0.05$). Rutin inhibited both extracellular and intracellular verotoxin production by 70-80% in all three EHEC strains tested, compared to the untreated controls ($P < 0.05$). RT-qPCR results revealed that RT down-regulated the transcription of verotoxin gens, *stx1* and *stx2* ($P < 0.05$). Verotoxin binding to Gb3 receptor was decreased in the presence of RT ($P < 0.05$). Verotoxin binding to Gb3 receptor was decreased in the presence of RT ($P < 0.05$). Further

experiments revealed that RT can competitively bind VT at the receptor binding sites, thereby possibly inhibiting toxin-induced pathology.

Caenorhabditis elegans, a free-living nematode with a rapid generation time, has been extensively used as an *in vivo* model in studies of microbial pathogenesis and innate immunity. *C. elegans* has been used as an infection model for several enteric pathogens, including EHEC, enteropathogenic *E. coli*, and *Salmonella*. The advantage of using *C. elegans* is that the pathogenic bacterium under study can be substituted in place of the normal feed of the nematode (*Escherichia coli* OP50) as the nutritional source, and the primary site of the infection is the intestine. Moreover, phenotypes such as animal survival, motility, and pathogen burden can be easily and noninvasively examined in *C. elegans*. Additionally, reports suggest that *C. elegans* intestinal cells share similar anatomic features with humans, which makes it an ideal model for the study of intestinal pathogens, including EHEC. Therefore, in objective 5, we used *C. elegans* as an *in vivo* model for studying the anti-EHEC activity of Se and RT.

A toxicity study was first conducted to determine the *in vivo* lethal doses of Se and RT in uninfected nematodes. The *in vitro* anti-virulence concentrations determined against EHEC in our previous studies found to be not lethal to the worms in the case of RT, whereas for Se the sub inhibitory concentration determined *in vitro* was found to be lethal. Further, in this study, RT increased the survivability of EHEC-infected worms ~ 65% compared to control. Furthermore, the ability of EHEC to colonize in the nematode was impeded after treating with RT. On the other hand, Se lacked anti-EHEC activity and the percentage survival of worms after treatment with the mineral was not significantly different from the infected control group.

Mechanisms of the pathogen is gaining much more interest in this decade of antibiotic resistance. In conclusion, this Ph.D. dissertation targeted to attenuate the virulence mechanisms

of EHEC, particularly the verotoxins, using two classes of natural antimicrobials, RT and Se. In addition, the efficacy of RT and Se for controlling EHEC in ground beef and food contact surfaces, respectively was investigated. The study expected to yield potential new strategies for reducing the risks due to this pathogen to humans.