INFLUENCE OF VITAMIN EXPOSURE ON *ESCHERICHIA COLI* O157:H7 ATTACHMENT, STRESS RESPONSE AND VIRULENCE

by

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Abstract

Fresh produce is a natural source of vitamins in our diet. Additionally, our enteric flora produces several vitamins, including biotin, cobalamin, folate, menaquinone, pantothenate, and riboflavin. The aim of this study was to determine whether enterically-produced or food-related vitamins may increase attachment of *Escherichia coli* O157:H7 to leafy green produce, and trigger expression of key stress and virulence genes, thereby enabling its gastrointestinal survival. Late logarithmic phase *E. coli* O157:H7 grown in M9 minimal medium was exposed to α-tocopherol, ascorbate, biotin, cobalamin, folate, menaquinone, pantothenate, or riboflavin. Following 1.5 and 3 h exposure, HeLa cell assays were performed to assess adherence, while the impact of ascorbic acid, cobalamin and pantothenate on Shiga toxin (Stx) production was quantified by Stx ELISA. Expression of stress response genes (*dnaK*, *osmC*, *rpoS*) was monitored using *lux*-promoter fusions. Expression of relevant stress response and virulence genes was examined by a quantitative real-time polymerase chain reaction. Lastly, to determine attachment behavior, treated *E. coli* O157:H7 cells were spotted onto spinach leaves. Treatments with α-tocopherol, biotin, cobalamin, and pantothenate significantly increased adherence to HeLa cells ($p<0.05$), though only pantothenate (50 mg/mL) produced a >1-$\log_{10}$ increase in adherence. Cobalamin treatment resulted in significantly increased Stx1 and 2 levels ($p<0.001$), while ascorbate and pantothenate led to lower levels ($p<0.001$) than the control. In general, exposure to vitamins affected expression of selected virulence and stress response genes. Lastly, vitamin-treated cells attached to the surface of spinach leaves similarly to controls, with the exception of ascorbate and pantothenate treatments where fewer cells attached over a period of 24 h.

Increases in *E. coli* O157:H7 cell concentration on leaves were observed up to 24 h in all treatments, except ascorbate (10 mg/mL) which caused cell numbers to decrease. This study suggests vitamins present in the enteric environment or food have the potential to influence stress response and virulence of *E. coli* O157:H7, though high concentrations above those found in food or enteric environments are required to elicit these. Ascorbic acid warrants further study as a potentially effective antimicrobial agent for decreasing *E. coli* O157:H7 contamination on the surface of spinach.

Preface

A part of Chapter 2, subsection 12, Shiga toxin quantification, is based on work conducted in collaboration with the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, ON, Canada, by Drs. Roger Johnson and Uma Silphaduang. I prepared bacterial lysates and supernatants to be tested, while Shiga toxin enzyme-linked immunosorbent assay (ELISA) was conducted by the collaborators. All statistical analyses were performed by me.

The rest of the research in this thesis was completed solely by the author, Ana Cancarevic.

The University of British Columbia (UBC) Department of Risk Management Services Laboratory Biological Safety Course was completed on June 10, 2011, prior to the start of the project. This project was approved by the UBC Biosafety Committee, Certificate Number B10-0010.

This thesis is original and contains work that has not been previously published.

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List of Abbreviations

A/E	Attaching and Effacing
AIEC	Adherent Invasive *Escherichia coli*
AI-3	Autoinducer 3
ANOVA	Analysis of Variance
AR	Acid Resistance
ATP	Adenosine Triphosphate
BCCDC	British Columbia Centre for Disease Control
bp	Base pair
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CFU	Colony Forming Unit
CoA	Coenzyme A
DAEC	Diffusely adherent *E. coli*
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-buffered Saline
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
GABA	γ-amino butyric acid
Gb3	Globotriaosylceramide
HeLa	Henrietta Lacks
HC	Hemorrhagic Colitis
H-NS	Histone-like protein global regulator
HUS	Hemolytic Uremic Syndrome
iFBS	Inactivated Fetal Bovine Serum
LB	Luria Bertani
LEE	Locus of Enterocyte Effacement
M9MM	M9 Minimal Medium

MEM	Minimum Essential Medium
mRNA	Messenger Ribonucleic Acid
MQ	Menaquinone
NADP	Nicotinamide Adenine Dinucleotide Phosphate
OD	Optical Density
ORF	Open Reading Frame
PAI	Pathogenicity Island
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PHAC	Public Health Agency of Canada
ppGpp	Guanosine-3',5'-bispyrophosphate
qPCR	Quantitative real-time Polymerase Chain Reaction
RLU	Relative Luminescence Units
RNA	Ribonucleic Acid
RNase	Ribonuclease
rRNA	Ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
RTX	Repeats in Toxin
SD	Standard Deviation
STEC	Shiga-toxin producing *E. coli*
Stx	Shiga Toxin
T3SS	Type Three Secretion System
TTP	Thrombocytopenic Purpura
USDA	United States Department of Agriculture
VBNC	Viable But Non-Culturable

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Dedication

To my family, who believed in me and inspired me to go beyond what I thought was possible.

Chapter 1: Literature review

1.1 Introduction

Escherichia coli, named after Theodor Escherich, a German pediatrician who discovered and described this bacterium in 1885, has been studied extensively in the world (Baylis & Betts, 2001; Shulman et al., 2007). *Escherichia coli* is Gram-negative, facultative anaerobic bacillus found naturally in mammalian intestinal tracts (Nataro & Kaper, 1998). In a human gut, *E. coli* has a symbiotic role as part of a normal and diverse microbiota that contributes to the fermentation of non-digestible nutrients, produces vitamins such as vitamin K (Almquist et al., 1938) and certain B vitamins required by the host (Burkholder & McVeigh, 1942), is immunostimulatory, and serves to block enterocyte binding of ingested pathogenic bacteria (Guarner, 2006; Salminen et al., 1998). Even though most *E. coli* are non-pathogenic, there is a subset of pathotypes with virulence features that are known to cause human disease. Based on disease outcomes, pathogenic *E. coli* can belong to: uropathogenic, neonatal meningitis, or diarrheagenic *E. coli* (Croxen & Finlay, 2009). Strains associated with intestinal disease are further classified in seven pathotypes: enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* [with enterohemorrhagic *E. coli* (EHEC) being an example], enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), and a new pathotype, adherent invasive *E. coli* (AIEC) (Croxen et al., 2013). Differences in pathogenicity relate directly to differing gene content, much of which is encoded within pathogenicity islands (PAIs), plasmids, or prophage elements (Croxen & Finlay, 2009). STEC possesses at least one Shiga toxin gene (*stx*$_1$ and/or *stx*$_2$) located in temperate lambdoid prophages and can cause mild to

bloody diarrhea and hemolytic uremic syndrome (HUS) (Croxen et al., 2013). EHEC is a locus of enterocyte effacement (LEE) PAI-positive subset of STEC, associated with hemorrhagic colitis (HC) and/or HUS. However, some LEE-negative serogroups associated with HC and HUS were termed EHEC in literature, such as O91:H21, O104:H4 (EHEC and EAEC hybrid), and O113:H21 (Croxen et al., 2013). The focus of this literature review will be on the *E. coli* O157:H7, a commonly reported LEE-positive EHEC.

1.2 Epidemiology and disease

Escherichia coli O157:H7 is a foodborne pathogen capable of causing hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and even death in humans (Karmali et al., 2010; Riley et al., 1983). Following consumption of contaminated food or water, disease onset typically occurs within 3-4 days, but may be as short as 1, or as long as 8 days. Clinical symptoms start with abdominal cramps and watery diarrhea, and may progress to bloody diarrhea (hemorrhagic colitis) after 2-3 days. Nausea and vomiting occur in approximately 50% of cases (Griffin et al., 1988). In 5 to 10% of infected individuals, hemolytic uremic syndrome, defined as a triad of symptoms including hemolytic anemia, thrombocytopenia and acute renal failure, may develop (Gyles, 2007; Mead & Griffin, 1998; Nataro & Kaper, 1998). Children less than 5 years of age and the elderly typically experience more severe disease outcomes. The highest rates of HUS (15.3%) have been reported in young children. Death occurs in approximately 0.6% of infections; in persons >60 years, higher mortality rates are reported with fatalities occurring in 3.1% of infected individuals (Gould et al., 2009). Another condition called thrombotic thrombocytopenic purpura (TTP) may occur in adults (Ruggenenti & Remuzzi, 1990). TTP presents in a similar manner to HUS, but affects the central nervous system rather than the kidneys (Boyce et al., 1995; Gyles, 2007;

Ruggenenti et al., 2001). The clinical neurological symptoms include: headache, aphasia, cranial nerve palsies, confusion, stupor, coma, and seizures (Liu et al., 2001).

Escherichia coli O157:H7 infections usually happen after consumption of contaminated food or water, though other routes of transmission may occur (Gyles, 2007). The most common route is foodborne, which contributed to 52% of all *E. coli* O157:H7 outbreaks in the United States (US) between 1982 and 2002. Other routes included: unknown (21%), person-to-person contact (14%), recreational water (6%), animal contact (3%), drinking water (3%), and lab-related infections (<1%) (Rangel et al., 2005). The most commonly reported foodborne vehicle is ground beef (Bell et al., 1994; Gyles, 2007; O'Brien et al., 1993; Rangel et al., 2005). However, other beef products such as roast beef, steak, sirloin tips, and salami have been reported as well (Rangel et al., 2005). In a Canadian *E. coli* O157:H7 outbreak in 2012, a few cases were associated with contaminated mechanically-tenderized beef that was found to be of higher risk for consumption compared to an intact beef cut (Catford et al., 2013). In addition to beef, leafy green vegetables (primarily lettuce and spinach), sprouts, unpasteurized milk, apple cider and apple juice-related outbreaks have been reported (Ackers et al., 1998; Baylis & Betts, 2001; Cody et al., 1999; Keene et al., 1997; Rangel et al., 2005; Wendel et al., 2009). In general, high rates of infections have been reported during the summer months (Griffin & Tauxe, 1991). Similarly, in British Columbia, Canada, the peak number of cases is registered in August and September (weeks 33 to 37; Figure 1.1; BCCDC, 2013).

The infectious dose of *E. coli* O157:H7 has been estimated to be less than 50 cells (Tilden et al., 1996). Such a low infectious dose can aid in easier spread of *E. coli* O157:H7 *via* food and other routes of transmission (Boyce et al., 1995).

1.3 Disease treatment

To date, no specific therapies effective against *E. coli* O157:H7 infections have been demonstrated. In general, antimicrobials known to damage DNA or disrupt bacterial cell walls may stimulate production or release of Shiga toxin (Karch et al., 1985; Walterspiel et al., 1992). In the past, antimicrobial treatments of *E. coli* O157:H7 infections have been controversial with regards to the development of HUS (Boyce et al., 1995; Karmali et al., 2010). While several studies report a higher risk of HUS in patients treated with antimicrobials during the diarrheal phase of the illness (Dundas et al., 2001; Wong et al., 2000; Wong et al., 2012), others suggest there is no effect on disease outcomes (Bell et al., 1994; Martin et al., 1990). In contrast, treatments with fosfomycin have been shown to prevent HUS in children, but only if administered early in the infection (Ikeda et al., 1999; Takeda et al., 1998). While the Public Health Agency of Canada (PHAC, 2014) advises antibiotics should not be used to treat patients with *E. coli* O157:H7 infection in Canada, data from the recent German *E. coli* O104:H4 outbreak suggest a combination of at least two antibiotics (meropenem and ciprofloxacin, and additionally rifaximin for patients on intensive care) reduced the severity of disease *i.e.* fewer seizures, abdominal surgeries, and deaths, and significantly shortened the bacterial shedding time (Menne et al., 2012). Recently, Bielaszewska et al. (2012) reported that some antibiotics, including meropenem, azithromycin, rifaximin, and tigecycline did not stimulate Shiga toxin 2 production in *E. coli* O104:H4 *in vitro*. However, further research is needed to evaluate effectiveness of these antimicrobials against different EHEC strains both *in vitro* and *in vivo*.

1.4 Outbreaks

One of the first documented *E. coli* O157:H7 outbreaks occurred in the US in 1982 when 47 people became ill after consuming contaminated undercooked hamburgers (Riley et al., 1983). Since then, foodborne outbreaks involving this organism have been recurring and, unfortunately, are significant sources of human morbidity and mortality. The largest *E. coli* O157:H7 outbreak occurred in Sakai city in Japan in 1996 when more than 6,000 school children were infected following consumption of contaminated radish sprouts that were included in a school lunch program (Hayashi et al., 2001).

In Canada, *E. coli* O157:H7 is the third most commonly reported foodborne pathogen, causing approximately 480 illnesses per year (Figure 1.2). The largest outbreak occurred in Ontario in 2000 when more than 2,300 people were infected and seven died after drinking *E. coli* O157:H7 contaminated water. Since 2003, the incidence rate has been in decline with 1.39 infections per 100,000 individuals reported in 2012 (Figure 1.3) (PHAC, 2014).

Interestingly, in British Columbia (BC), the incidence rate of STEC has remained above the Canadian average since 2004, ranging from 2.4 to 4.6 cases per 100,000 people. Among STEC, O157 was the predominant serogroup, causing 57.8% of the infections in 2012. Non-O157 STECs consisted mainly of three serogroups: O26, O103, and O121, and were linked to 35.3% illnesses (BCCDC, 2013).

In the US, approximately 63,153 cases of *E. coli* O157:H7 infections occur each year, resulting in 2,138 hospitalizations and 20 deaths (Scallan et al., 2011). As recently estimated, O157 STEC has been the most frequently reported, causing 50-74% of the human infections. Non-O157 STECs have been linked to the remaining 26-50% cases, among which six

serogroups, including O26, O45, O103, O111, O121, and O145, are the most prevalent (Brooks et al., 2005; Fey et al., 2000; Gould et al., 2013; Park et al., 2002). To address a rising concern for non-O157 STEC, the US Department of Agriculture (USDA) recently amended food safety regulatory policy by declaring these six non-O157 STEC serogroups as beef adulterants (USDA, 2012).

1.5 Reservoirs

The main reservoir of *E. coli* O157:H7 are ruminants, particularly cattle (Figure 1.4) (Ferens & Hovde, 2011; Gyles, 2007; Su & Brandt, 1995). Studies examining the prevalence of *E. coli* O157:H7 in cattle worldwide suggest a pervasive problem globally, with the organism infrequently not detected to 72% farms being positive (Chase-Topping et al., 2008), with a prevalence ranging from 0% (Bonardi et al., 1999) to 80% (Khaitsa et al., 2003). Generally, cattle are transiently infected, being colonized in the terminal rectal mucosa. However, the infection appears asymptomatic since bovines lack glycolipid globotriaosylceramide (Gb_3), the receptor recognised by Shiga toxin which ultimately leads to vascular damage in the human colon and kidneys (Pruimboom-Brees et al., 2000).

Typically, *E. coli* O15:H7 is shed in cattle faeces at 10 to 100 CFU/g, but concentrations as high as 10^7 CFU/g have been reported (Gyles, 2007). It has been suggested that high-shedding cattle, termed "supershedders", persistently shed the organism at levels $>10^4$ CFU/g, meaning that they are significant environmental and abbatoir-related sources of contamination. Shedding in cattle has been shown to be influenced by season, age and diet. In particular, increased shedding occurs in warmer months, at post-weaning, and in cattle fed with brewer's grains (Ferens & Hovde, 2011; Gyles, 2007). In addition to cattle,

E. coli O157:H7 has been reported in other food-producing animals, including sheep, goats, pigs, and even wild ruminants and birds (Ferens & Hovde, 2011; Gyles, 2007).

1.6 Virulence factors

Escherichia coli O157:H7 possesses genetic elements that enable its colonization of the human gut and subsequent disease processes. Genome sequencing of two *E. coli* O157:H7 clinical strains and a benign *E. coli* K-12 strain MG1665 revealed the presence of 4.1 Mb highly conserved core genetic material in all strains. In addition, sequencing showed pathogenic strains possessed an additional 1.4 Mb of DNA comprised largely of prophages and prophage-like elements. This genetic content included genes enabling *E. coli* O157:H7 to produce Shiga toxin(s) and enterohemolysin, and to create intimate attachment to the host cell (Hayashi et al., 2001; Perna et al., 2001).

1.6.1 Shiga toxins

Escherichia coli O157:H7 strains produce Shiga toxins (Stxs) Stx1 and/or Stx2 (Hayashi et al., 2001; Mauro & Koudelka, 2011; Nataro & Kaper, 1998). Historically, Shiga toxins have been also termed Vero toxins or Vero cytotoxins due to their cytotoxic effects on African green monkey kidney (Vero) cells (Cantey, 1985; Konowalchuk et al., 1977; Lingwood, 1996; Nataro & Kaper, 1998). In this thesis, the term "Shiga toxin" will be used.

Genes encoding Stx1 and Stx2 (stx_1 and stx_2) are carried in the two distinct temperate lambdoid prophages, 933J and 933W respectively, integrated in the bacterial chromosome (Nataro & Kaper, 1998; Schmitt et al., 1999; Serra-Moreno et al., 2008; Strockbine et al., 1986). Nucleotide sequencing revealed stx_1 is 99% homologous to *stx* of *Shigella dysenteriae* type 1, differing only in three nucleotides of three separate codons located in the A subunit (Strockbine et al., 1988). In contrast, stx_{2A} and stx_{2B} share 57% and

60% homology with *stx*$_{1A}$ and *stx*$_{1B}$ respectively (Jackson et al., 1987). While *stx*$_1$ is highly conserved among *E. coli* O157:H7 strains (represented by subtypes *stx*$_{1a}$, *stx*$_{1c}$ and *stx*$_{1d}$), *stx*$_2$ variants are more common, with seven subtypes being recognized: *stx*$_{2a}$, *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, *stx*$_{2f}$, and *stx*$_{2g}$ (Scheutz et al., 2012).

Epidemiological data show an association between *stx*$_2$ and higher risk for HUS development (Boerlin et al., 1999). In line with this, it has been reported that Stx2 is approximately 1,000 times more toxic to renal microvascular endothelial cells than Stx1 (Louise & Obrig, 1995).

Structurally, Shiga toxins are A-B toxins. The enzymatically active A subunit is surrounded by five B subunits responsible for recognition and binding to the glycolipid Gb3 receptor on the host cells (Schmitt et al., 1999). This binding results in holotoxin endocytosis, transportation to the Golgi apparatus, and eventually transport to the endoplasmic reticulum. During the early stage of holotoxin endocytosis, the A subunit is proteolytically cleaved into two fragments, A1 and A2, which stay linked by a disulfide bond. The A1 fragment possesses enzymatic activity, while A2 serves to associate the A1 portion with the B pentamer (O'Brien et al., 1992; Schmitt et al., 1999; Schüller, 2011). In the endoplasmic reticulum, the active A1 fragment is released from A2-B pentamer in a process that has not been well characterized, but that possibly involves enzyme protein disulfide isomerase, which accelerates reduction of a disulfide bond (Spooner & Lord, 2012). Following its release and translocation into the cytoplasm, the A1 fragment acts as an N-glycosidase on the 60S ribosomal subunit and cleaves a single adenine residue in the 28S ribosomal RNA, which leads to the inhibition of protein synthesis and eventual cell apoptosis (O'Brien et al., 1992; Schmitt et al., 1999; Schüller, 2011).

1.6.1.1 Regulation of *stx*$_1$ and *stx*$_2$

Three *stx* promoters located upstream of *stx* genes have been recognized: p_{stx} (*stx* specific), and p_R and p_R' (phage promoters) (Wagner et al., 2001b; Wagner et al., 2002). Studies suggest transcription of *stx*$_1$ and *stx*$_2$ are regulated differently.

Low-iron conditions induce the *stx*$_1$ promoter (p_{stx1}) by an iron-dependant Fur transcriptional repressor (Calderwood & Mekalanos, 1987). Following p_{stx1} induction, transcription of the late phage lysis genes is not stimulated even though *stx*$_1$ is induced. As a result, the bacterial cell remains intact, while Stx1 accumulates mainly in the bacterial periplasm (Wagner et al., 2002). Interestingly, no environmental triggers of promoter *stx*$_2$ have been detected (Mühldorfer et al., 1996).

Additionally, phage-inducing conditions play a role in regulation of both *stx*$_1$ and *stx*$_2$. For example, the DNA-damaging agent mitomycin C induces promoters: p_R and p_R' which stimulate expression of *stx*$_1$ and *stx*$_2$ (Wagner et al., 2001b; Wagner et al., 2002). Following DNA damage, the bacterial SOS response is activated and RecA-mediated autocleavage of the phage repressor CI occurs, leading to the transcription of early and late phage genes (Little, 1996; Waldor & Friedman, 2005). The phage lysis is regulated by two late phage promoters: p_R and p_R', which stimulate *stx*$_1$ and *stx*$_2$ as well. This ultimately leads to bacterial lysis and toxin release. While both p_R and p_R' play a role in *stx* expression, *stx*$_2$ induction is known to be highly dependent on p_R' (Wagner et al., 2001b; Wagner et al., 2002). Besides mitomycin C, other DNA-damaging agents such as H_2O_2 and neutrophils have been linked to the increased Stx2 production (Wagner et al., 2001a).

1.6.2 Attaching and effacing lesions

Following infection of the human host, *E. coli* O157:H7 intimately adheres to the enterocytes of the colon and creates an attachment and effacement (A/E) lesion (Nataro & Kaper, 1998). The A/E lesion formation can be divided into three general stages: the first stage is intimate attachment of bacteria to the host cell (mediated by the bacterial protein intimin), the second stage involves rearrangement of the cytoskeletal structures in the host cell underneath the attached bacteria resulting in the formation of pedestal-like structure, and the third stage is the loss of microvilli surrounding the cell, reduced absorptive capacity of the cell, and diarrhea (Armstrong, 2007; Gyles, 2007; Law, 2000; Paton & Paton, 1998). The A/E phenotype is encoded in a 43.4 Kb pathogenicity island (PAI) termed the locus of enterocyte effacement (LEE), which comprises of 54 open reading frames (ORFs) and is located on the *E. coli* O157:H7 chromosome (Perna et al., 1998). The LEE has five operons (LEE 1 to LEE 5), which include the following genes: *sep* and *esc* encoding type III secretion system, *eae* encoding intimin and *tir* encoding translocated intimin receptor (Tir) (LEE 5), *espABDF* encoding translocator and effector proteins (LEE 4), and *ler* (LEE 1) encoding regulator Ler, an H-NS-like protein that activates the expression of *LEE* genes (Garmendia et al., 2005; Gyles, 2007; Moxley, 2004; Nataro & Kaper, 1998).

Regulation of genes encoded by the LEE is complex and involves three LEE-encoded regulators: Ler, GrlA, and GrlR. While Ler plays a central role in transcription of most of the *LEE* genes, GrlA and GrlR serve as activator and repressors of *ler* respectively (Barba et al., 2005; Deng et al., 2004; Lio, 2004). Once produced, Ler replaces the H-NS protein from its promoter sites, and induces transcription of *grlR/A*, *LEE2*, *LEE3*, *LEE4*, and *LEE5* (encoding Eae and Tir) operon (Bustamante et al., 2001; Elliott et al., 2000; Haack et al., 2003).

1.6.2.1 Induction of *LEE* genes by environmental factors

Colonization of the human large intestine and *LEE* gene expression in *E. coli* O157:H7 are dependent on signals bacteria recognize in the enteric environment. Abe et al. (2002) reported *ler* transcription and production of EspA, EspB, intimin, and Tir were enhanced when *E. coli* O157:H7 was grown in Luria Bertani (LB) broth containing sodium bicarbonate ($NaHCO_3$). Since the highest effect on *ler* induction was seen with 44mM $NaHCO_3$, a concentration that is close to the physiological concentration of $NaHCO_3$ in the ileum, this study implies bicarbonate levels may be an important environmental trigger that promotes EHEC colonization in the human gut (Abe et al., 2002).

In addition to bicarbonate, studies suggest *E. coli* O157:H7 *LEE* genes can be stimulated by intra- and inter-kingdom signal molecules associated with the enteric environment. Specifically, in the colon, high concentrations of bacteria produce a quorum sensing compound called autoinducer-3 (AI-3) (Kaper & Sperandio, 2005). In *E. coli* O157:H7 this bacterial signaling molecule is detected by receptors QseC and QseE, which results in up-regulation of flagellum and *LEE* genes respectively (Kaper & Sperandio, 2005). Similarly, epinerphrine and norephinephrine, human hormones present in the gut, are involved in host-bacteria signaling. They contribute to flagellum and *LEE* gene expression by binding to the same AI-3 receptors (Kendall et al., 2007; Sperandio et al., 2003).

Finally, influence of other environmental factors such as temperature, osmolarity, and the presence of certain cations, and anions on the induction of *E. coli* O157:H7 *LEE* genes were investigated. Beltrametti et al. (1999) reported that Ca^{2+} and Mn^{2+}, as well as higher osmolarity (430 mM NaCl) in combination with temperatures of 37°C and 42°C stimulated expression of the *espA* promoter in M9 minimal medium. In contrast, presence of NH_4^+,

Mg^{2+}, chloride, iron, nitrite, sulphate, temperature (25, 37, or 42°C), and pH (6,7, and 8) had no effect on the promoter activity (Beltrametti et al., 1999).

1.6.3 Enterohemolysin

Enterohemolysin (Ehx) was recognized by Beutin et al. (1989) as a novel type of hemolysin frequently associated (89%) with STEC strains. Enterohemolysin gene (*hlyA*) is carried in a large 92 Kb plasmid, pO157, commonly found in *E. coli* O157:H7 strains (Burland et al., 1998; Schmidt et al., 1994). It is a pore-forming cytotoxin which acts by disrupting permeability of cytoplasmic membrane in erythrocytes (Bauer & Welch, 1996; Schmidt et al., 1996). Ehx belongs to the repeats in toxin (RTX) exotoxin family (Schmidt et al., 1995) commonly secreted by a wide variety of Gram-negative pathogenic bacteria, some of which include uropathogenic *E. coli* and *Vibrio cholera* (Welch, 2001). Uropathogenic *E. coli* strains produce α-hemolysin which lyses erythrocytes and creates large clear zones after 4 h incubation on blood-agar plates (Beutin, 1991). In contrast, *E. coli* O157:H7 produces enterohemolysin which generates small turbid zones of lysis visible only after overnight incubation on blood-agar plates with washed erythrocytes (Beutin, 1991). Schmidt & Karch (1996) reported that 88% of STEC strains isolated from patients with HUS produced Ehx, thus suggesting that Ehx might be an important factor in developing HUS; however the precise role of Ehx in human disease remains unknown.

1.7 Stress response

Bacterial survival and subsequent proliferation is dependent on the ability of the bacterium to effectively cope with different stresses they encounter in the environment. In general terms, stress can be defined as any deviation which alters optimal conditions and results in reduced growth rate of bacteria (Storz & Hengge-Aronis, 2000). Examples of

stresses found along a food chain, in food, or in food-related environments are: physical (heating, drying, high hydrostatic pressure, refrigeration, freezing, radiation, vacuum, and concentration of sugars and salts), chemical (presence of acids, alkali, chemical sanitizers *i.e.* chlorine, iodine, and quaternary ammonium compounds, oxidative treatments such as ozone and hydrogen peroxide, preservatives including benzoate, nitrite, and sorbate), and nutritional (starvation due to lack of nutrients) (Wesche et al., 2009). Different levels of stress can be distinguished based on the severity of the outcomes they cause in bacteria (Storz & Hengge-Aronis, 2000). Bacterial cells can adapt to suboptimal growth conditions, continue to grow, and even develop resistance to the particular stress or other stresses that had not been experienced (Bibek, 2003). Under more severe, sublethal or lethal stress, bacteria switch from growth to damage repair. Upon exposure to sublethal stress, cells may undergo injury that can be reversibly repaired, or may even enter a viable but non-culturable (VBNC) state. Finally, after exposure to lethal stress, cells will become unculturable under any circumstances (Bibek, 2003).

Bacteria can respond to stress conditions by mechanisms that involve sigma (σ) factors. Sigma factors are subunits of RNA polymerase that are capable of recognizing and binding to -35 and -10 promoter region sequences of specific genes. Each RNA polymerase holds only one sigma factor at a time. Once a bacterium senses stress in the environment, it employs a sigma factor to control the expression of different sets of genes under specific environmental conditions (Wilson et al., 2011). Sigma factors are distinguished by their molecular mass, *e.g.*, σ^{70} has molecular weight of 70 kDa (Wilson et al., 2011). In *E. coli*, seven sigma factors have been identified: vegetative (σ^{70} also known as RpoD) which regulates housekeeping genes for cell growth, nitrogen-limiting (σ^{54} or RpoN), general stress

response, also known as starvation and/or stationary phase sigma factor (σ^{38}, σ^{S}, or RpoS), heat shock (σ^{32}, σ^{H}, or RpoH), flagellar (σ^{28} or RpoF), extreme cytoplasmic or extreme heat (σ^{24} or RpoE), and ferric citrate sigma factor (σ^{19} or FecI) (Wick & Egli, 2004; Wilson et al., 2011). General stress response, as well as some of the specific stress response mechanisms of *E. coli* O157:H7 will be reviewed in the following sections.

1.7.1 General stress response

The general stress response present in many Gram-negative bacteria, including *E. coli* O157:H7, is orchestrated by RpoS (σ^{38} or σ^{S}) (Hengge-Aronis, 2002b). Previous studies reported RpoS is triggered upon bacterial entry into stationary phase, but also under growth limiting conditions such as starvation (Hengge-Aronis, 2002b), osmotic shock (Hengge-Aronis, 1996), cold/heat shock (Muffler et al., 1997; Sledjeski et al., 1996), and acid stress (Lee et al., 1995). In contrast to the action of the specific stress response proteins, which repair damage caused by the particular stress event, RpoS activity provides a more preventive approach. Once produced, this master regulator may act protectively not only against the encountered stress, but also to the other stress factors that have not been experienced yet (cross-protection) (Hengge-Aronis, 2002b). Generally, increased RpoS activity results in decreased bacterial susceptibility to multiple stresses, and therefore increased chance for bacterial survival (Hengge-Aronis, 2002b).

Genome-wide expression microarray analysis revealed RpoS controls the expression of 10% of *E. coli* genome (481 genes), out of which 140 genes were directly affected (Weber et al., 2005). Recent gene expression microarray analysis suggested the RpoS regulon might be considerably different in *E. coli* O157:H7 str. EDL933 and *E. coli* str. K12 (Dong & Schellhorn, 2009). In stationary phase, RpoS enhanced the expression of 50 EDL933-specific

O-island genes (including LEE-encoded regulators *ler* and *cesF*), while 49 EDL 933-specific O-island genes were negatively influenced (including LEE-encoded *eae*) (Dong & Schellhorn, 2009). Interestingly, studies suggest RpoS regulation of virulence genes can vary in different *E. coli* O157:H7 strains. While RpoS was reported to positively control *LEE3* and probably *tir* in *E. coli* O157:H7 str. 86-24 (Sperandio et al., 1999), it negatively influences expression of *LEE* genes in the Sakai strain (Iyoda & Watanabe, 2005; Tomoyasu et al., 2005).

1.7.1.1 Regulation of RpoS

Regulation of RpoS is complex and involves transcriptional, translational, and post-translational mechanisms (Battesti et al., 2011; Hengge-Aronis, 2002b). Regulation of *rpoS* at the transcriptional level has not been studied in as much detail as the regulation of RpoS at the translational and post-translational level. This is partially because most environmental triggers known to induce RpoS do not influence *rpoS* transcription (Hengge-Aronis, 2000). Additionally, studies suggest that much of the regulation is done in the post-transcriptional rather than in the transcriptional mechanisms. Therefore, post-transcriptional mechanisms have been more thoroughly examined (Hengge-Aronis, 2002b).

Transcription of *rpoS* is triggered by conditions of gradual decrease of growth rate during bacterial entry into stationary phase (Hengge-Aronis, 2000; Hengge-Aronis, 2002b). Three promoters involved in *rpoS* transcription have been identified: *nlpD*p1 and *nlpD*p2 located upstream, and *rpoS*p found within *nlpD* gene encoding a lipoprotein (NlpD) with an unknown function (Lange & Hengge-Aronis, 1994b). Even though all three promoters control the expression of *rpoS*, studies show *rpoS*p is the major promoter of *rpoS* transcription during bacterial entry into stationary phase (Lange et al., 1995).

Translation of *rpoS* mRNA to RpoS is stimulated by growth at moderately low temperature (*e.g.,* at 20°C) (Sledjeski et al., 1996), a shift from neutral to acidic conditions (*e.g.,* from pH 7 to pH 5) (Hengge-Aronis, 2002b), growth to a certain cell density (c.a. 1 to $2x10^8$ cells/mL) (Lange & Hengge-Aronis, 1994a), and high osmolarity (Muffler et al., 1996b). Exponentially grown cells contain relatively high levels of *rpoS* mRNA (Hengge-Aronis, 2002b). Under non-stress conditions, it is proposed that *rpoS* mRNA forms a stable secondary structure, which inhibits translation by making its translational initiation region unavailable to ribosomes (Hengge-Aronis, 2002a). Translation may be initiated by alterations in the secondary structure (Abee & Wouters, 1999). Muffler et al. (1996a) found that HF-I, a RNA-binding protein, is crucial for *rpoS* mRNA translation. Additionally, histone-like proteins, HU and H-NS, were found to promote and inhibit *rpoS* mRNA translation respectively (Balandina et al., 2001; Barth et al., 1995). Furthermore, a few small regulatory RNAs (*i.e.*, DsrA, OxyS, and RprA), were reported to influence *rpoS* mRNA translation through stimulatory (*e.g.*, DsrA and RprA) or repressing (OxyS) functions (Hengge-Aronis, 2002b).

Besides the regulation through transcription and translation, RpoS can also be controlled at the post-translational level. RpoS is highly unstable under non-stress conditions (Abee & Wouters, 1999). In the growing cell, it is rapidly cleaved by ClpXP protease, which maintains it at low levels (Schweder et al., 1996). However, the cellular level of RpoS can be increased by reduction or inhibition of its degradation in response to stress conditions, such as starvation (Lange & Hengge-Aronis, 1994a), downshift from neutral to acidic pH (*e.g.,* pH 7.7 to pH 4.4) (Bearson et al., 1996), upshift in growth temperature from 30°C to 42.5°C

(Muffler et al., 1997), and hyperosmolarity (*e.g.*, addition of 0.3M NaCl) (Muffler et al., 1996b).

1.7.2 Acid stress response

Escherichia coli belongs to the neutralophiles, a group of microorganisms that prefer neutral pH for growth, *i.e.* pH 5 to 8.5 (Foster, 2000). Interestingly, *E. coli* are capable of surviving in extremely low pH conditions. Gorden & Small (1993) reported 80% of non-pathogenic *E. coli* (n=10) and 66% of EIEC (n=9) strains were acid resistant (*i.e.* able to persist at ≥10% of the starting population) in LB broth acidified to pH 2.5 with HCl for at least 2 h. Similarly, Waterman & Small (1996) evaluated acid resistance of 58 clinical and foodborne STEC strains, and found the majority of strains (n=45) to be acid resistant in LB containing HCl (pH 2.5) after 2 h.

However, highly acidic external environments can have dramatic consequences on *E. coli*. If the environmental pH gets extremely low, H^+ ions can leak through the cell membrane, acidifying the internal pH (Foster, 2000). Additionally, weak organic acids present in food *i.e*. acetate, butyrate, propionate, and lactate, can cross cell membranes and lower the internal pH upon dissociation (Abee & Wouters, 1999; Foster, 2000). As a result of the intracellular acidification, bacteria may experience DNA damage *e.g.*, release of purine bases (adenine and guanine) (Lindahl & Nyberg, 1972); and RNA, probably due to loss of Mg^{2+}, which is necessary for ribosome integrity (Hurst, 1977). Furthermore, organic acid (*e.g.*, lactic acid), disrupts the outer membrane of *E. coli* O157:H7 and affects its permeability (Alakomi et al., 2000).

To cope with acid stress, *E. coli* has developed complex strategies. Specifically, three acid resistance (AR) systems have been identified: oxidative (AR1), glutamate-dependent

(AR2), and arginine-dependant system (AR3) (Foster, 2000; Foster, 2004; Lin et al., 1995). The AR1 is the least understood, and occurs when stationary phase *E. coli* grown in LB buffered to pH 5.5 are transferred to minimal medium at pH 2.5. The acid adapted cells are able to survive low pH conditions, unlike the cells previously grown in LB at pH 8. Although the exact mechanism of survival obtained by this system is unclear, it appears that this acid resistance system is RpoS dependant and glucose repressed (Foster, 2000; Foster, 2004). The AR2, glutamate-dependant system, is the most efficient of all three systems in attaining survival at pH 2 (Castanie-Cornet & Foster, 2001). In this system, internal pH is increased due to activity of two glutamate decarboxylases (GadA and GadB), which use up H^+ ions to convert glutamate to CO_2 and γ-amino butyric acid (GABA). GABA is subsequently exported through a glutamate/GABA membrane antiporter (GadC) in exchange for a new glutamate substrate. Similarly, the AR3 system employs arginine decarboxylase (AdiA) to convert arginine to agmatine, which then gets exported through AdiC antiporter (Foster, 2000; Foster, 2004). Although mechanisms behind AR2 and AR3 systems appear simple, studies suggest complex gene regulation is involved.

Genes encoding glutamate decarboxylase A (*gadA*) and B (*gadB*) are 98% homologous and mapped on different loci of chromosomal DNA (Smith et al., 1992). While *gadA* is monocystronic, *gadB* creates an operon with *gadC* (Castanie-Cornet & Foster, 2001). To date, 11 proteins are known to be involved in regulation of this acid resistance system, some of which include GadE, GadX, GadW, and RpoS (Foster, 2004). GadE is a key activator of *gadA*/*BC*, and binds to 20-bp nucleotide sequence upstream of the transcriptional start sites of both genes, known as *gad* box (Ma et al., 2003). The other known regulators mainly influence transcription of *gadA/BC* by regulating the expression of *gadE* (Foster,

2004). GadX and GadW are DNA binding proteins (Ma et al., 2002), which directly activate transcription of *gadE*, and thus indirectly enhance transcription of *gadA/BC* (Sayed et al., 2007). Interestingly, direct activation of *gadA/BC* promoters by GadX/W was successfully demonstrated *in vitro* (Tramonti et al., 2006), but showed little effect *in vivo* (Sayed et al., 2007). RpoS is required for the expression of GadX. Specifically, RpoS was found to activate *gadY* encoding GadY, a small-RNA regulator that stabilizes *gadX* mRNA and thus contributes to increased GadX synthesis (Opdyke et al., 2004).

In minimal medium, *gadA/BC* is induced in cells while entering stationary phase or by acidic pH during exponential phase growth. In exponential phase, induction of *gadA/BC* was mainly due to increased transcription of *gadE* during growth in minimal salts glucose medium (pH 5.5) supplemented with 0.7 mM glutamate. However, in stationary phase cells, GadE and either GadX or GadW are needed for the maximal expression of *gadA/BC* (Ma et al., 2003).

1.7.3 Oxidative stress response

Oxidative stress has been characterized as a disturbance in the prooxidant-antioxidant balance in favour of prooxidants (Sies, 1991). This circumstance results in intracellular accumulation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), and superoxide anion (O_2^-) (Cabiscol et al., 2000; Farr & Kogoma, 1991). While H_2O_2 and O_2^- are generated endogenously upon contact of molecular oxygen and enzymes carrying electrons for transfer to other substrates (Imlay, 2003), OH· is produced from H_2O_2 in the presence of iron *via* the Fenton reaction (Cabiscol et al., 2000; Park et al., 2005). The excess of intracellular ROS, particularly OH·, is harmful for the cell and can seriously damage biomolecules *e.g.*, lead to breakage of DNA, peroxidation of

polyunsaturated fatty acids, and protein oxidation (Cabiscol et al., 2000; Storz & Zheng, 2000).

To deal with H_2O_2 and O_2^- caused oxidative stress, *Escherichia coli* have developed defence mechanisms mediated by transcriptional regulators OxyR and the two-step SoxRS system. Both OxyR and SoxR are present in "unstressed cells" in their inactive forms, but are rapidly activated upon specific oxidative stresses occurs (Cabiscol et al., 2000). In oxidised form, SoxR will bind to the *soxS* promoter and initiate transcription, probably by making conformational change that leads to stronger binding of RNA polymerase to the promoter (Hidalgo & Demple, 1994). Once produced, SoxS stimulates transcription of defense and repair genes against O_2^- stress (Cabiscol et al., 2000). Activation of OxyR and SoxRS affects genes with antioxidant roles in *E. coli* (Table 1.1) (Storz & Zheng, 2000). In addition to OxyR and SoxRS, general stress response regulator (RpoS or σ^S), can help decrease oxidative stress as well (Table 1.1) (Storz & Zheng, 2000).

1.8 Attachment to the leaf surface

In recent years, numerous produce outbreaks have been associated with *E. coli* O157:H7 contamination of fresh, minimally processed leafy green vegetables, primarily lettuce and spinach (Centers for Disease Control and Prevention, 2013; Rangel et al., 2005). Although plants can become contaminated with *E. coli* O157:H7 at any point along the produce continuum, the most significant risks of transmission is believed to occur in the field, from manure-contaminated soil or irrigation water (Solomon et al., 2002). Furthermore, studies demonstrated *E. coli* O157:H7 can attach to damaged and intact leaf surfaces (Seo & Frank, 1999), or enter the root system and eventually reach edible parts of plants (Solomon et al., 2002).

Attachment is the initial step in bacterial colonization on the leaf surface (Brandl, 2006). The leaf surface is covered with a hydrophobic, waxy layer called cuticle (Romberger et al., 1993). Cuticle has a low permeability for water, gases, and solutes, restricts water and nutrient diffusion from the leaf interior, provides protection against injuries, and limits wetting on the leaf surface (Eichert & Fernández, 2012; Lindow & Brandl, 2003; Romberger et al., 1993). Generally, nutrients are available in minor quantities and are more or less exposed outside of the cuticle or embedded in the epicuticlar waxes (Derridj, 1996). However, studies suggest a few locations on the leaf surface could be abundant in carbohydrates, such as areas in the proximity of veins, near stomates, in crevices between epidermal cells, and at base and tip of trichomes (Leveau & Lindow, 2001; Miller et al., 2001). Interestingly, large bacterial aggregates are commonly observed at these anatomical features of leaves (Leben, 1988; Romantschuk et al., 1996; Timmer et al., 1987), probably due to better accessibility to carbohydrates and water (Lindow & Brandl, 2003).

Generally, mechanisms by which foodborne pathogens colonize leaf surface are complex and not understood well (Critzer & Doyle, 2010). Biological and physiological characteristics of both microorganisms and plants can contribute to establishment and persistence of bacteria on leafy green vegetables. Studies have shown that attachment of *E. coli* O157:H7 to leaf surfaces is influenced by age, zone of contamination, and physiological condition of the plant. In particular, increased colonization of *E. coli* O157:H7 was seen on abaxial side (Zhang et al., 2009) on young (Brandl & Amundson, 2008) cut (Seo & Frank, 1999) or injured leaves (Aruscavage et al., 2008). Elemental analysis of exudates on the leaf surface revealed young (inner) leaves are richer in total nitrogen and carbon compared to middle leaves (Brandl & Amundson, 2008). It is also presumed that bacteria can

use nutrients that leak out from damaged or cut plant tissues for growth (Brandl, 2006; Critzer & Doyle, 2010).

In addition to biological and physiological characteristics of the leaf, characteristics of *E. coli* O157:H7 can also contribute to its attachment to the plant surface. Studies suggest that the attachment depends on the ability of *E. coli* O157:H7 to produce curli, flagella, and the type three secretion system (T3SS) (Critzer & Doyle, 2010; Patel et al., 2011; Xicohtencatl-Cortés et al., 2009). Production of curli, which are adhesive, long, thin, aggregative amyloid fibers, has been shown to be sufficient for *E. coli* K12 strains binding to plant surfaces (sprouts), but not necessary for *E. coli* O157:H7, thus suggesting that this foodborne pathogen may have additional mechanisms that contribute to the attachment (Jeter & Matthysse, 2005). In a recent study, Macarisin et al. (2012) found that curli producing *E. coli* O157:H7 str. EDL 933 achieved significantly stronger association with spinach leaf surface after 24 h incubation compared to the curli deficient mutant. During that time, relative attachment strength to spinach (ratio of strongly attached and total number of attached bacteria) increased. Additionally, Macarisin et al. (2012) noted that cellulose, a component of the extracellular matrix, was not required for the strong attachment of *E. coli* O157:H7 to the spinach leaf surface.

Flagella may affect *E. coli* O157:H7 interaction with the leaf surface, in addition to their role in motility and biofilm formation (Wood et al., 2006). Xicohtencatl-Cortés et al. (2009) have found that mutations in *fliC* flagellin-encoding gene resulted in *E. coli* O157:H7 str. EDL 933 having impaired adherence to spinach and lettuce. In line with this, quantitative real-time PCR analysis revealed increased expression of *fliC* in *E. coli* O157:H7 EDL 933 after inoculation on Romaine lettuce at 15°C (Carey et al., 2009).

Furthermore, it has been suggested that the T3SS may play an important role in *E. coli* O157:H7 str. EDL 933 colonization of the leaf surface, although the mechanisms by which this occurs have not been elucidated (Xicohtencatl-Cortés et al., 2009). Comparison of attachment of T3SS mutants with wild type EDL 933 to leafy greens revealed that mutations in the *escN* gene (which encodes an ATPase associated with T3SS function), but not the *eae* gene (which encodes the intimin adhesin) significantly reduced colonization (Xicohtencatl-Cortés et al., 2009).

Conflicting results have been reported regarding additional surface properties of *E. coli* O157:H7, such as hydrophobicity. While Patel et al. (2011) found that more hydrophobic *E. coli* O157:H7 strains attached to the surface of lettuce at significantly higher numbers, Hassan & Frank (2004) suggest hydrophobicity, as well as surface charge, do not play important roles in *E. coli* O157:H7 attachment to the surface of lettuce. Moreover, attachment of *E. coli* O157:H7 to the leaf surface has been found to be dependent on contact time, with higher numbers attaching after prolonged exposure (Takeuchi & Frank, 2000). Also, *E. coli* O157:H7 survival and growth on the lettuce surface may be altered by the presence of epiphytic microflora (Cooley et al., 2006).

1.9 Vitamins

In 1911, Casimir Funk, a Polish biochemist, named an anti-beriberi compound from rice husk extract “vitamin”, as it was believed to contain amino groups and was vital to life (Combs, 2012; Smolin & Grosvenor, 1997). Nowadays, vitamins can be defined as organic substances naturally found in foods that are not synthesized by the hosts in amounts that satisfy their needs, and therefore are essential in minute quantities for normal physiological functions. Their absence in a diet results in a specific deficiency syndrome (Combs, 2012).

To date, thirteen groups of substances have been recognized as vitamins, some of which contain more than one chemically related compound (termed vitamers) with similar metabolic activities (Combs, 2012). Traditionally, vitamins are classified as water-soluble (B and C) or fat-soluble (A, D, E, and K) based on their ability to dissolve in polar and non-polar solvents respectively (Combs, 2012). Vitamin groups, their vitamers, primary physiological functions, deficiency, and toxicity syndromes are summarized in Table 1.2.

Vitamins can be present in foods in different quantities; certain types of food may contain higher amounts of particular vitamins than others. For example, citrus fruits are known to be rich in vitamin C, fish and meat contain B vitamins, leafy greens are high in folate, vitamins A and K, milk provides riboflavin and vitamin D, while vegetable oils are a great source of E vitamin (Smolin & Grosvenor, 1997) (Table 1.3). Vitamin content in food is influenced by preparation, processing, and storage. In particular, increased vitamin losses occur during blanching (leaching of water-soluble vitamins), with exposure to air (ascorbic acid and fat-soluble vitamins), enzymes (ascorbic acid is sensitive to the activity of oxidases and peroxidases), heat (ascorbic acid, cobalamin, folate, pantothenic acid, riboflavin, thiamine, and vitamin A), light (ascorbic acid, fat-soluble vitamins, riboflavin, and pyridoxine), and metal elements such as copper and iron (ascorbic acid and fat-soluble vitamins) (Klein, 1987; Reddy & Love, 1999). Microwaving can indirectly improve retention rates of heat labile vitamins as heating time can be shortened due to rapid temperature increase (Ball, 2005). Freezing has no effect on vitamin losses, while impact of irradiation can be different (*e.g.*, have no effect on vitamins K, D, and riboflavin, or result in decrease of ascorbic acid, thiamin, and vitamin A) (Ball, 2005).

To restore vitamins lost during processing or improve nutritional content, food can be enriched or fortified. In Canada, addition of folic acid to white flour, enriched pasta, and cornmeal has been mandatory since 1998 (Anonymous, 1998). Additionally, legal requirements include fortification of all forms of milk with vitamin D to prevent rickets in children and osteomalacia in adults, and addition of vitamin A to skim and partly skimmed milk to replenish its loss due to the decrease in fat content during the separation process. To ensure people who live in isolated communities that do not have access to fresh fruits and vegetable, whole, skimmed and partially skimmed evaporated milk are required to be fortified with vitamin C (CFIA, 2013).

Besides being in foods, vitamins can be introduced in a diet in a form of single- or multi-vitamin supplements. This is particularly important for certain categories of people that cannot reach their daily vitamin needs by consuming food. In Canada, women that could become pregnant, are pregnant, or breastfeeding are recommended to take a multivitamin containing 0.4 mg of folic acid per day to reduce risk to baby developing neural tube defects (Health Canada, 2009). Additionally, to prevent osteoporosis and bone fractures, elderly (over the age of 50) are advised to supplement their diet with 10 µg of vitamin D every day (Health Canada, 2011).

Absorption of orally ingested biotin, niacin, pantothenic acid, thiamin, riboflavin, vitamin B_6, and fat soluble vitamins occurs mainly in the small intestine (Smolin & Grosvenor, 1997). Ileum, the latter portion of small intestine, is a place of absorption for ascorbic acid (Smolin & Grosvenor, 1997) and cobalamin (Booth & Mollin, 1959). The capacity to absorb vitamins, taken in a single dose, declines as the intake increases (Bender, 2003). For example, absorption of a single oral dose of 1.5 g and 12 g ascorbic acid was

found to be 50% and 16% of the ingested dose respectively (Kübler & Gehler, 1970), while an average absorption capacity of the doses below 180 mg ranged from 71% (Kübler & Gehler, 1970) to 80-90% (Kallner et al., 1977).

Studies show that vitamins, including biotin, cobalamin, folate, menaquinone, pantothenate, and riboflavin can be enterically produced by gut flora (Guarner, 2006; Hill, 1997; Suttie, 1995). A close estimate of vitamin concentration in a gut can be obtained from the values measured in faeces collected from animals and humans (Table 1.4). The availability of enterically synthesized vitamins to the host may be limited by vitamin release from bacterial cells (Mitchell & Isbell, 1942), and competition for vitamin absorption by other microorganisms in a gut (Hill, 1997). For the purpose of this literature review, only vitamins used in this study will be described in the following sections.

1.9.1 Vitamin E

Vitamin E can be found in variety of oils (safflower, sunflower, and soybean), foods made of these oils (mayonnaise, salad dressings), some nuts (almonds), seeds, and vegetables (Thompson & Manore, 2005). From ingested foods, vitamin E is absorbed in the small intestine together with fatty acids, then transferred and stored in adipose tissue and cell membrane lipids. The most important role of vitamin E is as an antioxidant. It stabilizes free radicals in the lipid fraction of the cell membranes by providing them with electrons, thus preventing further oxidative damage to the cell. Once oxidized, vitamin E can be either excreted from the cell, or returned back to its active form by ascorbic acid (Thompson & Manore, 2005).

A biologically active form of vitamin E, tocopherol, can be found in foods and supplements in four different compounds: α, β, γ, and δ, with α-tocopherol being the most

active one. Vitamin E is unstable in the presence of oxygen and metal ions, and can be destroyed by the exposure to ultraviolet light or heat (Thompson & Manore, 2005).

1.9.2 Vitamin C

Vitamin C is consumed in a diet through different fruits, primarily citrus (orange, lemon, lime) and fresh vegetables (peppers, broccoli, spinach, cauliflower). In the human body, vitamin C functions mainly as an antioxidant. It prevents damage of cells and tissues by donating electrons to free radicals similarly to vitamin E, but in extracellular fluid rather than in the cell membrane (Thompson & Manore, 2005). It is also involved in regeneration of vitamin E and collagen synthesis. Side effects of vitamin C overconsumption rarely occur as the excess amounts are usually excreted in urine. However, if overdose through excess supplementation happens (*e.g.*, >2,000 mg per day), vitamin C may act as a "prooxidant", by shifting the balance of exchange reactions towards oxidation, which promotes production of free radicals. Ascorbic acid and dehydroascorbic acid are two biologically active forms of vitamin C. Heat is known to destroy vitamin C (Thompson & Manore, 2005).

1.9.3 Riboflavin

Milk, enriched bread, and grain products are good sources of riboflavin (Thompson & Manore, 2005). It serves in oxidation-reduction reactions of carbohydrate and fat metabolism, as a component of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) coenzymes, and as a part of antioxidant glutathione peroxidase (Thompson & Manore, 2005).

1.9.4 Pantothenic acid

Pantothenic acid is widely distributed in foods; thus deficiencies rarely occur. It plays a key role in energy production as coenzyme A (CoA), a part of the acetyl CoA complex

(Smolin & Grosvenor, 1997). Good sources of pantothenic acid include meat, egg yolk, potatoes, and whole grains (Thompson & Manore, 2005). As a supplement, it is available in a form of calcium pantothenate (Smolin & Grosvenor, 1997).

1.9.5 Biotin

Biotin functions as a coenzyme for several enzymes involved in carbohydrate, fat, and protein metabolism. Good food sources of biotin are egg yolks, liver, nuts, and yogurt (Thompson & Manore, 2005).

1.9.6 Folate

Folic acid is naturally found in leafy green vegetables such as spinach and Romaine lettuce, lentils, liver, and asparagus. A good source of folate in Canada is bread, pasta and cereals due to mandatory fortification. Folic acid is important for DNA synthesis and amino acid metabolism (Thompson & Manore, 2005).

1.9.7 Cobalamin

Cobalamin can be introduced in a diet through animal products, mainly beef and poultry (Smolin & Grosvenor, 1997). Cobalamin is a part of a coenzyme that is involved in the formation of blood. Additionally, it is required for normal functioning of the nervous system as it helps maintain the sheath that coats nerve fibres, thus allowing normal conduction of nervous signals (Thompson & Manore, 2005).

1.9.8 Vitamin K

Vitamin K is a fat-soluble vitamin, naturally found in leafy vegetables (spinach, broccoli, and cabbage) in a form of phylloquinone (vitamin K_1), or enterically produced by large intestine microorganisms as menaquinone (vitamin K_2) (Thompson & Manore, 2005).

1.9.9 Previous research on vitamin influence on *Escherichia coli*

The requirement of vitamins in microorganisms is associated with their ability to synthesize vitamins in the amounts that satisfy their needs. Burkholder and McVeigh (1942) demonstrated that *E. coli* is able to grow in a vitamin free medium at 36°C, and produce B vitamins, including biotin, riboflavin, thiamine, and nicotinic acid. Following 48 h incubation, concentration of biotin and riboflavin were 1 ng/mL and 0.05 ng/mL culture, respectively. Additionally, Miller (1944) reported that *E. coli* grown in bacto-peptone medium at 37°C for 20 h synthesized biotin (5 ng/mL) and folic acid (18 ng/mL). Similarly, Almquist et al. (1938) were the first to report that *E. coli* can synthesize an antihemorrhagic factor in a medium that contained no vitamin K. This factor was later characterized as vitamin K_2, and was found to be tightly bound to cytoplasmic membrane in *E. coli* (Bishop & King, 1962). Newton et al. (1971) showed *E. coli* K-12 required menaquinone for pyrimidine biosynthesis during growth in anaerobic conditions, but not in aerobic. Pantothenic acid is a precursor for biosynthesis of CoA (Brown, 1959b), an important cofactor in many metabolic pathways such as synthesis of fatty acids in *E. coli* (Magnuson et al., 1993). Brown (1959a) demonstrated that addition of calcium pantothenate (0.02 mg/mL) in growth medium resulted in 180-fold and 9-fold increase in the amount of pantothenic acid and CoA in *E. coli* respectively during 18-24 h incubation at 37°C. He also showed that CoA is the main pantothenic acid-containing compound present in *E. coli*. Additionally, Jackowski & Rock (1981) reported *E. coli* is able to synthesize pantothenate or absorb it, if supplied in the medium. They also found that *E. coli* regulates its intracellular pantothenate pool to <1µM by excreting the excess amounts outside the cell. Interestingly, *E. coli* does not synthesize *de novo* nor require vitamin B_{12} for growth (Raux et al., 1996). However, addition of cobalamin

in minimal medium accelerates *E. coli* growth (Davis & Mingioli, 1950). *E. coli* absorbs vitamin B_{12} rapidly and in large amounts from media (Davis & Mingioli, 1950; Sampson & Gotschlich, 1992). This effective mechanism of absorption consists of an initial, rapid phase (completed within 1 min), followed by a slower, energy-dependant phase that may be linear for 20-30 min, until a plateau is reached (Di Girolamo & Bradbeer, 1971). The absorbed cobalamin may serve as a cofactor in an enzymatic methylation of homocysteine to methionine (Davis & Mingioli, 1950) or for utilization of ethanolamine as a nitrogen source (Scarlett & Turner, 1976).

While certain vitamins are beneficial, others may have harmful effects for *E. coli.* Destructive effects of ascorbic acid in conjunction with H_2O_2 on bacteria have been reported by Ericsson & Lundbeck (1955). Additionally, Miller (1969) showed that a mixture of ascorbic acid and H_2O_2 resulted in a powerful antibacterial system against Gram-negative bacteria, probably by a mechanism dependent on a formation of free radicals. Specifically, around a 3-log reduction in the number of *Salmonella pullorum* cells was seen after treatment with ascorbic acid (0.02 mg/mL) containing H_2O_2 (1-10µmol/mL) in an acetate buffer (pH 5.0) at 37°C for 30 min. Presence of lysozyme (10µg/mL) and a pH shift to from 5.0 to 7.0 enhanced the bacterial death during the treatment, while addition of sodium thiosulfate (a free radical scavenger) abolished the effect. It has been suggested that free radicals generated in the system may disturb the integrity of the bacterial cell membrane, leading to the bacterial death and an increased susceptibility to lysozyme in the remaining cells (Miller, 1969). Destructive effects of ascorbic acid on *E. coli* O157:H7 have been documented (Burnham et al., 2001; Derrickson-Tharrington et al., 2005). In a study conducted by Burnham et al. (2001), numbers of stationary phase *E. coli* O157:H7 inoculated on apple

slices were reduced by 8.0 to 8.3 log CFU/g after immersion in ascorbic acid (34 mg/mL; w:v) at room temperature for 15 min and subsequent dehydration at 62.8°C for 6 h. Similarly, in a study carried out by Derrickson-Tharrington et al. (2005), numbers of stationary phase *E. coli* O157:H7 inoculated on apple slices decreased by 6.7 to 7.1 log CFU/g after immersion in ascorbic acid (2.8%, pH 2.6) at 25±2°C for 10 min and subsequent drying at 62.8°C for 6 h. Interestingly, when the above mentioned ascorbic acid treatment was replaced by a treatment with citric acid (1.7%, pH 2.2), commercial lemon juice with preservatives (50%, pH 2.5) or commercial lemon juice without preservatives (50%, pH 2.7), the numbers of surviving *E. coli* O157:H7 cells were similar and not significantly different among treatments. Influence of vitamin E treatment on surface characteristics of *E. coli* was examined by Uberos et al. (2001). Following 12 h treatment with 0.05 mg/mL (w/v) disodium α-tocopherol phosphate at 36±1°C, hydrophobicity of *E. coli* was significantly reduced, as measured by adhesion to hydrocarbon (*p*-xylene), while adherence to nitrocellulose filters increased.

1.10 Research objectives and hypotheses

The objectives of this research were to determine whether *Escherichia coli* O157:H7 attachment to leafy green produce, stress response gene expression, and virulence may be influenced by exposure to food-related vitamins (*i.e.* ascorbic acid and α-tocopherol), or enterically-produced vitamins (*i.e.* biotin, cobalamin, folate, MQ, pantothenate, and riboflavin). For this purpose *E. coli* O157:H7 str. EDL933 was used, since this strain had caused serious human health illnesses in the past and its genome has been previously sequenced (Perna et al., 2001). I hypothesize that exposure of *E. coli* O157:H7 to:

1. Enterically produced or food-related vitamins promotes adhesion to enterocytes;

2. Enterically produced vitamins (specifically, cobalamin and pantothenate) or ascorbic acid stimulates production of Stx1 and Stx2;
3. Enterically-produced vitamins (specifically, biotin, cobalamin, and pantothenate) results in enhanced transcription of fimbriae or LEE virulence genes;
4. Enterically-produced vitamins (specifically, cobalamin, and pantothenate) or ascorbic acid results in enhanced transcription of Shiga toxin virulence genes;
5. Enterically produced or food-related vitamins stimulates transcription of general stress response gene;
6. Enterically-produced vitamins (specifically, cobalamin and pantothenate) or ascorbic acid induces transcription of selected acid and oxidative stress response genes;
7. Enterically produced vitamins (specifically, biotin, cobalamin, pantothenate, and riboflavin), or food-related vitamins result in enhanced attachment to leafy green produce surface.

Table 1.1. Antioxidant activities in *Escherichia coli*. Adapted from Storz & Zheng (2000).

Gene	Regulation	Activity	Function	Additional regulator(s)
***oxyR* regulon**				
katG	+	Hydroperoxidase I	Eliminate H_2O_2	σ^S
ahpCF	+	Alkyl hydroperoxide reductase	Eliminate of H_2O_2	
gorA	+	Glutathione reductase	Maintain thiol-disulfide balance	σ^S
grxA	+	Glutaredoxin 1	Maintain thiol-disulfide balance	
trxC	+	Thioredoxin 2	Maintain thiol-disulfide balance	
fur	+	Ferric uptake repressor	Prevent OH· generation *via* Fenton reaction	SoxRS
dps	+	Nonspecific DNA binding protein	DNA protection	σ^S
oxyS	+	Regulatory RNA	Repress *rpoS* mRNA translation; protect against mutagenesis	
agn43	-	Outer membrane protein	Biofilm formation in *E. coli* grown in minimal medium	
fhuF	-	Ferric reductase	Iron reduction	
***soxRS* regulon**				
sodA	+	Manganese superoxide dismutase	Dismutate O_2^- to H_2O_2	
nfo	+	Endonuclease IV	DNA repair	
zwf	+	Glucose-6-phosphate dehydrogenase	Increase reducing power of the cell	
fumC	+	Fumarase C	O_2^- resistant isozyme of fumarase	σ^S
acnA	+	Aconitase A	O_2^- resistant isozyme of aconitase	σ^S
tolC	+	Outer membrane protein	Exclusion of O_2^- generating compounds	
fur	+	Ferric uptake repressor	Prevents OH· generation *via* Fenton reaction	OxyR
micF	+	RNA regulator of *ompF*	Exclusion of O_2^- generating compounds	
acrAB	+	Multidrug efflux pump	Exclusion of O_2^- generating compounds	
fnsA	+	Nitroreductase A	Reduce O_2^- generation from organic nitro compounds	
fpr	+	Ferredoxin/flavodoxin reductase	Maintain reduced state of Fe-S clusters	
fldA	+	Flavodoxin	Maintain reduced state of Fe-S clusters	
fldB	+	Flavodoxin	Maintain reduced state of Fe-S clusters	
ribA	+	GTP cyclohydrolase	Unclear	
Other defense activities				
katE	+	Hydroperoxidase II	Eliminate H_2O_2	σ^S
xthA	+	Exonuclease III	DNA repair	σ^S
sodC	+	Copper-zinc superoxide dismutase	Dismutate O_2^- to H_2O_2	σ^S

Table 1.2 The groups of vitamins, their vitamers, physiological functions, deficiency disease and syndromes. Adapted from Combs (2012) and Thompson & Manore (2005).

Vitamin group and vitamers	Physiological functions	Deficiency syndromes	Toxicity Symptoms/Side effects
Vitamin A			
Retinol Retinal Retinoic acid	Visual pigments, epithelial cell differentiation	Night blindness, impaired immunity	Spontaneous abortions and birth defects, loss of appetite, blurred vision, hair loss, nausea, diarrhea, liver and nervous system damage
Vitamin D			
Cholecalciferol (D_3) Ergocalciferol (D_2)	Calcium homeostasis, bone metabolism, regulates blood calcium levels, maintains bone health, cell differentiation	Rickets (in children) leading to bone weakness and deformities, osteomalacia leading to bone weakness and increased fractures; osteoporosis leading to increased rate of fractures	Hypercalcemia, weakness, loss of appetite, diarrhea, vomiting, extreme thirst, increased bone loss, formation of calcium deposits in kidney
Vitamin E			
α-tocopherol β- tocopherol γ-tocopherol δ- tocopherol	Cell membrane antioxidant	Red blood cell hemolysis, anemia, impairment of nerve transmission	Inhibition of blood clothing, increased risk of hemorrhage stroke, intestinal discomfort
Vitamin K			
Phylloquinones (K_1) Menaquinones (K_2) Menadione (K_3)	Blood clothing, calcium metabolism, coenzyme in the synthesis osteocalcin involved in maintaining bone density and in coagulation of blood (prothrombin, and procoagulants, factors VII, IX, and X)	Reduced the ability of blood to clot properly *i.e.* clotting can be delayed or not able to occur; effect on bone health is controversial	No toxicity/side effects known
Vitamin C			
Ascorbic acid Dehydroascorbic acid	Reductant in hydroxylations in the formation of collagen and carnitine, and in the metabolism in drugs and steroids, regenerates oxidized vitamin E; assists in the collagen synthesis	Scurvy, sore gums, painful joints, depression, weakness	Nausea and diarrhea, nosebleeds, abdominal cramps, increased oxidative damage

Table 1.2 Continued.

Group and vitamers	Physiological functions	Deficiency syndromes	Toxicity Symptoms/Side effects
Vitamin B_1			
Thiamin	Coenzyme for decarboxylations of 2-keto acids (*e.g.*, pyruvate) and transketolations; as a part of coenzyme thiamine pyrophosphate plays a critical role in glucose breakdown; coenzyme in the branched-chain amino acids (leucine, isoleucine, and valine) metabolism	Beriberi: muscle vesting and nerve damage	No toxicity/side effects known
Vitamin B_2			
Riboflavin	Coenzyme in redox reactions of fatty acids and the tricarboxylic acid cycle; coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide involved in carbohydrates and fats metabolism	Ariboflavinosis: sore throat, swelling of mouth and throat	No toxicity/side effects known
Vitamin B_3 (niacin)			
Nicotinic acid Nicotinamide	Coenzyme for several dehydrogenases; coenzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (NADP)	Pellagra, pigmented rash, vomiting, depression, apathy, headache and fatigue	Flushing, liver dysfunction and damage, glucose intolerance, blurred vision and edema of eyes
Vitamin B_5			
Pantothenic acid	Coenzyme in fatty acid metabolism; component of coenzyme A that assist with fatty acid metabolism	Rare, only seen in people fed diets with virtually no pantothenic acid	No toxicity/side effects known
Vitamin B_6			
Pyridoxol Pyridoxal Pyridoxamine	Coenzyme in amino acid metabolism; Part of coenzyme pyridoxal phosphate involved in amino acid metabolism, carbohydrate metabolism, and synthesis of red blood cells	Seborrheic dermatitis, mycrocytic anemia, convulsions, depression, and confusion	Sensory neuropathy, lesions of the skin
Vitamin B_7			
Biotin	Coenzyme for carboxylations; Component of coenzymes involved in fat, protein, and carbohydrate metabolism	Red, scaly skin rash, depression, hallucinations, burning, tingling, tickling	No toxicity/side effects known

Table 1.2 Continued.

Group and vitamers	Physiological functions	Deficiency syndromes	Toxicity Symptoms/Side effects
Vitamin B_9			
Folic acid Polyglutamyl folacins	Coenzyme in single-carbon metabolism; coenzyme tetrahydrofolate involved in DNA synthesis and amino acid metabolism	Macrocytic anemia, weakness and fatigue, difficulty concentrating, headache, shortness of breath, neural tube defects in the developing fetus	A masking of symptoms of cobalamin deficiency, neurological damage
Vitamin B_{12}			
Cobalamin	Coenzyme in the metabolism of propionate, amino acids, and single-carbon units; part of coenzymes that assist with formation of blood, nervous system function, and homocysteine metabolism	Pernicious anemia, pale skin, fatigue, shortness of breath, poor concentration, and disorientation	No toxicity/side effects known

Table 1.3 Vitamin content of certain foods. Data were obtained from Canadian nutrient file (Health Canada, 2012).

Food category and type	Vitamin												
	A[a] µg/g	D µg/g	E[b] mg/g	K µg/g	C mg/g	B_1 mg/g	B_2 µg/g	B_3 mg/g	B_5 mg/g	B_6 mg/g	B_7 µg/g	B_9[e] µg/g	B_{12} µg/g
Dairy													
Milk skim	0.6	0.01	<0.01	ND	ND	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01[c]	0.05	<0.01
Milk whole[f]	0.5	0.01	<0.01	<0.01	ND	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01[c]	0.05	<0.01
Eggs													
Egg whole	2	0.01	0.02	<0.01	ND	<0.01	<0.01	<0.01	0.02	<0.01	0.2[c]	0.7	0.02
Fruit													
Orange	<0.01	ND	<0.01	ND	0.5	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01[c]	0.3	ND
Grains/ Flour													
Wheat bleached	ND	ND	<0.01	<0.01	ND	<0.01	<0.01	0.06	<0.01	<0.01	-	0.3	ND
Whole wheat	0.05	ND	<0.01	0.02	ND	<0.01	<0.01	0.05	0.01	<0.01	-	0.4	ND
Leafy Greens													
Romaine lettuce	4	ND	<0.01	1	0.2	<0.01	<0.01	<0.01	<0.01	<0.01	0.02[d]	1	ND
Spinach	5	ND	0.02	5	0.3	<0.01	<0.01	0.01	<0.01	<0.01	0.03[d]	2	ND
Meat													
Ground beef[g]	ND	<0.01	-	<0.01	ND	<0.01	<0.01	0.05	<0.01	<0.01	-	0.08	0.02
Seeds/oil													
Sunflower seeds[h]	ND	ND	0.4	0.03	0.01	<0.01	<0.01	0.04	0.07	0.01	0.03[d]	2	ND
Vegetable oil[i]	ND	ND	0.4	0.05	ND	ND	ND	ND	ND	ND	-	ND	ND
Vegetables													
Broccoli	0.3	ND	<0.01	1	0.9	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01[c]	0.6	ND

[a]Retinol activity equivalents; [b]α-tocopherol; [c]Reported by Staggs et al. (2004); [d]Reported by Hoppner et al. (1994); [e]Naturally occurring; [f]Pasteurized homogenized 3.3% milk fat; [g]Lean; [h]Oil roasted; [i]Sunflower, vegetable oil, linoleic: 60% and over; "ND"- not detected *i.e.* 0.0000; "-" - no data; Note: Biotin content is not available in the Canadian Nutrient File.

Table 1.4 Summary of studies examining vitamin concentration in tissues or faeces of animals or humans.

Vitamin name and concentration or range	Sample type, origin and size	Assay*	Reference
α - Tocopherol			
Not detected (<0.08 mg/g)	Faeces of humans (n=14; wet)	HPLC	Nierenberg et al., 1987
Ascorbic acid			
0.08-0.2 mg/g	Rectum of humans (n=15)	HPLC	Waring et al., 1996
0.05 mg/g	Colon (cecum) of mice (n=24)	MS	Finlay Brett, personal communication
0.03 mg/g	Faeces of humans (n=1)	-	Van Eekelen, 1936
Biotin			
0.00006-0.00009 mg/g	Ileum of humans (n=3)	Microbiological	Taylor et al., 1942
0.00008-0.0001 mg/g	Colon mucosa of humans (n=3)	Microbiological	Taylor et al., 1942
0.0003-0.0009 mg/g	Faeces of rats (n=8)	Microbiological	Mitchell & Isbell, 1942
0.001 mg/g	Faeces of rats (n=6; hard)	Microbiological	Sukemori et al., 2003
Cobalamin			
0.00002 mg/g**	Faeces of humans (n=18; wet)	LC-MS	Allen & Stabler, 2008
0.0001 mg/g	Faeces of humans (n=2, dried)	Microbiological	Girwood, 1950
0.001 mg/g	Faeces of rabbits (n=6, hard, dried)	Microbiological	Kulwich et al., 1953
0.003 mg/g	Faeces of rats (n=6; hard)	Microbiological	Sukemori et al., 2003
Folic acid			
0.001-0.002 mg/g***	Ileum of humans (n=3)	Microbiological	Taylor et al., 1942
0.002-0.003 mg/g***	Colon mucosa of humans (n=3)	Microbiological	Taylor et al., 1942
0.004-0.008 mg/g***	Faeces of rats (n=8)	Microbiological	Mitchell & Isbell, 1942
0.02 mg/g	Faeces of rats (n=6; hard)	Microbiological	Sukemori et al., 2003
Menaquinone			
0.0004-0.009 mg/g	Faeces of humans (estimated)	-	Hill, 1997
Pantothenic acid			
0.004-0.006 mg/g	Ileum of humans (n=3)	Microbiological	Taylor et al., 1942
0.004-0.01 mg/g	Colon mucosa of humans (n=3)	Microbiological	Taylor et al., 1942
0.01 mg/g	Faeces of rabbits (n=6, hard, dried)	Microbiological	Kulwich et al., 1953
0.02-0.05 mg/g	Faeces of rats (n=8)	Microbiological	Mitchell & Isbell, 1942
0.01 mg/g	Faeces of rats (n=6)	Microbiological	Brækkan, 1955
Riboflavin			
0.003-0.005 mg/g	Ileum of humans (n=3)	Microbiological	Taylor et al., 1942
0.001-0.002 mg/g	Colon mucosa of humans (n=3)	Microbiological	Taylor et al., 1942
0.01 mg/g	Faeces of rabbits (n=6, hard, dried)	Microbiological	Kulwich et al., 1953
0.01-0.02 mg/g	Faeces of rats (n=8)	Microbiological	Mitchell & Isbell, 1942

"-" data not available; * Using high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), mass spectrometry (MS), or microbiological assays.** Cobalamin only; concentration of cobalamin and cobalamin analogues was 0.001 mg/g; *** Milligrams of material of "potency" 40,000.

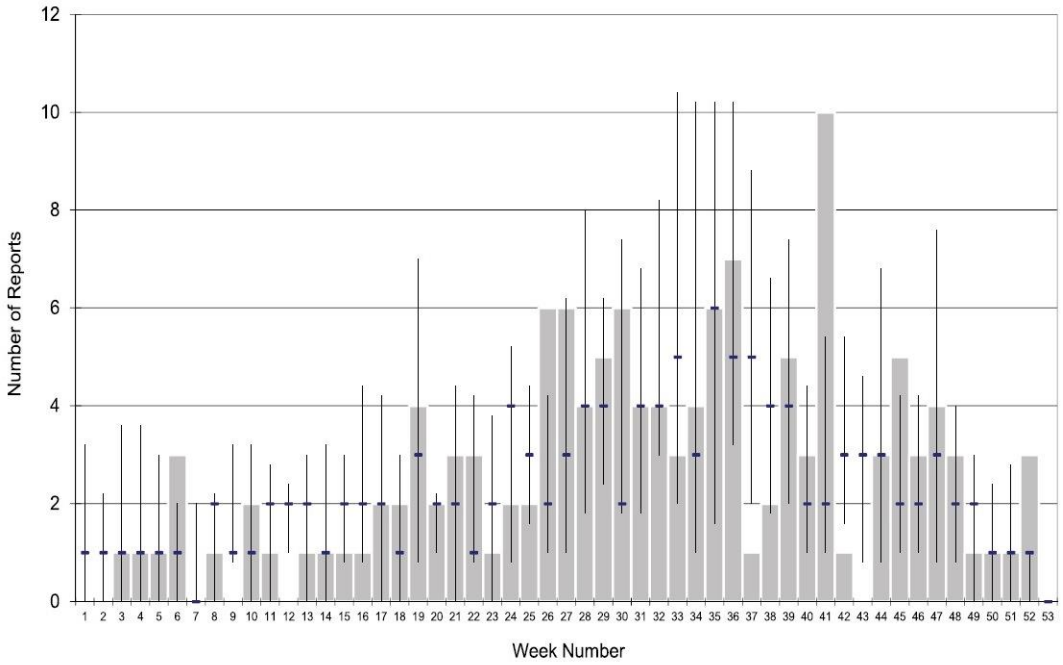

Figure 1.1 Seasonality of Shiga toxin-producing *Escherichia coli* (STEC) in British Columbia. In bars are presented numbers of STEC cases reported in weekly intervals in 2012. Historic median is indicated with horizontal lines; vertical lines represent 10^{th} and 90^{th} percentiles around the median (2003 to 2011). Adapted from British Columbia Centre for Disease Control (BCCDC, 2013) British Columbia Annual Summary of Reportable Diseases 2012, from: http://www.bccdc.ca/util/about/annreport/default.htm.

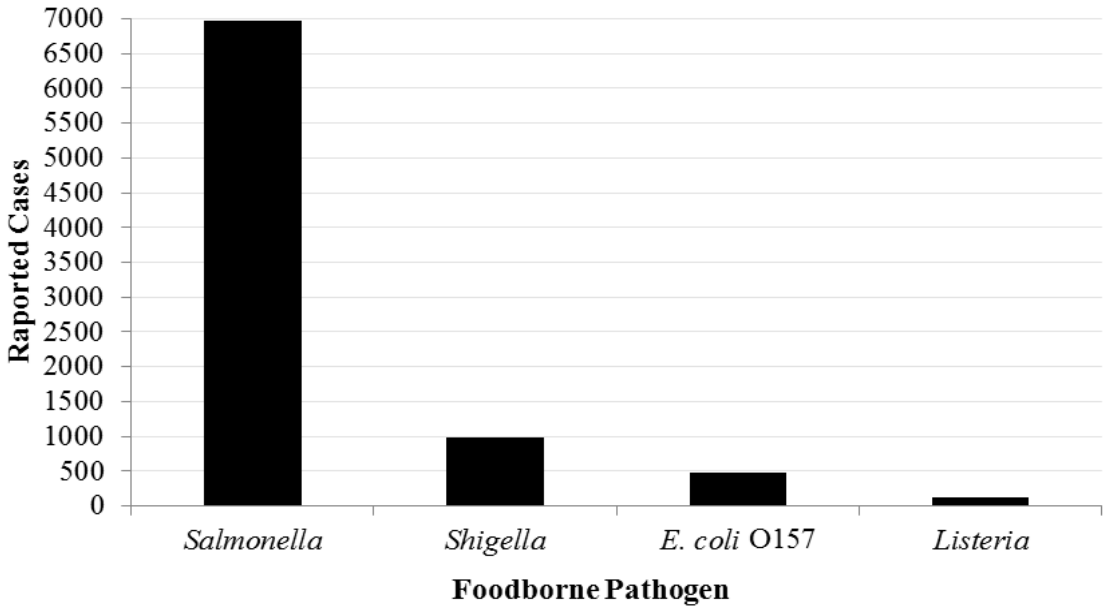

Figure 1.2 The number of cases for the most frequently reported foodborne pathogens in Canada in 2012. Data were collected through National Enteric Surveillance Program (NESP). Adapted from Public Health Agency of Canada (PHAC, 2014), from: http://www.phac-aspc.gc.ca/fs-sa/fs-fi/ecoli-eng.php.

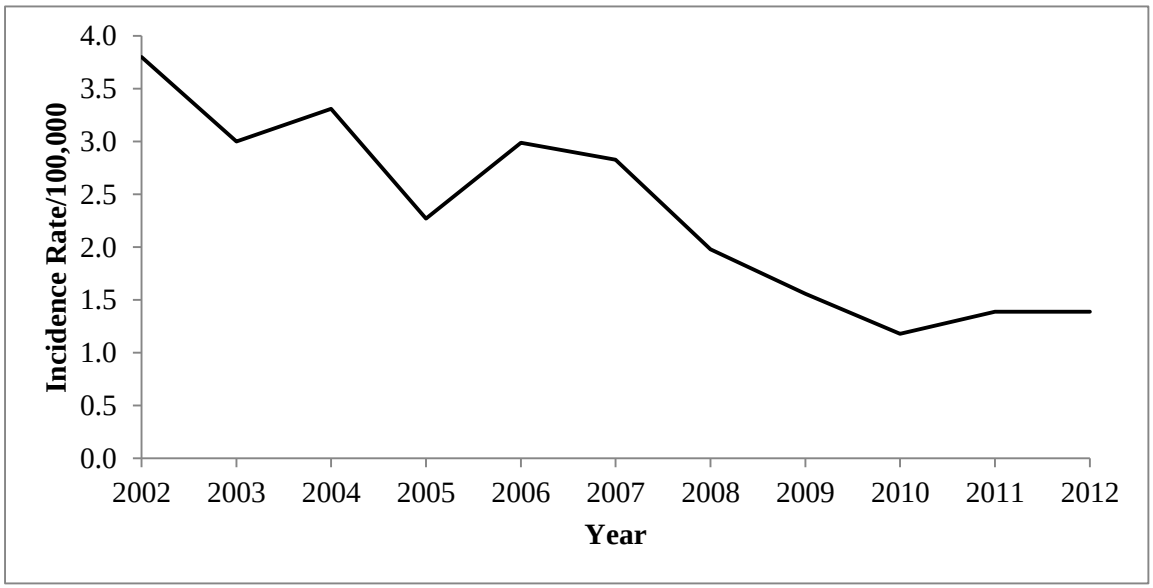

Figure 1.3 The rate of *E. coli* O157:H7 infection per 100,000 people in Canada from 2002 to 2012. Data were collected through National Enteric Surveillance Program. Adapted from Public Health Agency of Canada (PHAC, 2014), from: http://www.phac-aspc.gc.ca/fs-sa/fs-fi/ecoli-eng.php.

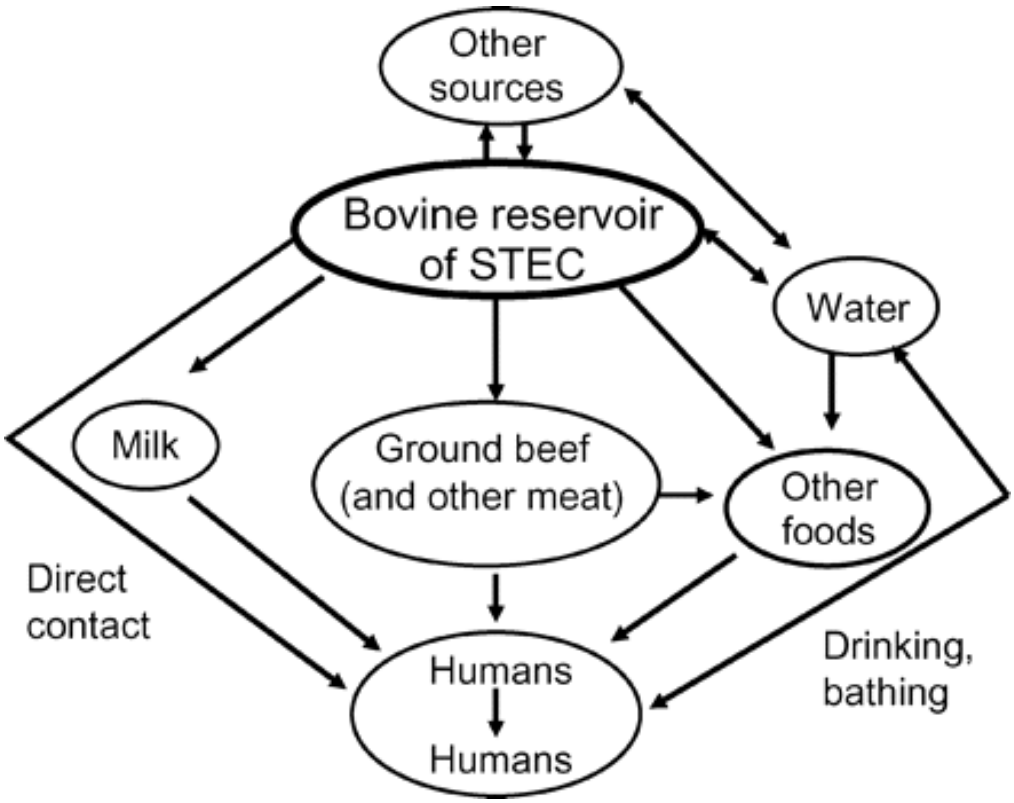

Figure 1.4 Cattle are the main source of Shiga toxin-producing *Escherichia coli* (STEC), including *E. coli* O157:H7. Reprinted from Gyles (2007), with the permission from the Journal of Animal Science.

Chapter 2: Influence of vitamin exposure on *Escherichia coli* O157:H7 virulence using tissue culture, Shiga toxin enzyme linked immunosorbent assay, and quantitative real-time polymerase chain reaction

2.1 Introduction

Escherichia coli O157:H7 is a foodborne pathogen capable of causing diarrhea, hemorrhagic colitis and hemolytic uremic syndrome in humans (Karmali et al., 2010). Following human host infection, *E. coli* O157:H7 intimately adheres to enterocytes of the colon and creates a typical attachment and effacement (A/E) lesion (Nataro & Kaper, 1998). Additionally, *E. coli* O157:H7 may produce Shiga toxins (Stxs) Stx1 and/or Stx2 (Hayashi et al., 2001; Mauro & Koudelka, 2011; Nataro & Kaper, 1998), which can cause an inhibition of protein synthesis and eventual apoptosis of the host cell (O'Brien et al., 1992; Schmitt et al., 1999; Schüller, 2011). Pathogenesis, specifically expression of locus of enterocyte effacement (LEE) genes, is coordinated by unique environmental signals that *E. coli* O157:H7 recognizes in the colon, such as bicarbonate levels, hormones or quorum sensing molecules (Abe et al., 2002; Sperandio et al., 2003). In the enteric environment, commensal bacteria produce a variety of vitamins, including biotin, folic acid, cobalamin, pantothenate, riboflavin, and menaquinone (MQ) (Guarner, 2006; Hill, 1997). Also, vitamins can become constituents of the enteric environment through our diet by consumption of foods or vitamin supplements. It is possible that enterically produced or food-related vitamins serve as environmental triggers for the expression of virulence genes in *E. coli* O157:H7. However, no data regarding vitamin impact on *E. coli* O157:H7 virulence exist.

The goal of this research was to determine whether food-related or enterically produced vitamins influence virulence of *E. coli* O157:H7 by using tissue culture assays to assess

adherence and invasion into HeLa cells, and a Shiga toxin enzyme-linked immunosorbent assay to evaluate levels of Shiga toxins produced. For experiments in which differences between control and vitamin-exposed cultures were observed, quantitative real-time polymerase chain reaction (qPCR) was used to examine the expression of relevant genes. When combined, these approaches provide a novel insight into whether vitamins are triggers for enhanced virulence of *E. coli* O157:H7.

2.2 Materials and methods

2.2.1 Bacterial strain, culture and growth conditions

Escherichia coli O157:H7 str. EDL 933 was maintained in 1 mL Luria Bertani (LB) broth [Becton, Dickinson, and Company (BD), Sparks, Maryland, USA] containing 20% (v/v) glycerol (Fisher Scientific, Fair Lawn, New Jersey, USA) at -80°C. Frozen cells were streaked on LB agar (Fisher Scientific) and incubated at 37°C for 24 h. A colony was picked and inoculated in 3 mL M9 minimal medium (M9MM) (BD) supplemented with 0.4% (w/v) D-glucose, 100 µM calcium chloride, and 2 mM magnesium sulfate (Fisher Scientific). Cells were grown with shaking (170 rpm) at 37°C. After 16 h, 1 mL culture was transferred in 100 mL M9MM and grown until late exponential phase was reached [optical density at 600 nm (OD_{600}) of 0.50 ± 0.03 (Spectronic 20, Bausch & Lomb); c.a. 2.4 x 10^8 CFU/mL] at which time treatments were applied.

2.2.2 Vitamins

Vitamins, including α-tocopherol, ascorbic acid, calcium pantothenate, cobalamin, folic acid, menaquinone, and riboflavin (Sigma-Aldrich, St. Louis, MO, USA) were stored according to manufacturer's instructions. To prepare stock solutions, water soluble vitamins (ascorbic acid, biotin, calcium pantothenate, cobalamin, folic acid, and riboflavin) were dissolved at maximum

concentrations in distilled water (Table 2.1), filter sterilized with a 0.2 µm filter (Sarstedt Inc., Montreal, QC), and stored in 1.5 mL sterile polypropylene tubes (Eppendorf, Mississauga, ON); fat soluble vitamins (α-tocopherol and menaquinone) were dissolved at maximum concentration in 80% ethanol (Table 2.1) in sterile glass tubes with screw cups to avoid adsorption to plastic surfaces (Anonymous, 2009a), kept overnight at 4°C, and placed at -20°C for long-term storage. Vitamin stock solutions were serially diluted using sterile distilled water (water soluble vitamins) or 80% ethanol (fat soluble vitamins) according to Table 2.1. All stock solutions were wrapped with aluminum foil and stored in dark at -20°C for up to 6 months, with the exception of ascorbic acid solutions that were freshly prepared prior to experiments. Prior to experiments, all solutions were defrosted, mixed by vortexing, and kept on ice. Vitamin and control treatments were added to *E. coli* O157:H7 late exponential phase cells in a 1:10 ratio (*e.g.*, 10 µL vitamins were added to 90 µL cells).

2.2.3 Vitamin treatments

Escherichia coli O157:H7 late exponential phase cells (90 µL; OD_{600} of 0.50 ± 0.03) were exposed to 10 µl vitamin or control (Table 2.1) in 96-well plates covered with a low evaporation lid (Corning Inc.). Cells were incubated statically at 37°C in 5% CO_2 for 1.5 and 3 h. At each time point, adherence and invasion assays were performed. To determine if vitamin exposure influenced cell numbers, vitamin (maximum concentrations) and control treated cells were serially diluted in 1X phosphate-buffered saline (PBS, Fisher Scientific) at 1.5 and 3 h exposure, spread plated on LB agar, incubated aerobically at 37°C for 24 h, and colony forming units assessed. Experiments were run in three biological and three technical replicates.

For the Shiga toxin quantification assay, *Escherichia coli* O157:H7 cells (4.5 mL; OD of 0.50 ± 0.03) were treated with 500 µl of ascorbic acid (100 mg/mL), cobalamin (10 mg/mL),

calcium pantothenate (50 and 500 mg/mL) or control in 15 mL sterile tubes with loosely screwed caps (Corning Inc.). Cells were incubated statically at 37°C for 3 h at which time Shiga toxin quantification using enzyme-linked immunosorbent assay was performed and number of cells enumerated by serially diluting cultures in 1 X PBS (Fischer Scientific) and plating on LB agar. Experiment was run in two biological and two technical replicates. An untreated mutant of *E. coli* O157:H7 EDL 933 lacking stx_1 and stx_2 ($\Delta stx_1 \Delta stx_2$), kindly provided by Dr. Brett B. Finlay, was used as a negative control.

2.2.4 Growth promoting and bactericidal effects of vitamin treatments in *E. coli* O157:H7 during 24 h

Escherichia coli O157:H7 was grown as described in section 2.2.1. After 18 h, 1 mL culture was added to 100 mL M9MM, then 180 µL cells were transferred per well in a 96-well plate and treated with 20 µL ascorbic acid (10 and 100 mg/mL), biotin (1 mg/mL), riboflavin (0.1 mg/mL), folic acid (1mg/mL), or control (double distilled water) (Table 1.2). The plate was covered with a clear Microseal® B adhesive sealer (Bio-Rad, Mississauga, ON), incubated statically at 37°C for 24 h, and absorbance at 600 nm monitored (after shaking) in an Infinite® M200 PRO (Tacan Group Ltd.) spectrophotometer.

In a separate experiment, 180 µL *E. coli* O157:H7 prepared as above were treated with 20 µL of calcium pantothenate (50 and 500 mg/mL), cobalamin (10 mg/mL), α-tocopherol (10 mg/mL), menaquinone (0.5 mg/mL), or respective controls (Table 1.2) in a 96-well plate covered with a low evaporation lid, statically at 37°C. To retain humidity over 24 h, 96-well plates were placed in a Ziploc bag. Following 0, 2, 4, 6, 8, 12, and 24 h treatments, cells were serially diluted in 1 X PBS, plated on LB agar, plates incubated at 37°C for 24 h, and colony

forming units assessed. Three biological and three technical replicates were run for each treatment and control.

2.2.5 HeLa cells and culture conditions

HeLa cells were kindly provided by Dr. Brett B. Finlay (University of British Columbia, Vancouver, BC). Monolayers were grown in cell culture dishes (Corning Inc., Corning, NY, USA) containing minimum essential medium (MEM) with L-glutamine and Earle's salt (Gibco, Grand Island, New York, USA) supplemented with 1% (v/v) MEM GlutaMAX^{TM}-I (Gibco), 1% (v/v) MEM non-essential amino acids (100X) (Gibco), 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 100 IU/mL penicillin, and 100 µg/mL streptomycin (PenStrep, Gibco). Cultures were incubated in 5% CO_2 at 37°C and grown to 70-80% confluence. Cells were passaged a maximum of 20 times after thawing.

2.2.6 Adherence and invasion assays

Adherence and invasion assays for vitamin and control treated *E. coli* O157:H7 were performed using HeLa cells as described by Abe et al. (1997), with slight modification. Briefly, 1 mL of 10^5 HeLa cells/mL were laid down in 24-well culture plate (Corning Inc.) and grown for 24 h (5% CO_2, 37°C). HeLa cells were inoculated with 1.5 or 3 h vitamin treated *E. coli* O157:H7 using a 100:1 ratio followed by incubation in 5% CO_2 at 37°C for 6 h. Tissue culture medium was replaced with 500 µL of fresh antibiotic free medium prior to and 3 h after bacterial inoculation. After 6 h infection, HeLa cells were washed 10 times with 1X Dulbecco's phosphate-buffered saline (DPBS) with magnesium and calcium (HyClone, Thermo Scientific, Toronto, ON), treated with 1% Triton X-100 for 10 min, diluted in DPBS, and plated on LB agar. To assess invasion, the experiment was repeated as described above with some changes. After 6 h infection, HeLa cells were washed three times with DPBS, treated with

10 µg/mL gentamicin (Sigma-Aldrich) in MEM for 1.5 h to eliminate adhered bacteria, washed 10 times with DPBS with magnesium and calcium, treated with 1% Triton X-100, diluted, and plated on LB agar. Experiments were performed in three biological and three technical replicates. As expected, exposure to vitamins had no effect on invasion (data not presented).

2.2.7 Vitamin mediated cytotoxicity in HeLa cells

Vitamin and control treatments (Table 2.1.) were diluted in sterile double distilled water in a 1:10 ratio (*e.g.*, 20 µL vitamins to 180 µL water) and added to HeLa cells as described in section 2.2.7. Following 6 h incubation, lactate dehydrogenase (LDH) presence in supernatant was evaluated using a CytoTox96® non-radioactive cytotoxicity assay (Promega Corporation, Madison, WI) according to manufacturer's instructions. Briefly, 150 µL of vitamin or control treated HeLa cells were transferred to 96-well plate and centrifuged at 1,800 rpm for 10 min. Supernatant (50 µL) was transferred to new 96-well flat-bottom plate, 50 µL/well of substrate mix added, and the plate incubated at room temperature, in dark, for 30 min. Following the incubation, 50 µL/well of stop solution was added and absorbance (A) measured at 490 nm in an Infinite® M200 PRO (Tacan Group Ltd., Männedorf, Switzerland) 96-well plate reader. Percent of cytotoxicity was determined by normalizing absorbance values of vitamin treated and control cells to a 100% cytotoxicity (total cell lysis) and untreated control as follows:

$$\text{Cytotoxicity (\%)} = \frac{(A_{treated\ cells} - A_{media}) - (A_{untreated\ cells} - A_{media})}{(A_{total\ cell\ lysis} - A_{media\ with\ lysis\ buffer})} \times 100$$

Total cell lysis and media with lysis buffer were obtained by the addition of lysate buffer to the wells containing HeLa cells or media only, one hour before supernatant collection. The rest of the assay was performed as described above. The assays were run in triplicate.

2.2.8 Calcium chloride

To determine whether adherence of calcium pantothenate treated *E. coli* O157:H7 to HeLa cells was influenced by pantothenic acid or calcium, equimolar calcium solution was prepared from calcium chloride, and run as control in parallel with double distilled water. Calcium chloride dihydrate (Fisher Scientific) was dissolved in sterile double distilled water, filter sterilized with a 0.2 µm filter (Sarstedt Inc.), and stored in 15 mL sterile tubes at room temperature until use (Corning Inc.). To verify that amounts of calcium in calcium chloride and calcium pantothenate solutions were equimolar, ionic calcium was directly measured in calcium pantothenate (5 mg/mL) and corresponding calcium chloride solution using a plastic Orion 9320 membrane half-cell electrode (Thermo Scientific, Waltham, MA) with a single junction Orion 9001 reference electrode (Thermo Scientific). Calibration of the calcium electrode was prepared by creating a standard curve from serial dilutions of 0.1M (4008 ppm) calcium standard (0.1M $CaCl_2$; Thermo Scientific) (Figure 2.1). Measured amounts of Ca^{2+} in calcium pantothenate (5 mg/mL) and corresponding $CaCl_2$ solutions were 7.5 mM (301 ppm) and 8.0 mM (319 ppm) Ca^{2+} respectively, with standard deviation <1% in three independent measurements.

2.2.9 Modified M9MM

Upon addition of calcium pantothenate to *E. coli* O157:H7 (OD_{600} of 0.50 ± 0.03) to achieve final concentration of approximately >5 mg/mL calcium pantothenate, a precipitate formed due to reaction of calcium with phosphate buffer system present in M9MM. To avoid this, modified M9MM was prepared using 0.1 M HEPES (pH 7.4) instead of phosphate buffer as previously described (Deng et al., 2005; Li et al., 2000). Also, modified M9MM was not supplemented with calcium, to ensure calcium amounts in the medium were minimal in

experiments comparing calcium and calcium pantothenate influence on *E. coli* O157:H7 adherence to HeLa cells. Compositions of original and modified M9MM are presented in Table 2.2.

2.2.10 Comparison of calcium pantothenate and calcium chloride influence on *E. coli* O157:H7 adherence to HeLa cells

To be consistent with previous experiments, *E. coli* O157:H7 was grown in original M9MM (section 2.2.1). After 16 h, 1 mL culture was transferred to 100 mL modified M9MM or to 100 mL original M9MM, and grown until late log phase (OD_{600} of 0.50 ± 0.03), at which point cells were treated with calcium pantothenate (50 mg/mL), calcium chloride (with equimolar calcium), or control (double distilled water) as in section 2.2.2. Following 1.5 and 3 h treatment, adherence to HeLa cells was assessed (as in section 2.2.6.). To assess bacterial growth at 1.5 and 3 h, cells were serially diluted in 1 X PBS, plated on LB agar, plates incubated at 37°C for 24 h, and colony forming units counted. The experiment was repeated in three biological and three technical replicates. Prior to logarithmic transformation, numbers of adhered *E. coli* O157:H7 were normalized based on bacterial plate counts at the moment of infection. Briefly, number of adhered vitamin treated *E. coli* O157:H7 (CFU/mL) was divided by a ratio of average number of vitamin and control treated *E. coli* O157:H7 (CFU/mL) at the moment of infection.

2.2.11 pH

Measurement of pH for *E. coli* O157:H7 (OD_{600} of 0.50 ± 0.03) grown in original or modified M9MM, after addition (1:10 ratio) of ascorbic acid (1, 10, and 100 mg/mL), calcium pantothenate (50 and 500 mg/mL), calcium chloride (equimolar calcium to 500 mg/mL calcium pantothenate), or control were carried out using an Accumet® Basic AB15 pH Meter (Fisher Scientific). The pH measurements were run in triplicate for treatments with a potentially acidic

character, such as ascorbic acid and calcium pantothenate, and/or for the treatments with a final vitamin concentration of ≥10 mg/mL. Adjustment of pH for folic acid stock solutions (Table 2.1) to 7.1 was carried out with potassium hydroxide and hydrochloric acid (Fisher Scientific) before the treatments.

2.2.12 Shiga toxin quantification using enzyme-linked immunosorbent assay

To determine whether vitamin treatments impacted Shiga toxin (Stx) levels, 1.5 mL of vitamin and control treated *E. coli* O157:H7 EDL 933 cells (section 2.2.3) were centrifuged at 10,000 x *g* at room temperature for 5 min. The supernatant was recovered and used to determine levels of total secreted Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). To assess levels of cell associated Stx1 and Stx2, collected pellets were topped up to 1.5 mL with 1X PBS containing 0.5 mg/mL polymyxin B sulphate (Fisher Scientific) to disrupt membrane integrity, vortexed for 30 sec, incubated at 37°C for 60 min, and vortexed again for 30 sec (Zhang et al., 2010). Lysed cells were centrifuged at 16,000 x *g* at 4°C for 5 min and supernatant collected. Cell-associated and secreted Stx samples were frozen at -80°C for subsequent capture enzyme-linked immunosorbent assays (ELISA).

The concentrations of cell associated and secreted Stx1 and Stx2 present in culture supernatants were determined by a Stx-ELISA as described previously (Atalla et al., 2000; Chen et al., 1998; Ziebell et al., 2008) at the Laboratory for Foodborne Zoonoses (LFZ), Guelph, Ontario, Canada. Briefly, flat-bottom wells of eight-well immunostrips (Nunc Maxisorb Immuno-Module; Thermo Scientific) in 96-well frames were coated with 100 µl of 2 µg/ml rabbit anti-Stx antibodies (LFZ) in carbonate-bicarbonate buffer (pH 9.6) and then post-coated by the addition of 200 µl/well of a stabilizing/blocking solution (LFZ). The purified Stx standards and test and control samples (100 µl/well) were incubated in duplicate at 22°C for 30

min, and then washed five times with 300 µl of 0.01 M PBS (pH 7.4) containing 0.1% Tween 20. Bound Stx1 and Stx2 were detected by serial incubations at 22°C with 100 µl/well of monoclonal antibodies directed against either Stx1 or Stx2/2c (2 µg/ml; LFZ) for 30 min, followed by 100 µl/well of horseradish peroxidase-labeled anti-mouse IgG (Jackson ImmunoResearch, PA) at 0.6 µg/ml for 30 min. Wells were washed after each of these incubations as mentioned above. Then, 100 µl/well of 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, Oakville, Ontario, Canada) was added to induce color development. After 10 min, the reaction was stopped with 100 µl/well of 0.2 M sulfuric acid. Absorbance was measured with a microplate spectrophotometer (ELx 808 Ultra microplate reader; Bio-Tek Instruments) at dual wavelengths of 450 and 630 nm. Concentrations of cell-associated and excreted Stx1 and Stx2 in tested samples were calculated from their mean ODs of two experimental runs, using linear equations of the respective standard curves generated from Stx1 and Stx2 standard solutions (Figure 2.2). Shiga toxin 1, purified from *E. coli* strain JB28 (Huang et al., 1986) as previously described by Lingwood et al. (1987), was kindly provided by Clifford Lingwood, Hospital for Sick Children, while Stx2 was prepared and purified at LFZ from *E. coli* strain R82pJES120DH5α as previously described by Tesh et al. (1994).

2.2.13 Evaluation of virulence gene expression in vitamin treated *E. coli* O157:H7 using qPCR

Adherence to HeLa cells and Stx ELISA results were validated using quantitative real-time polymerase chain reaction (qPCR) on a CFX96 Touch™ real-time PCR thermal cycler (Bio-Rad, Mississauga, ON). A two-step strategy was performed where mRNA was isolated and subsequently converted to complementary DNA (cDNA), which was then used as a qPCR template. For validation of adherence assay, 5.4 mL of late log phase *E. coli* O157:H7 cells

(three biological replicates) grown in M9MM (as described in section 2.2.1) were exposed to 600 µl biotin (1 mg/mL), cobalamin (10 mg/mL), calcium pantothenate (50 mg/mL), riboflavin (0.1 mg/mL) or control (double distilled water) treatment in pre-warmed glass tubes, statically in 5% CO_2 at 37°C. For validation of Stx ELISA assay, 4.5 mL of late log phase *E. coli* O157:H7 cells (three biological replicates) grown in M9MM (as described in section 2.2.1) were exposed to 500 µl ascorbic acid (100 mg/mL), cobalamin (10 mg/mL), calcium pantothenate (50 mg/mL), or control (double distilled water) treatment in 15 mL plastic tubes (Corning Inc.), aerobically and statically at 37°C.

After 3 h, the number of bacteria was assessed by serial dilution and plating on LB agar. In parallel, the culture was treated with RNAprotect Bacteria Reagent (Qiagen, Mississauga, ON) in 1:2 ratio, incubated at room temperature (15-25°C) for 15 min, and pelleted by centrifugation (5,000 x *g*; Eppendorf 5415 R) at room temperature for 10 min. RNA was extracted using RNeasy Mini Kit (Qiagen) with on-column DNase digestion (RNase-free DNase set, Qiagen) according to manufacturer's instructions. RNA purity was assessed spectrophotometrically (NanoDrop® ND-1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA) using A_{260}:A_{280} and A_{260}:A_{230} ratios of >1.8 but < 2.2 as indicators of acceptable quality (Wan et al., 2013). Gel electrophoresis of total RNA on a 1% agarose gel (70 V for 1 h) was used to confirm RNA integrity as defined by the presence of clear, non-degraded 16S and 23S rRNA bands and an absence of genomic DNA band (Wieczorek et al., 2012) visualized on a Chemi Doc™ MP Imaging System (Bio-Rad). cDNA was synthesized, from high quality total RNA, using a QuantiTect® Reverse Transcription Kit (Qiagen) according to manufacturer's instructions. Briefly, 2 µg of total RNA was first treated with gDNA wipeout buffer at 42°C for 2 min to eliminate any residual genomic DNA. Subsequently, Quantiscript Reverse Transcriptase,

Quantiscript RT Buffer and RT Primer Mix were added (total reaction volume of 40 µL), and cDNA was synthesized at 42°C for 30 min. Following cDNA synthesis, reverse transcriptase was heat inactivated at 95°C for 3 min.

Oligonucleotides (Tables 2.4. and 2.5) used in qPCR were based on *E. coli* O157:H7 str. Sakai sequence and previously published by Allen (2007), or on *E. coli* O157:H7 str. EDL 933 sequence (AE005174.2, NCBI gene bank). Regarding the latter, oligonucleotides were designed using PrimerQuest® (http://www.idtdna.com/Scitools) (IDT Coralville, IA, USA). All oligonucleotides were assessed for efficiency from cDNA reactions using five-fold dilution series in nuclease-free water (Qiagen) over a minimum of five points. Oligonucleotide pairs were determined to be acceptable if they had a quantification (or threshold) cycle number (C_t) >15 and <38, efficiencies >85 but <110%, a single amplicon as revealed by melting curve analysis, yielded a single band on a 2.5% agarose gel, and had a standard curve coefficient of determination (R^2)≥0.985 (Taylor et al., 2010), as determined by CFX Manager™ Software, Version 3.0 (Bio-Rad).

Real-time polymerase chain reaction was performed to quantify mRNA transcript levels for a selected gene in vitamin treated *E. coli* O157:H7, relative to the expression of the gene in the control treated cells. Reactions were carried out in a final volume of 20 µL, containing 1 µL cDNA template, 10 µL SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), and 0.25 µM forward and reverse oligonucleotides. Reaction mixtures were first denatured at 95°C for 3 min, and subsequently cycled 39 times as follows: 95°C for 10 sec, annealing at 58°C for 3 sec, and extension at 72°C, 12 sec. Melting curves were generated by increasing temperature from 65°C to 95°C in 0.5°C increments each for 5 sec. Relative changes in the expression levels for genes of interest were normalized against two housekeeping genes (*rpoA*, encoding α-subunit of RNA

polymerase, and *tufA*, encoding elongation factor Tu; with the acceptable gene stability value (M) <0.5 and coefficient of variation (CV) <0.25) (Hellemans et al., 2007), and were analyzed by the normalized expression ($\Delta\Delta C_t$) mode in CFX Manager™ Software, Version 3.0 (Bio-Rad). Experiments were run in three biological and two technical replicates. Ct standard deviation (SD) for all genes and treatments was ≤0.3. Run-to-run variation within a series of qPCR measurements for a given gene was avoided by using sample maximization strategy (Hellemans et al., 2007), which requires all samples (or as many as possible) to be analysed for a given gene in the same run (Figure 2.3). In this strategy, no inter-run calibration was required and there was no need to repeat the measurement of the reference gene in each run (Hellemans et al., 2007).

2.2.14 Statistical analysis

Number of *E. coli* O157:H7 following vitamin and control treatment and adhering to HeLa cells were analyzed with repeated measures one-way Analysis of Variance (ANOVA) with index of biological replicates being blocking factor. To determine which vitamin treatments were significantly (p<0.05) different than control, Holm-Sidak's multiple-comparison test was used. Prior to analysis, data were transformed, where applicable, to logarithmic values. Number of *E. coli* O157:H7 (Log_{10} CFU/mL) following folic acid (0.1 mg/mL) and control treatment were analyzed using a paired *t*-Test (p<0.05).

For the experiment comparing calcium pantothenate, calcium chloride and control treatment on the number of *E. coli* O157:H7 and adhering to HeLa cells, data were analyzed using repeated measures one-way ANOVA with Tukey's multiple-comparison test. *P* values of <0.05 were considered significant.

To analyze Shiga toxin production data, repeated measures one-way ANOVA with Holm-Sidak's test (p<0.05) was used as described above. Prior to analysis, Stx data were

normalized by dividing cell associated or secreted Stx1 and Stx2 (ng/mL) with mean numbers of *E. coli* O157:H7 EDL 933 cells (CFU/mL) for the respective treatments. Results were expressed as pg of Stx1 or Stx2 per 10^6 CFU of *E. coli* O157:H7.

Statistical analyses were performed using Prism6 (GraphPad Software, Inc., La Jolla, CA, USA).

2.3 Results

2.3.1 Examination of growth promoting and bactericidal effects of vitamin treatments on *E. coli* O157:H7

Growth curves were used to assess influence of vitamins on *E. coli* O157:H7 (Figure 2.4). Specifically, absorbance at 600 nm was measured during 24 h treatment with ascorbic acid (1, 10 mg/mL), biotin (0.1 mg/mL), folic acid (0.1 mg/mL), riboflavin (0.01 mg/mL), or respective controls. For biotin (0.1 mg/mL) and riboflavin (0.01 mg/mL) treatments, growth curves were similar to control during 24 h. However, during folic acid (0.1 mg/mL) treatment, the growth curve was similar to the control in the first 4 h, then somewhat distinct over the next 14 h, and finally the difference was ameliorated in the last 6 h. Treatments with ascorbic acid (1, 10 mg/mL), resulted in no growth observed in the first 16 h and minimal growth in the next 8 h, which was distinct from the growth seen in the control.

Plate counts were used to determine the effects of exposure to selected water soluble vitamins [calcium pantothenate (5, 50 mg/mL), cobalamin (1 mg/mL), and control (double distilled water) (Figure 2.5a) or fat soluble vitamins [α-tocopherol (1 mg/mL), menaquinone (0.05 mg/mL), and control (8% ethanol) (Figure 2.5b)] had upon the bacterium. For cobalamin (1 mg/mL) treatment, the growth curve was similar to the control over a period of 24 h. However, during calcium pantothenate (5 mg/mL) treatment, the growth curve was similar to the

control in the first 4 h, and then static over the next 20 h. Treatment with calcium pantothenate (50 mg/mL) resulted in the lowest numbers of *E. coli* O157:H7 during 24 h. During treatment with α-tocopherol (1 mg/mL), menaquinone (0.05 mg/mL), and the control (8% ethanol), numbers of bacteria remained unchanged during the first 12 h, and slightly increased over the other 12 h. Both α-tocopherol (1 mg/mL) and menaquinone (0.05 mg/mL) treatments produced lower numbers of bacteria compared to control at 24 h.

Plate counts were also used to measure numbers of *E. coli* O157:H7 at the moment of infection of HeLa cells. Following 1.5 and 3 h treatment with calcium pantothenate (50 mg/mL) and 3 h treatment with ascorbic acid (10 mg/mL), the CFU for *E. coli* O157:H7 was significantly (p<0.01) lower than in control (double distilled water) treatment (Figure 2.6a). No significant differences were observed in the CFU for *E. coli* O157:H7 during treatments with other vitamins (Figure 2.6b and c). Conditions during the treatments (incubation at 37°C, 5%CO_2, static) were the same as in HeLa assay.

Finally, plate counts were used to assess number of *E. coli* O157:H7 during production of Stx. After 3 h treatment with ascorbic acid (10 mg/mL), calcium pantothenate (5 and 50 mg/mL), and cobalamin (1 mg/mL), numbers of *E. coli* O157:H7 were not significantly different than in control (double distilled water) treatment (Figure 2.11). Conditions during the treatments (incubation at 37°C, static) were the same as in Stx ELISA assay.

2.3.2 Adherence of *E. coli* O157:H7 to HeLa cells

Following 1.5 h exposure to calcium pantothenate (50 mg/mL) and cobalamin (0.01, 0.1, 1 mg/mL), a significant (p<0.05) increase in adherence to HeLa cells was observed. At this time, no significant differences were observed during exposure to other vitamins (Figures 2.7 and 2.8). At 3 h exposure, significantly increased adherence was observed for *E. coli* O157:H7 exposed to

α-tocopherol (1 mg/mL), biotin (0.01, 0.1 mg/mL,), calcium pantothenate (5, 50 mg/mL), and cobalamin (0.01, 0.1, 1 mg/mL) (Figures 2.7 and 2.8). As expected, exposure to vitamins had no effect on invasion (data not presented).

2.3.3 Vitamin mediated cytotoxicity in HeLa cells

Lactate dehydrogenase release from HeLa cells was negligible (data not presented). The highest cytotoxicity (3.5%) was detected in 0.1 mg/mL ascorbic acid treatment. This cytotoxicity was only 2% higher than in control treatment.

2.3.4 Comparison of calcium pantothenate and calcium chloride influence on *E. coli* O157:H7 adherence to HeLa cells

Right after treatment of *E. coli* O157:H7 with calcium pantothenate (50 mg/mL), the pH of the growth medium dropped from 6.9 to 5.2 (Table 2.3). Also, a precipitate formed due to the reaction of calcium from calcium pantothenate and phosphates from M9 minimal medium. To control for the precipitate formed and to evaluate whether the increased adherence to HeLa cells was caused by calcium or pantothenic acid, *E. coli* O157:H7 was treated with either calcium pantothenate (50 mg/mL), calcium chloride (equimolar Ca^{2+}), or control (double distilled water) for 1.5 and 3 h in original and modified M9 minimal medium (Table 2.2), followed by assessment of adherence of the *E. coli* O157:H7 to HeLa cells. Following 1.5 and 3 h treatment with calcium pantothenate (50 mg/mL) and calcium chloride (equimolar Ca^{2+}) in original M9MM, a significant ($p<0.01$) increase in *E. coli* O157:H7 adherence to HeLa cells was observed (Figure 2.9). Treatment with calcium pantothenate (50 mg/mL) resulted in 0.8 and 1-log higher adherence than the control *E. coli* O157:H7 after 1.5 and 3 h treatments. At the same time, calcium chloride (equimolar Ca^{2+}) yielded 0.5 and 0.9-log higher adherence. In modified M9MM, a significant ($p<0.05$ and $p<0.01$) increase in *E. coli* O157:H7 adherence to HeLa cells

was observed for 1.5 and 3 h calcium chloride (equimolar Ca^{2+}) treatments compared to the control, while treatment with calcium pantothenate (50 mg/mL) was not significantly different than the control. However, treatments in modified M9 minimal medium showed only <0.5-log increase in adherence to HeLa cells. pH of calcium pantothenate and calcium chloride treatments in modified M9 minimal medium were similar to the control (Table 2.3).

Exposure to calcium pantothenate (50 mg/mL), calcium chloride (equimolar calcium), or control (double distilled water) in original M9MM had no effect on invasion of *E. coli* O157:H7 in HeLa cells (data not presented). Invasion of *E. coli* O157:H7 in HeLa cells following the same treatments in modified M9MM was not assessed

2.3.5 Shiga toxin production

Following 3 h treatment with cobalamin (1 mg/mL), significantly more cell associated Stx1 (p<0.001) and secreted Stx2 (p<0.0001) were produced by *E. coli* O157:H7 compared to the respective control (Figure 2.12). Precisely, 3.7-fold more cell associated Stx1 and 4-fold more secreted Stx2 were produced compared to control treatments. Exposure to ascorbic acid (10 mg/mL) and calcium pantothenate (50 mg/mL) resulted in no significantly different levels of Stx1 within the cell, and no secreted Stx2. Exposure to lower concentration of calcium pantothenate (5 mg/mL) did not yield a significantly different amount of cell associated Stx1, while secreted Stx2 levels were significantly lower (p<0.001) compared to the control. Also, some amounts of secreted Stx1 were found in the supernatant of cobalamin (1mg/mL) treated cells. The amount of cell associated Stx2 in ascorbic acid (10 mg/mL) treated cells was minor, and somewhat more pronounced in cobalamin (1 mg/mL) treated cells. No secreted Stx1 and cell associated Stx2 were observed for *E. coli* O157:H7 exposed to other vitamin and control

treatments. As expected, *E. coli* O157:H7 EDL 933 Δ*stx*$_1$Δ*stx*$_2$ mutant (negative control), did not produce any Stx1 and Stx2 that could be detected by ELISA.

2.3.6 Evaluation, using qPCR, of virulence gene expression in vitamin treated *E. coli* O157:H7 under conditions in HeLa assay

Following 3 h treatment with biotin (0.1 mg/mL), calcium pantothenate (5 mg/mL), and cobalamin (1 mg/mL), the CFU/ml of *E. coli* O157:H7 was not significantly different than in the control (double distilled water) (Figure 2.13). Conditions during the treatments were the same as in the HeLa assay (incubation at 37°C, 5%CO_2, static).

At 3 h treatment with biotin (0.1 mg/mL), *eae*, which encodes intimin, was down-regulated (Table 2.6). In contrast, treatment with calcium pantothenate (5 mg/mL) stimulated expression of *eae*, while cobalamin (1 mg/mL) treatment resulted in up-regulation of *espA*, which encodes the translocator EspA.

2.3.7 Evaluation, using qPCR, of virulence gene expression in vitamin treated *E. coli* O157:H7 under conditions in Stx ELISA assay

Following 3 h treatment with ascorbic acid (10 mg/mL), calcium pantothenate (5 mg/mL), and cobalamin (1 mg/mL), the CFU/ml of *E. coli* O157:H7 was not significantly different than in the control (double distilled water) (Figure 2.14). Conditions during the treatments were the same as in the Stx ELISA assay (incubation at 37°C, static).

At 3 h treatment with ascorbic acid (10 mg/mL), *stx*$_{1a}$ was down-regulated 2.5-fold, while *stx*$_{2a}$ was 5.1-fold induced (Table 2.7). In the same time, exposure to cobalamin (1 mg/mL) resulted in 2.0-fold down-regulation of *stx*$_{1a}$ with no effect on *stx*$_{2a}$ expression. Calcium pantothenate (5 mg/mL) treatment had no effect on the expression of *stx*$_{1a}$ and *stx*$_{2a}$.

2.4 Discussion

Generally, vitamin concentrations used in this study were the highest that could be prepared, so the influence on *E. coli* O157:H7 can be easily observed. In addition to the highest vitamin concentration, two 10-fold dilutions were applied to ensure a wide range of vitamin concentrations were covered (Table 2.1).

2.4.1 Growth promoting and bactericidal effects of vitamins on *E. coli* O157:H7

Right after the overnight *E. coli* O157:H7 culture was diluted (1:100) in M9MM, lag phase cells were exposed to different vitamins or respective controls, and CFU/mL determined or OD_{600} monitored over a 24 h period. The starting amount of the CFU/mL was relatively high (around 7 log CFU/mL; Figure 2.5b), which enabled testing for potential bactericidal effects by the vitamins. In vitamin treatments with growth promoting effects, more growth would have been expected to occur if lower initial CFU/mL were prepared instead. Vitamin concentrations exceeded the concentrations naturally found in foods (spinach) from around 3-fold for ascorbic acid (1 mg/mL) to >5,000-fold for calcium pantothenate (50 mg/mL) (Table 1.3), or in human faeces from 2-fold for riboflavin (0.01 mg/mL) to 50,000-fold for cobalamin (1 mg/mL) (Table 1.4). As expected, *E. coli* O157:H7 cells exposed to biotin (0.1 mg/mL; Figure 2.4a), riboflavin (0.01 mg/mL; Figure 2.4a), folic acid (0.1 mg/mL; Figure 2.4b), or cobalamin (1 mg/mL; Figure 2.5b) resulted in growth curves similar to those of the respective controls. Exposure to ascorbic acid (1, 10 mg/mL) inhibited *E. coli* O157:H7 growth during the first 16 h, with a minor increase over the next 8 h (Figure 2.4a). This was not surprising, as an inhibitory effect of ascorbic acid (2.8%, pH 2.6, 10 min) on *E. coli* O157:H7 growth has been previously reported (Derrickson-Tharrington et al., 2005). Surprisingly, calcium pantothenate (5, 50 mg/mL) resulted in lower numbers of *E. coli* O157:H7 compared to the control after 4 h

treatment (Figure 2.5a). Over a 24 h period, the lowest CFU/mL was achieved with calcium pantothenate (50 mg/mL) treatment. The lower pH of calcium pantothenate (5 mg/mL, pH 6.7; 50 mg/mL, pH 5.2) might have contributed to this (Table 2.3). However, the difference in the CFU/mL between 5 and 50 mg/mL calcium pantothenate treatments was not as pronounced as the difference in the CFU/mL between control (pH 6.9) and calcium pantothenate 5 mg/mL (pH 6.7), suggesting that characteristics of the treatments rather than pH might be the cause for this (Figure 2.5a). Pantothenic acid is known to be a precursor for biosynthesis of CoA (Brown, 1959b), an important cofactor in many metabolic pathways such as synthesis of fatty acids in *E. coli* (Magnuson et al., 1993). Additionally, *E. coli* is able to synthesize or absorb pantothenate if supplied in the medium and regulate its intracellular pool to <1μM by excreting the excess amounts outside the cell (Jackowski & Rock, 1981). The cells had already reached saturation with the calcium pantothenate at the lower concentration (5 mg/mL, about 20 mM) and this could be why there was not a huge change in the CFU/ml between treatments with the two different calcium pantothenate concentrations. Treatments with fat soluble α-tocopherol (1 mg/mL) and menaquinone (0.05 mg/mL), as well as the control (8% ethanol) resulted in unchanged numbers of *E. coli* O157:H7 during the first 12 h, probably due to the same amount of ethanol present in the treatments (Figure 2.5b). This was expected, as ethanol (c.a. 5%) has been previously shown to have an inhibitory effect on *E. coli* growth, by allowing the exponential growth, but depressing the specific growth rate (Sawada & Nakamura, 1987). However, at 24 h, exposure to α-tocopherol (1 mg/mL) and menaquinone (0.05 mg/mL) resulted in lower numbers of bacteria compared to control (8% ethanol), suggesting potential inhibitory effects of these vitamins on *E. coli* O157:H7 growth.

2.4.2 Adherence of *E. coli* O157:H7 to HeLa cells

While the CFU/mL of *E. coli* O157:H7 cells treated with α-tocopherol (1 mg/mL), biotin (0.1 mg/mL), and cobalamin (1 mg/mL) at 1.5 and 3 h (Figure 2.6a and c) were not significantly different than control, significantly more bacteria adhered to HeLa cells for α-tocopherol (1 mg/mL, 3 h, Figure 2.7), biotin (0.01, 0.1 mg/mL; 3 h, Figure 2.7), and cobalamin 0.01, 0.1, 1 mg/mL; 1.5 and 3 h, Figure 2.8). Treatments with ascorbic acid (10 mg/mL, 3 h) and calcium pantothenate (50 mg/mL, 1.5 and 3 h) resulted in the decreased CFU/mL (Figure 2.6a), while adherence was not different with ascorbic acid (10 mg/mL, 1.5 and 3 h) and significantly increased with calcium pantothenate (5 mg/mL, 3 h; 50 mg/mL, 1.5 and 3 h) (Figure 2.7). Exposure to several vitamins, including folic acid (0.1 mg/mL; Figure 2.6b), menaquinone (0.5 mg/mL; Figure 2.6c), and riboflavin (0.01 mg/mL; Figure 2.6a) did not affect both the CFU/mL of *E. coli* O157:H7 and the adherence to HeLa cells (Figure 2.8). Vitamin treatments that resulted in a significantly increased adherence of *E. coli* O157:H7 at a range of concentrations after 3 h (biotin, calcium pantothenate, and cobalamin; Figures 2.7 and 2.8), were selected for assessment of relative virulence gene expression at the moment of HeLa cells infection using qPCR. Following 3 h treatment with biotin (0.1 mg/mL), virulence genes were not expressed differently than in the control, except that *eae*, which encodes intimin was minimally (1.6-fold) down-regulated (Table 2.6). Further research examining production of the type three secretion system proteins rather than relative gene expression is needed in order to validate increased adherence to HeLa cells as a consequence of these treatments. At 3 h treatment calcium pantothenate (5 mg/mL) up-regulated *eae*, which encodes intimin, while cobalamin (1 mg/mL) stimulated *espA*, which encodes a translocator protein EspA (Table 2.6). Both intimin and EspA are involved in formation of the attachment and effacement lesion,

particularly in the initial stage of the intimate attachment of *E. coli* O157:H7 to the host cell (Garmendia et al., 2005; Gyles, 2007; Nataro & Kaper, 1998). Therefore up-regulation of *eae* and *espA* might explain increased adherence during HeLa infection (Figures 2.7 and 2.8).

Among all vitamin treatments, exposure to calcium pantothenate (50 mg/mL) resulted in the largest (1.1- and 1.5-log CFU/mL) difference of adhering bacteria compared to the control at 1.5 and 3 h respectively (Figure 2.7). However, right after treatment of *E. coli* O157:H7 with calcium pantothenate (50 mg/mL), the pH of the growth medium dropped from 6.9 to 5.2 (Table 2.3). Also, a precipitate formed due to the reaction of calcium from calcium pantothenate and phosphates from M9 minimal medium. To control for the precipitate formed and to evaluate whether the increased adherence to HeLa cells was caused by calcium or pantothenic acid, *E. coli* O157:H7 was treated with either calcium pantothenate (50 mg/mL), calcium chloride (equimolar Ca^{2+}) or control (double distilled water) for 1.5 and 3 h in original and modified M9 minimal medium, followed by assessment of the adherence to HeLa cells. In original M9MM, similar levels of bacteria adhered to HeLa cells after 3 h in both calcium pantothenate (50 mg/mL) and calcium chloride (equimolar Ca^{2+}) treatments, suggesting that Ca^{2+}, rather than pantothenic acid increased the adherence (Figure 2.9). This was in line with the previous studies reporting that Ca^{2+} stimulates expression of *espA* promoter of EHEC in M9 minimal medium (Beltrametti et al., 1999) and increases secretion of EspA and EspB translocator proteins in EPEC (Kenny et al., 1997). In modified M9 minimal medium (calcium and phosphate free), no precipitate was formed after addition of calcium pantothenate and calcium chloride, and pH was minimally affected (Table 2.3). However, the previous 1- and 0.9-log increase in adherence at 3 h calcium pantothenate (50 mg/mL) and calcium chloride (equimolar Ca^{2+}) treatments was decreased to 0.2- and 0.5-log CFU of adherent *E. coli* O157:H7 respectively (Figure 2.9), thus

indicating that in addition to Ca^{2+}, more complex pH and/or media related features may play a role in *E. coli* O157:H7 adherence as well.

2.4.3 Shiga toxin production

Following 3 h treatment with ascorbic acid (10 mg/mL), calcium pantothenate (5, 50 mg/mL), cobalamin (1 mg/mL), or the control (double distilled water) Stx1 was mainly cell associated, while Stx2 was mainly secreted (Figure 2.12). The exceptions were ascorbic acid (10 mg/mL) and cobalamin (1 mg/mL) treatments that resulted in minor amounts of cell associated Stx2, and cobalamin (1 mg/mL) with minimal Stx1 secreted. This was is in line with previous studies reporting different distributions of Stx1 and Stx2, with Stx1 being mainly cell associated and Stx2 mostly released from the cell (Shimizu et al., 2009; Strockbine et al., 1986). Ascorbic acid (10 mg/mL) and calcium pantothenate (5, 50 mg/mL) treatments resulted in no significantly different Stx1 and significantly (p<0.001) less Stx2 produced compared to control (Figure 2.12). Following calcium pantothenate (5 mg/mL) treatment, stx_{1a} expression (Table 2.7) and Stx1 production (Figure 2.12) were not significantly different than in the control. While ascorbic acid (10 mg/mL) resulted in 2.5-fold down-regulation of stx_{2a} (Table 2.7), Stx1 levels were not different than the control (Figure 2.12). Additionally, 5.1-fold up-regulation of stx_{2a} after ascorbic acid (10 mg/mL; Table 2.7) treatment and no significant effect on regulation of stx_{2a} after calcium pantothenate (5 mg/mL; Table 2.7) were not seen at the Stx2 protein level (Figure 2.12). On the contrary, Stx2 production by *E. coli* O157:H7 exposed to both vitamin treatments was lower than in the control. It might happen that the initial up-regulation at transcriptional level in those treatments was decreased at translational or post-translational level. Cobalamin (1 mg/mL) treatment resulted in 3.7- and 4-fold increase in the amounts of cell associated Stx1 and secreted Stx2 respectively. Increased Stx2 production has been previously

observed in treatments with DNA-damaging agents such as H_2O_2 and neutrophils (Wagner et al., 2001a). Also, in a separate study, growth curve of the cobalamin (1 mg/mL) treated *E. coli* O157:H7 was similar to that of the control (Figure 2.5a). Additionally, after 3 h treatment with cobalamin (1mg/mL), stx_{1a} was 2.0-fold down-regulated, while and stx_{2a} was not significantly affected (Table 2.7). Therefore, further studies looking into mechanisms by which cobalamin (1 mg/mL) stimulates production of Stx1 and Stx2 in *E. coli* O157:H7 are needed. Also, it would be interesting to assess if lower cobalamin concentrations (*e.g.*, those of human colon) might have similar effect on Stx1 and Stx2 production as well as on stx_1 and stx_2 expression in *E. coli* O157:H7.

2.5 Conclusion

The approaches used in this study provide a novel insight into whether vitamins are triggers for enhanced virulence of *E. coli* O157:H7. Exposure of *E. coli* O157:H7 to α-tocopherol, biotin, calcium pantothenate, and cobalamin increased the capacity of the bacteria to adhere to HeLa cells. The largest *in vitro* adherence (1.5-log increase in CFU/mL than control) was observed at 3 h treatment with calcium pantothenate (50 mg/mL). However, Ca^{2+} (in addition to pH and/or more complex media related features) contributed to this phenomenon. Furthermore, calcium pantothenate and cobalamin triggered the expression of *eae* and *espA*, which encode intimin and translocator EspA respectively. Adherence data for the treatments with α-tocopherol, biotin, calcium pantothenate, or cobalamin could be validated by determining the levels of type three secretion proteins produced by *E. coli* O157:H7.

Cobalamin (1 mg/mL, 3 h) treatment resulted in 3.7- and 4-fold increased cell associated Stx1 and secreted Stx2 by *E. coli* O157:H7 compared to the control. Interestingly, gene expression data did not correspond to the levels of Stx1 and Stx2 produced. Only DNA-

damaging agents such as H_2O_2, neutrophils, and mitomycin C have been previously linked to the increased Stx2 production. In this study, the CFU/mL of *E. coli* O157:H7 treated with cobalamin (1 mg/mL, 1.5 and 3 h) was not different than control and growth curve was not negatively affected over a 24 h period. It would be worth investigating if the virulence gene expression can be triggered by calcium pantothenate and cobalamin at concentrations naturally found in a human gut, and if cobalamin at those concentrations might have similar effect on Stx1 and Stx2 production. Determining kinetic profiles of Stx1 and Stx2 production, and stx_1 and stx_2 expression over a longer period of time may contribute to better understanding of cobalamin influence on *E. coli* O157:H7 virulence. The mechanism by which cobalamin stimulates production of both Stx1 and Stx2 remains unknown.

Finally, exposure of *E. coli* O157:H7 to ascorbic acid (10 mg/mL, 3 h) did not influence adherence to HeLa cells and resulted in lower Stx2 levels compared to control. Since destructive effects of ascorbic acid on *E. coli* O157:H7 have been previously reported, our results may allow for further investigation of the use of ascorbic acid as a novel pathogen mitigation strategy for different foods.

Table 2.1 Stock solutions of fat and water soluble vitamins used in the study.

Vitamin		Control
Name (Group)	**Concentration mg/mL (w:v)**	
α-Tocopherol (Vitamin E)	10, 1, 0.1	80% ethanol
Ascorbic acid (Vitamin C)	100, 10, 1	Distilled water
Biotin (Vitamin B_7)	1, 0.1, 0.001	Distilled water
Calcium pantothenate (Vitamin B_5)	500, 50, 5	Distilled water
Cobalamin (Vitamin B_{12})	10, 1, 0.1	Distilled water
Folic acid (Vitamin B_9)*	1, 0.1, 0.01	Distilled water**
Menaquinone (Vitamin K_2)	0.5, 0.05, 0.005	80% ethanol
Riboflavin (Vitamin B_2)	0.1, 0.01, 0.001	Distilled water

pH adjusted with KOH and HCl to: *7.1 and **7.4.

Table 2.2 Components of original and modified M9 minimal medium (M9MM).

Component name	Supplier	Stock concentration	Final concentration*		Reference
			M9MM	Modified M9MM	
M9 minimal salts	Becton Dickinson	5 X	1 X	-	Anonymous, 2009b
M9 minimal salts, modified**	Fisher Scientific	5 X	-	1 X	Sambrook & Russell, 2001
HEPES, pH 7.4	Sigma Aldrich	1 M	-	0.1 M	Deng et al., 2005
Glucose	Fisher Scientific	20%	0.4%	0.4%	Li et al., 2000
Casamino acids, vitamin assay***	Becton Dickinson	10%	-	0.1%	Li et al., 2000
Magnesium sulfate	Fisher Scientific	1 M	2 mM	2 mM	Sambrook & Russell, 2001
Calcium chloride	Fisher Scientific	1 M	0.1 mM	-	Anonymous, 2009b

*All components were dissolved in sterile double distilled water to reach final concentrations;

**M9 minimal salts modified (pH 7.1) is a phosphate free (no disodium phosphate and monopotassium phosphate); contains 94 mM NH_4Cl and 43 mM NaCl (Fisher Scientific, Fair Lawn, NJ); pH was adjusted with 10M NaOH;

***Casamino acids vitamin assay is an acid digest of casein with markedly reduced or eliminated vitamins. It is an amino acid source, recommended for use in microbiological assay media and studies of the growth requirements of microorganisms (Anonymous, 2009b).

Table 2.3 pH of late log phase *E. coli* O157:H7 grown in original or modified M9 minimal medium (M9MM), after treatment with vitamins, calcium chloride or control.

Treatment, final concentration	pH	
	M9MM	**Modified M9MM**
Control (ddH_2O)	6.9	6.7
Ascorbic acid, 0.1 mg/mL	7.2	NA
Ascorbic acid, 1 mg/mL	6.4	NA
Ascorbic acid, 10 mg/mL	3.5	NA
Ca-pantothenate, 5 mg/mL	6.7	NA
Ca-pantothenate, 50 mg/mL	5.2	6.9
$CaCl_2$*	4.8	6.8

*Equimolar Ca^{2+} to Ca-pantothenate 50 mg/mL;
"NA" Not applicable.

Table 2.4 Oligonucleotides (n=9) used in qPCR to assess expression of virulence genes in biotin, calcium pantothenate, or cobalamin treated *E. coli* O157:H7 EDL 933 relative to the control, under conditions used in the adherence to HeLa cell assay (37°C, 5% CO_2, 3 h, static).

Gene	Primer	Sequence (5'→3')	Length (bp)	Tm (°C)	Physiological function	Reference
eae	1	TCAACGGTAAGTCTCAAACG	20	52.5	Intimin	Allen (2007)
eae	2	CGCGATACTGGTATTTTCTG	20	51.0		
ecpD	1	GTATCCAACCAGTTCTGA	18	48.4	Putative fimbrial chaperone protein	This study
ecpD	2	GTGCTTATGTTTCCCTTG	18	48.4		
espA	1	TTAGCTGAAATAGCCGCCTT	20	54.3	Translocator protein EspA	This study
espA	2	AAGAATGCGAAAGCCAAACT	20	53.2		
hcpA	1	CGTCGCAGGTATCTAATC	18	49.6	Major pilin subunit	This study
hcpA	2	GAACTGATGGTGGTTATTG	19	48.3		
ler	1	CGACCAGGTCTGCCCTTCT	19	59.4	Regulator of *LEE* genes	Allen (2007)
ler	2	GCGCGGAACTCATCGAAA	18	55.9		
lpfA	1	TTTCATCGTGGTGGTATC	18	49.1	Major fimbrial subunit	This study
lpfA	2	ACTTCTGGCACTGTATTC	18	49.1		
rpoA	1	GTCAATTCCAGATCGTCAAC	20	51.2	α-subunit of RNA polymerase	Allen (2007)
rpoA	2	TTCATTCGGAAGAAGATGAG	20	49.6		
tir	1	CGTGGTCAGCTCATTAACTC	20	53.2	Translocated intimin receptor	Allen (2007)
tir	2	GTCTCAACACCATTCCTCTG	20	52.9		
tufA	1	ATCGTGTTCCTGAACAAATG	20	50.9	Elongation factor Tu	Allen (2007)
tufA	2	TCTACACGACCGGTAACAAC	20	54.2		

Table 2.5 Oligonucleotides (n=4) used in qPCR to assess virulence gene expression in ascorbic acid, calcium pantothenate, or cobalamin treated *E. coli* O157:H7 EDL 933 relative to the control, under conditions used in Stx ELISA quantification assay (37°C, 3h, static).

Gene	Primer	Sequence (5'→3')	Length (bp)	Tm (°C)	Physiological function	Reference
rpoA	1	GTCAATTCCAGATCGTCAAC	20	51.2	α-subunit of RNA polymerase	Allen (2007)
rpoA	2	TTCATTCGGAAGAAGATGAG	20	49.6		
stx_{1a}	1	TAATGTCGCATAGTGGAACC	20	52.2	Shiga-like toxin 1 A subunit	Allen (2007)
stx_{1a}	2	CAGTATTAATGCCACGCTTC	20	51.6		
stx_{2a}	1	CATATCTGGCGTTAATGGAG	20	50.6	Shiga-like toxin 2 A subunit	Allen (2007)
stx_{2a}	2	TCAGTATAACGGCCACAGTC	20	54.2		
tufA	1	ATCGTGTTCCTGAACAAATG	20	50.9	Elongation factor Tu	Allen (2007)
tufA	2	TCTACACGACCGGTAACAAC	20	54.2		

Table 2.6 List of *E. coli* O157:H7 EDL 933 genes, relatively up- or down-regulated compared to the control, after treatment with biotin, calcium pantothenate, or cobalamin under conditions applied in adherence to the HeLa cell assay (37°C, 5% CO_2, 3 h, static).

Functional category and gene		**Physiological function**	**Fold change for the treatment***		
			Biotin, 0.1 mg/mL	**Calcium pantothenate, 5 mg/mL**	**Cobalamin, 1 mg/mL**
Fimbriae					
	lpfA[†]	Major fimbrial subunit	1.2	-1.3	-1.1
Virulence					
	eae	Intimin	**-1.6**	**1.8**	1.5
	espA	Translocator protein EspA	-1.2	ND	**2.0**
	ler	Regulator of *LEE* genes	-1.3	-1.2	-1.2
	tir	Translocated intimin receptor	-1.1	1.0	-1.4

[*]Relative changes in the expression levels for genes of interest were normalized against two housekeeping genes: *rpoA*, encoding α-subunit of RNA polymerase, and *tufA*, encoding elongation factor Tu. Housekeeping genes had acceptable gene stability value (M)<0.5 and coefficient of variation (CV)<0.25. Bold are results >1.5-fold up- ("+") or down- ("-") regulated;

[†]PCR Efficiency determined using chromosomal (Chr) DNA for *lpfA*, and for respective housekeeping genes *tufA* and *rpoA*;

"ND" Not determined as more than one technical replicate had Ct >37, or no amplification detected; Ct standard deviation (SD) for all genes and treatments was ≤0.3

Note: *ecpD* encoding a putative fimbrial chaperone protein was excluded from analysis as acceptable PCR efficiency could not be reached neither with cDNA nor Chr DNA for the selected oligonucleotide pair; *hcpA* encoding a major pilin subunit had acceptable PCR efficiency with Chr. DNA, but was excluded as Ct SD was >0.3.

Table 2.7 Shiga toxin genes (stx_{1a} and stx_{2a}) of *E. coli* O157:H7 EDL 933, relatively up- or down-regulated compared to the control, following treatment with ascorbic acid, calcium pantothenate, or cobalamin under conditions used in Stx ELISA assay (37°C, 3 h, static).

Gene	**Physiological function**	**Fold change for the treatment***		
		Ascorbic acid, 10 mg/mL	**Calcium pantothenate, 5 mg/mL**	**Cobalamin, 1 mg/mL**
stx_{1a}	Shiga-like toxin 1 A subunit	**-2.5**	-1.4	**-2.0**
stx_{2a}	Shiga-like toxin 2 A subunit	**5.1**	1.5	-1.3

Relative changes in the expression levels for genes of interest were normalized against two housekeeping genes: rpoA*, encoding α-subunit of RNA polymerase, and *tufA*, encoding elongation factor Tu. Housekeeping genes had acceptable gene stability value (M)<0.5 and coefficient of variation (CV)<0.25.Bold are results >1.5-fold up- ("+") or down- ("-") regulated. Ct standard deviation for all genes and treatments was ≤0.3.

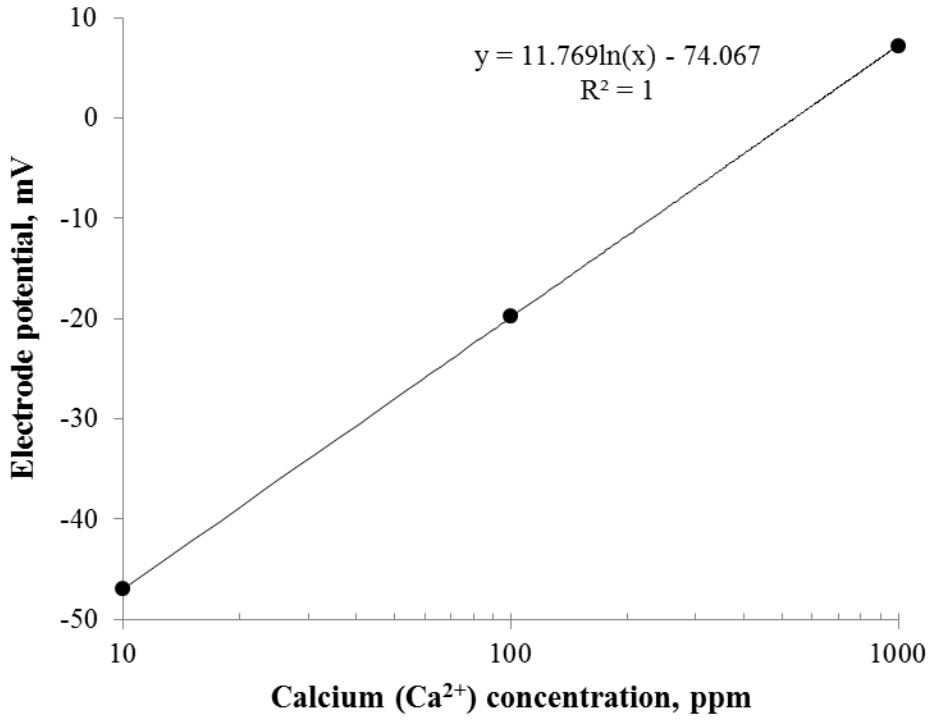

Figure 2.1 Direct calibration curve of Orion 9320 calcium electrode (Thermo Scientific, Waltham, MA) using standard calcium (Ca^{2+}) solutions.

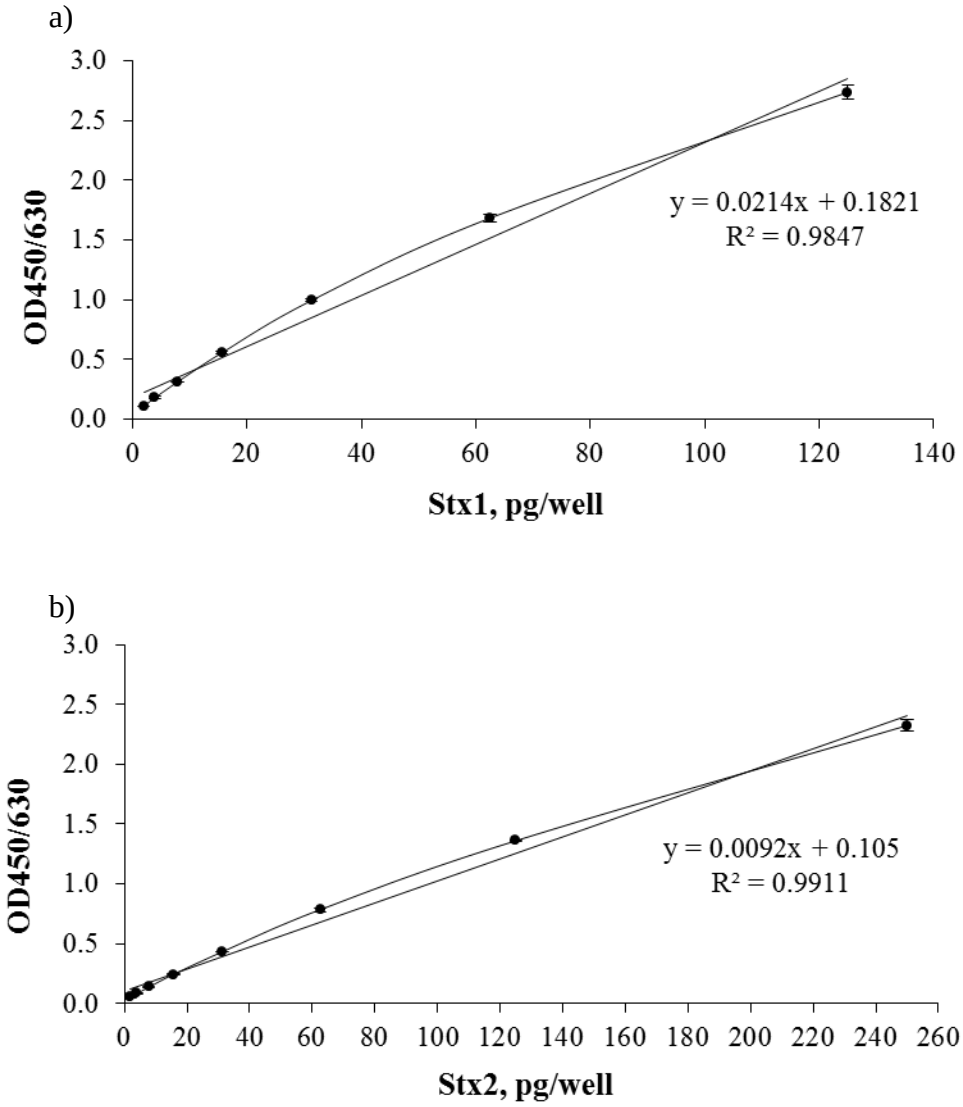

Figure 2.2 Standard curves used in Stx ELISA assay to quantify a) Shiga toxin 1 (Stx1) and b) Shiga toxin 2 (Stx2). Mean optical densities (OD) 450/630nm for Stx1 and Stx2 standards ranging from 1.95 to 250 pg/well (two replicates) are presented with points, while standard deviation is indicated with horizontal lines.

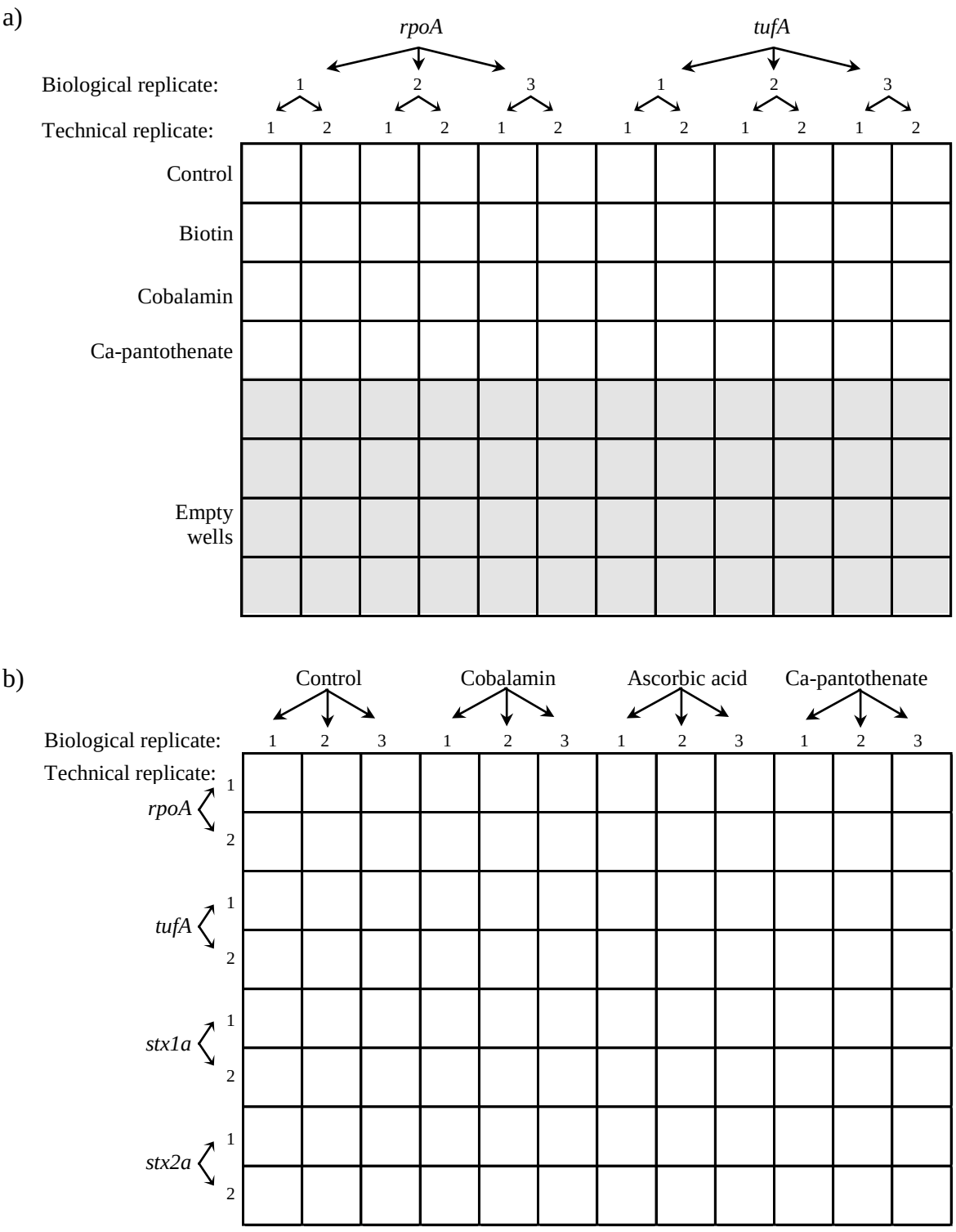

Figure 2.3 Example of sample maximization strategy used to set up quantitative real-time polymerase chain reaction plates with vitamin treated and control samples (three biological and two technical replicates) in experiments run under conditions mimicking a) adherence to HeLa, or b) Stx ELISA assay.

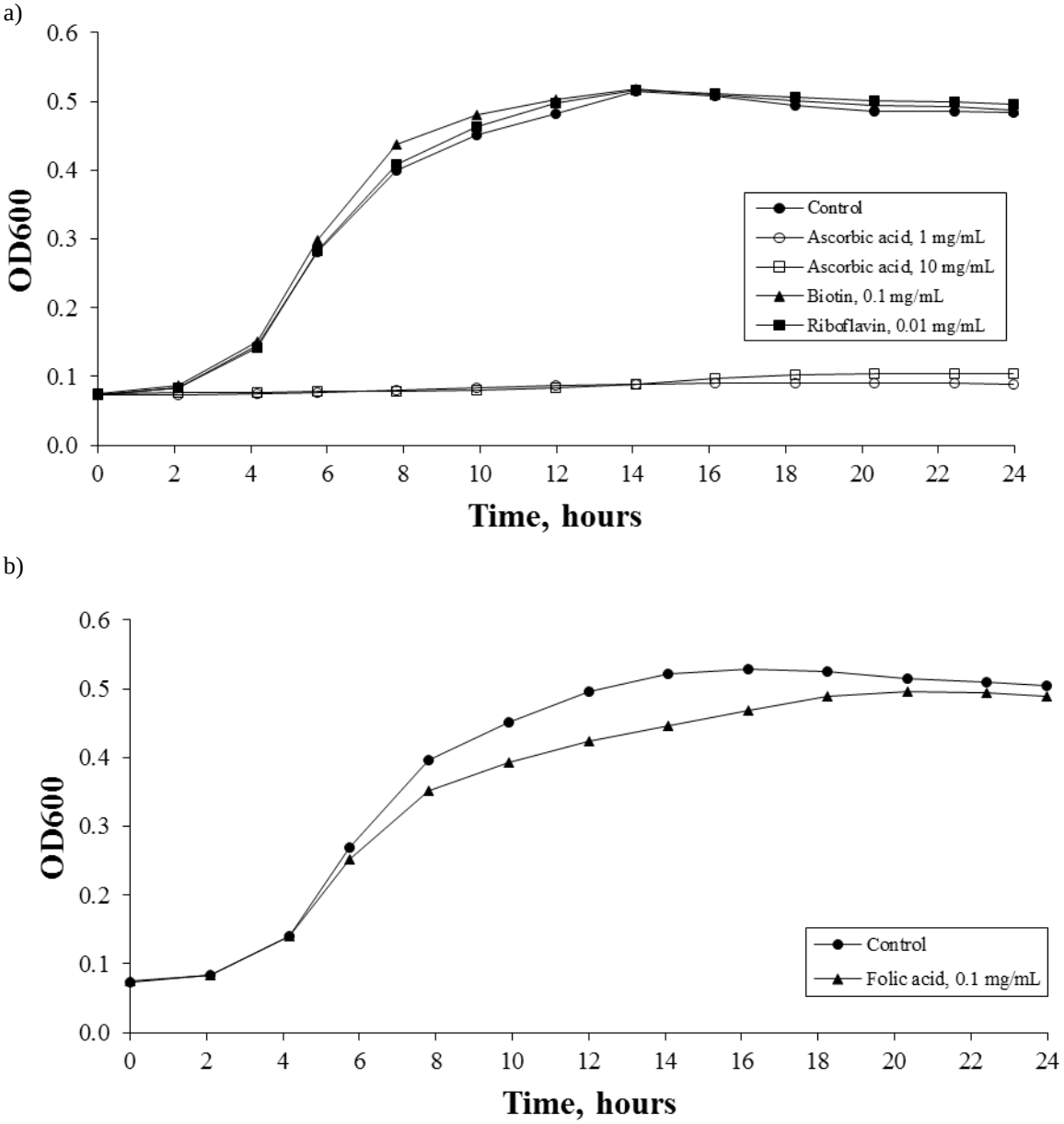

Figure 2.4 Growth curve of *E. coli* O157:H7 treated with a) water soluble vitamins, or b) folic acid and their controls: a) double distilled water, or b) potassium hydroxide (pH 7.1; adjusted with hydrochloric acid), in M9 minimal medium at 37°C. Vitamins or respective controls were added to bacteria at 0 h and absorbance (OD_{600}) was measured using spectrophotometer for 24 h. Each value indicates mean of three biological replicates. Standard deviation ranged from 0.00 to 0.04, with the highest level in control for folic acid (0.1 mg/mL) treatment at 6 h.

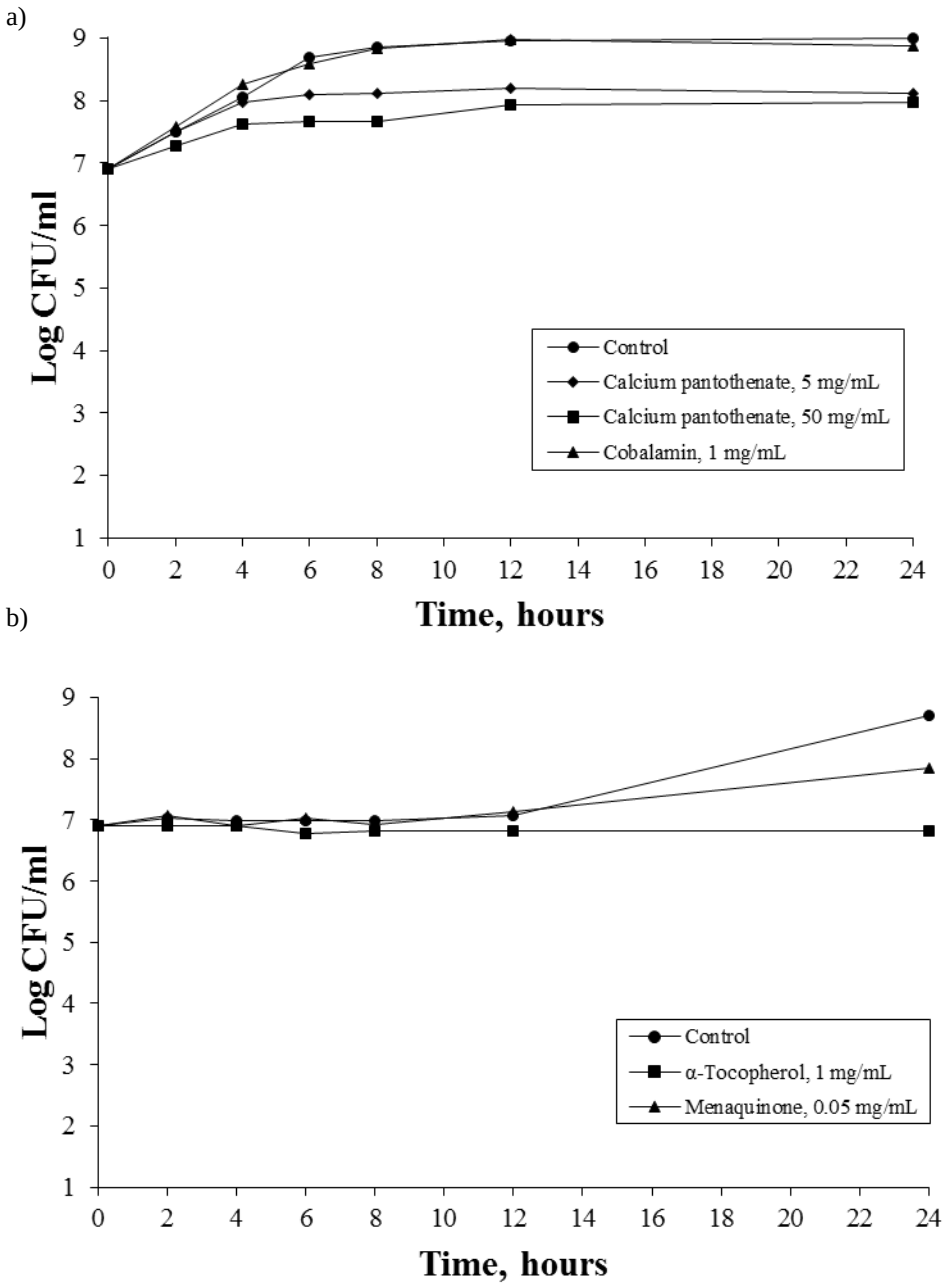

Figure 2.5 Number (log CFU/ml) of *E. coli* O157:H7 in M9 minimal medium during exposure to a) water soluble, or b) fat soluble vitamins, or their controls a) double distilled water, or b) 8% ethanol, at 37°C, static. *E. coli* O157:H7 was plated on Luria Bertani Agar at 0, 2, 4, 6, 8, 12, and 24 h. Mean of three biological replicates are presented with points. Standard deviation ranged from 0.0 to 0.4, with the highest levels in control (double distilled water, 4 h), α-tocopherol (1 mg/mL, 12 h), and control (8% ethanol, 12 h).

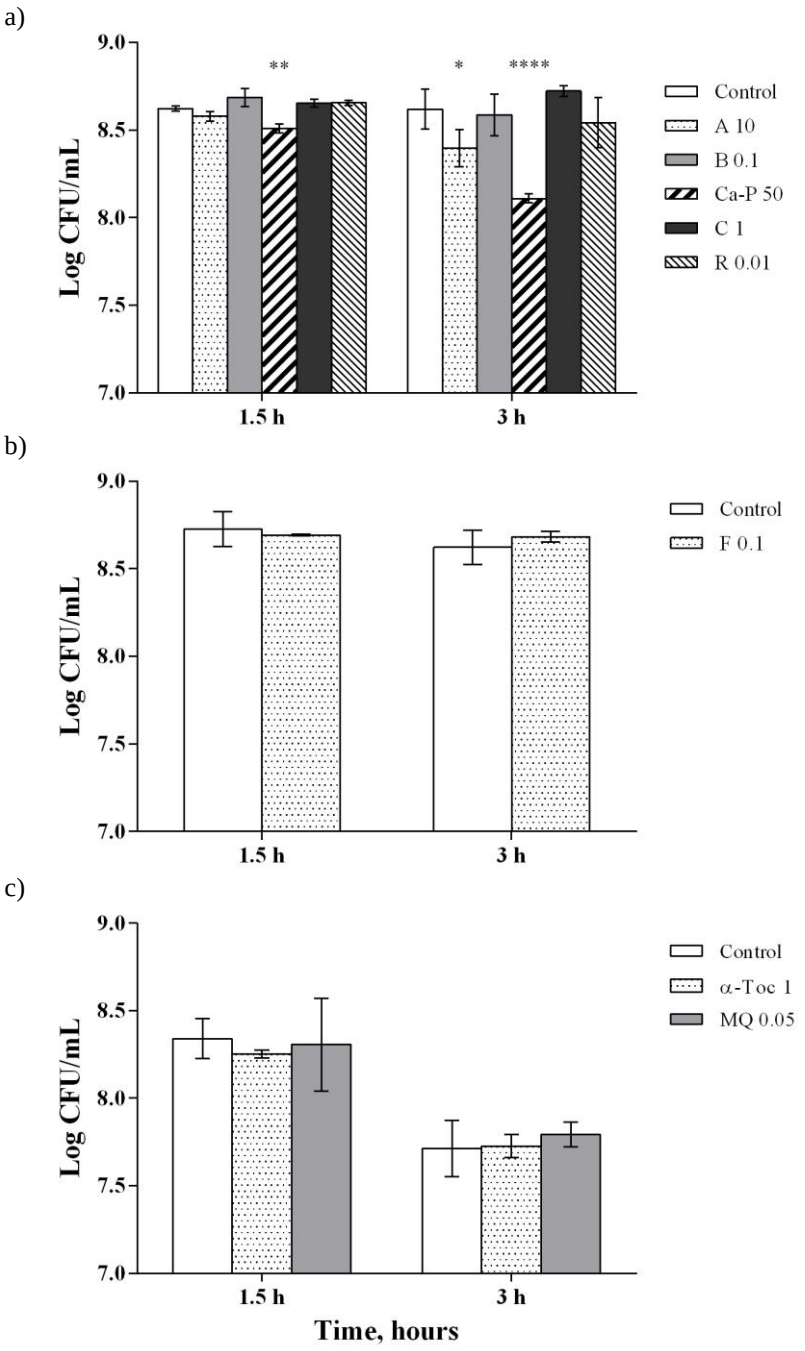

Figure 2.6 Number (Log CFU/mL) of *E. coli* O157:H7 after 1.5 and 3 h exposure to maximum concentration of (a) water soluble vitamins [ascorbic acid (A), biotin (B), calcium pantothenate (Ca-P), cobalamin (C), and riboflavin (R)], (b) folic acid (F), and (c) fat soluble vitamins [α-tocopherol (α-Toc), and menaquinone (MQ)]; and controls: (a) double distilled water, (b) potassium hydroxide (pH 7.1; adjusted with hydrochloric acid), and (c) 8% ethanol, at 37°C, 5% CO_2, static. Vitamin initial is followed by the vitamin concentration (mg/mL). Bars indicate means of three biological replicates and vertical lines standard deviations (±SD). Significant difference between numbers of vitamin and control treated *E. coli* O157:H7 (Log CFU/mL) at the same time point were determined by repeated measures one-way ANOVA with Holm-Sidak's multiple comparison test: *(p<0.05), **(p<0.01), ****(p<0.0001). Number of *E. coli* O157:H7 following folic acid (1 mg/mL) and control treatment at the same time point, were analyzed using a paired *t*-Test (p<0.05).

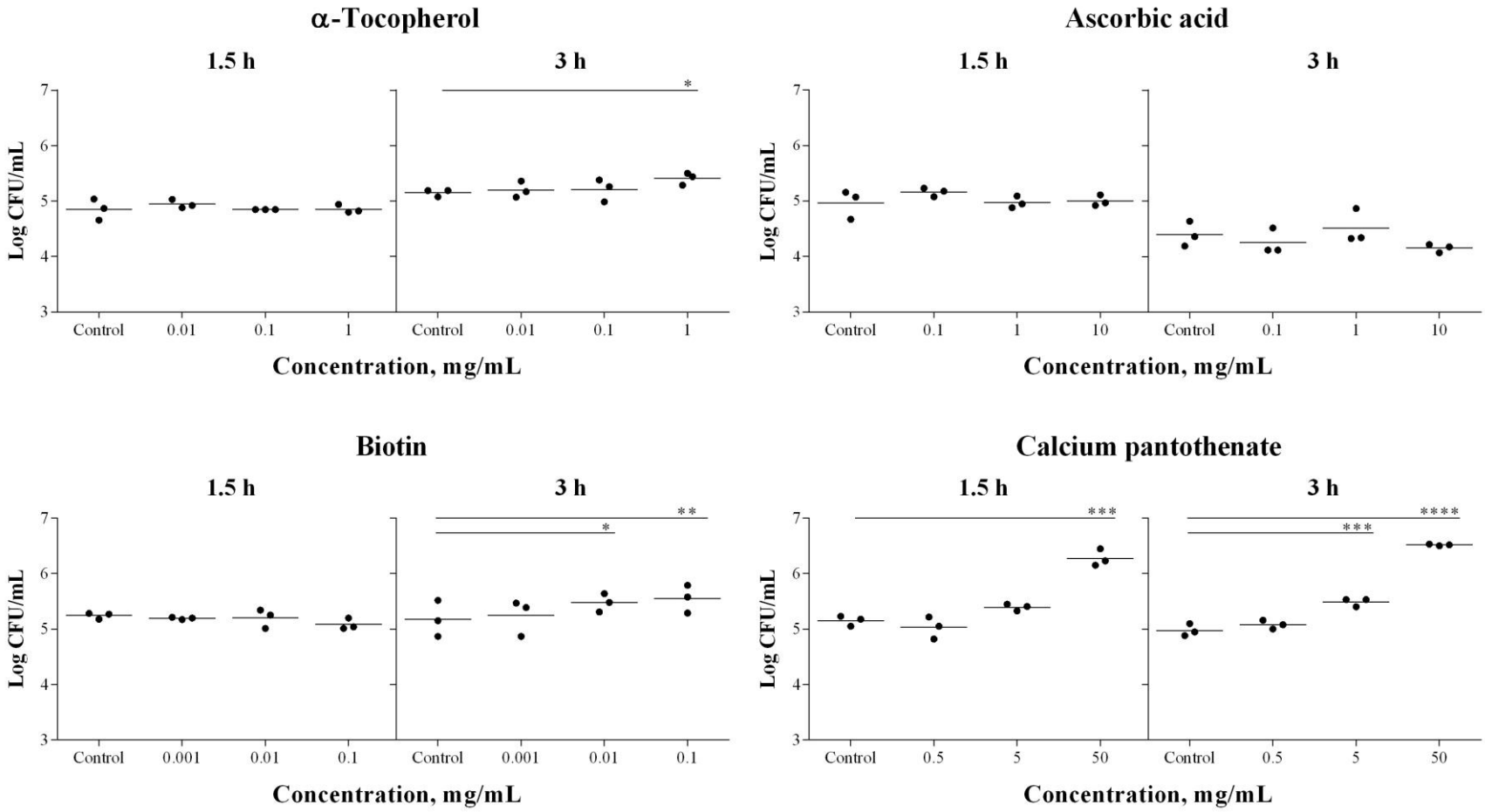

Figure 2.7 Number of adhered *E. coli* O157:H7 (Log CFU/mL) to HeLa cells following 1.5 and 3 h vitamin exposure. Horizontal lines indicate means of three biological replicates. Significant differences were determined using repeated measures one-way ANOVA with Holm-Sidak's multiple-comparison test: *(p<0.05); **(p<0.01); ***(p<0.001); ****(p<0.0001).

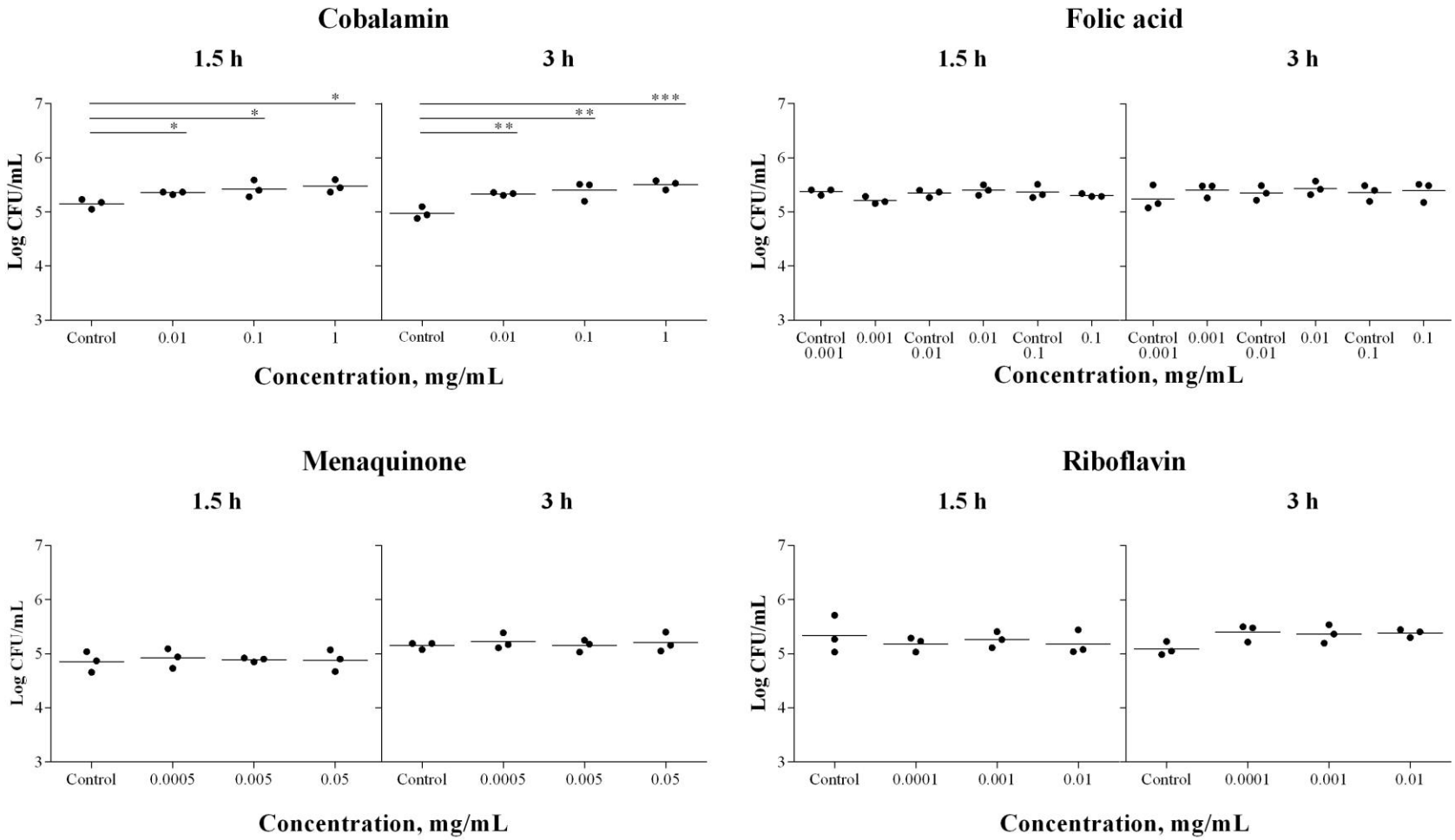

Figure 2.8 Number of adhered *E. coli* O157 (Log CFU/mL) to HeLa cells following 1.5 and 3 h vitamin exposure. Horizontal lines indicate means of three biological replicates. Significant differences were determined using repeated measures one-way ANOVA with Holm-Sidak's multiple-comparison test: *(p<0.05); **(p<0.01); ***(p<0.001).

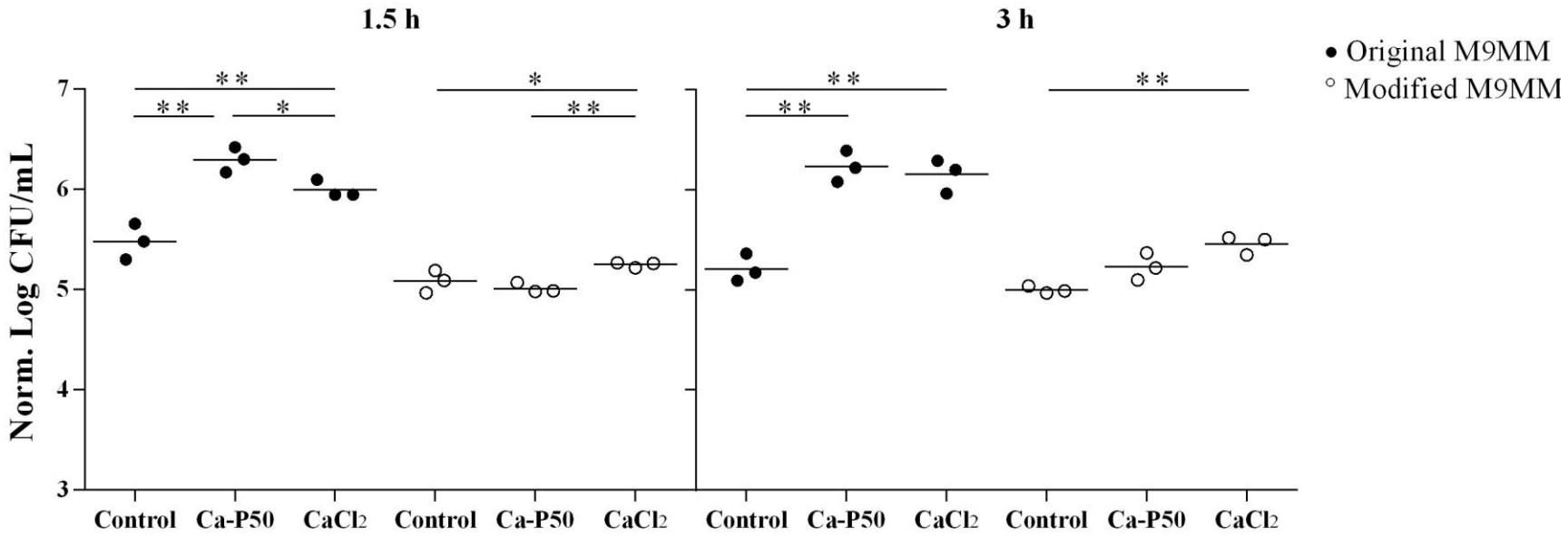

Figure 2.9 Normalized number (Norm. Log CFU/mL) of *E. coli* O157:H7 adhering to HeLa cells after 1.5 and 3 h treatment with calcium pantothenate (50 mg/mL), $CaCl_2$ (equimolar Ca^{2+}), or control (double distilled water) in original and modified (calcium and phosphate free) M9 minimal medium. Horizontal lines indicate medians of three biological replicates. Normalized number (Norm. Log CFU/mL) of *E. coli* O157:H7 adhering to HeLa cells, following calcium pantothenate, calcium chloride, and control treatment at the same time point and for the same M9 medium, were analyzed using repeated measures one-way ANOVA followed by Tukey's multiple-comparison test: *(p<0.05), **(p<0.01). Note: prior to logarithmic transformation, numbers of adhered and *E. coli* O157:H7 were normalized based on bacterial plate counts at the moment of infection. Briefly, number of adhered vitamin treated *E. coli* O157:H7 (CFU/mL) was divided by a ratio of mean number of vitamin and control treated *E. coli* O157:H7 (CFU/mL) at the moment of infection.

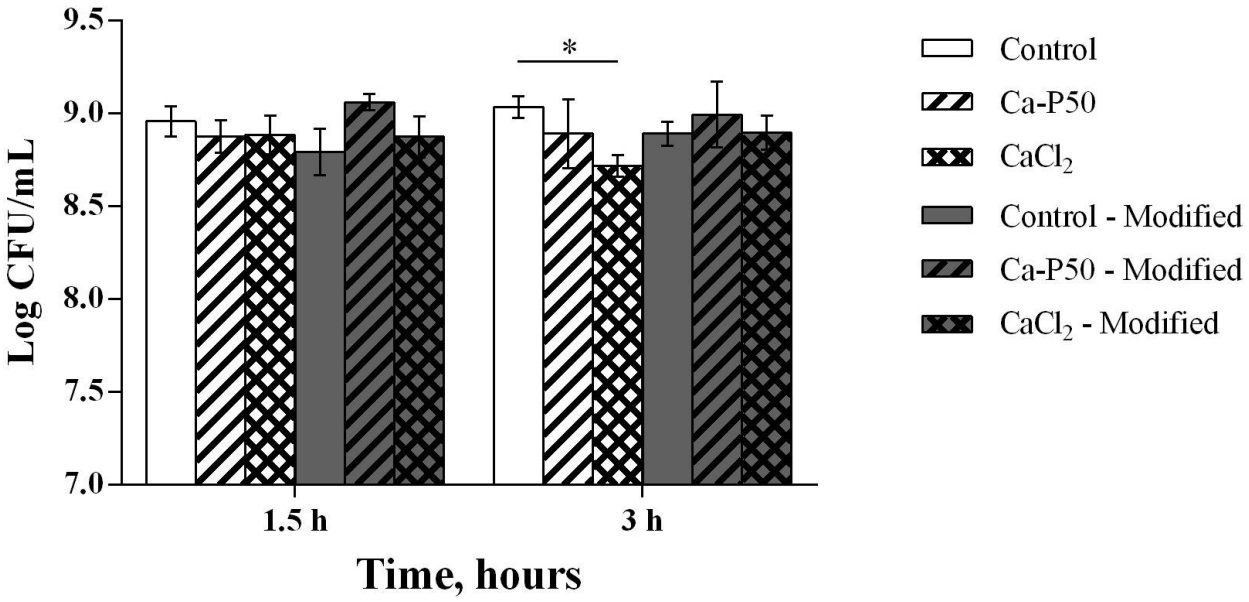

Figure 2.10 Number (Log CFU/mL) of *E. coli* O157:H7 after 1.5 and 3 h treatment with calcium pantothenate (50 mg/mL; Ca-P50), $CaCl_2$ (equimolar Ca^{2+}), or control (double distilled water) in original and modified (calcium and phosphate free) M9 minimal medium. Bars indicate means of three biological replicates and vertical lines standard deviations (±SD). Statistical analysis of the number of calcium pantothenate, calcium chloride, and control treated *E. coli* O157:H7 (Log CFU/mL) for the same M9 medium and at the same time point was performed on three biological replicates using repeated measures one-way ANOVA with Tukey's multiple-comparison test: *(p<0.05).

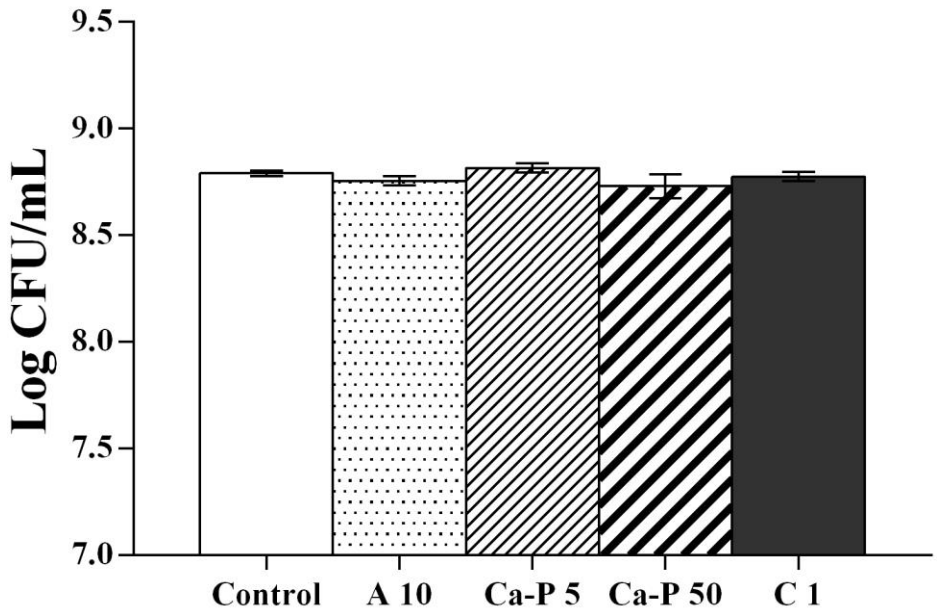

Figure 2.11 Number (Log CFU/mL) of *E. coli* O157:H7 after 3 h exposure to ascorbic acid (10 mg/mL, A 10), calcium pantothenate (5 mg/mL; Ca-P 5, and 50 mg/mL; Ca-P 50), cobalamin (1 mg/mL; C 1), or control (double distilled water) at 37°C, static. Bars indicate means of two biological replicates and vertical lines standard deviations (±SD). Repeated measures one-way ANOVA with Holm-Sidak's test revealed no significant (p>0.05) difference between numbers (Log CFU/mL) of vitamin and control treated *E. coli* O157:H7.

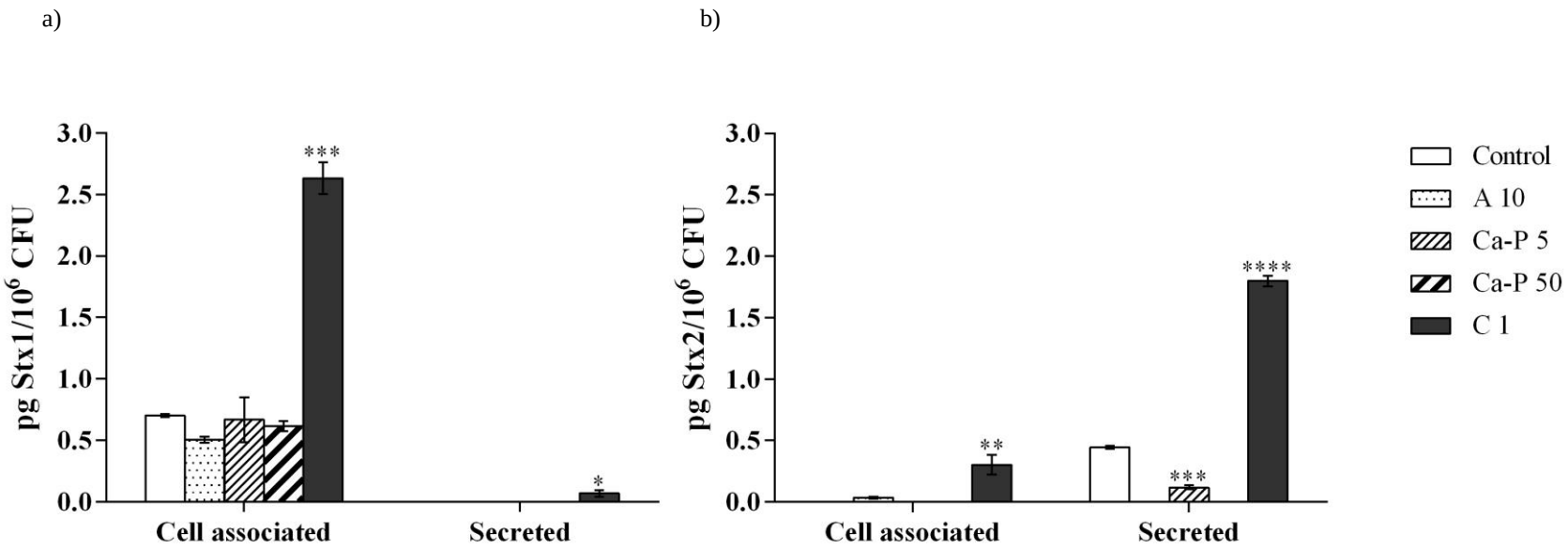

Figure 2.12 Amounts of cell associated and secreted a) Shiga toxin 1 (pg Stx1/10^6 CFU) or b) Shiga toxin 2 (pg Stx2/10^6 CFU) by *Escherichia coli* O157:H7 EDL 933 after 3 h treatment with ascorbic acid (10 mg/mL; A 10), calcium pantothenate (5, 50 mg/mL; Ca-P 5; Ca-P 50), cobalamin (1 mg/mL; C 1) or control (double distilled water) using Stx ELISA. Bars indicate means and vertical lines standard deviations (±SD) of two biological replicates. Significant differences between the amounts of Stx1 (pg Stx1/10^6 CFU) or Stx2 (pg Stx2/10^6 CFU) produced after vitamin and control treatment, were separately determined for cell associated and secreted Stx using repeated measures one-way ANOVA with Holm-Sidak's multiple-comparison test: *(p<0.05), **(p<0.01); ***(p<0.001); ****(p<0.0001)..

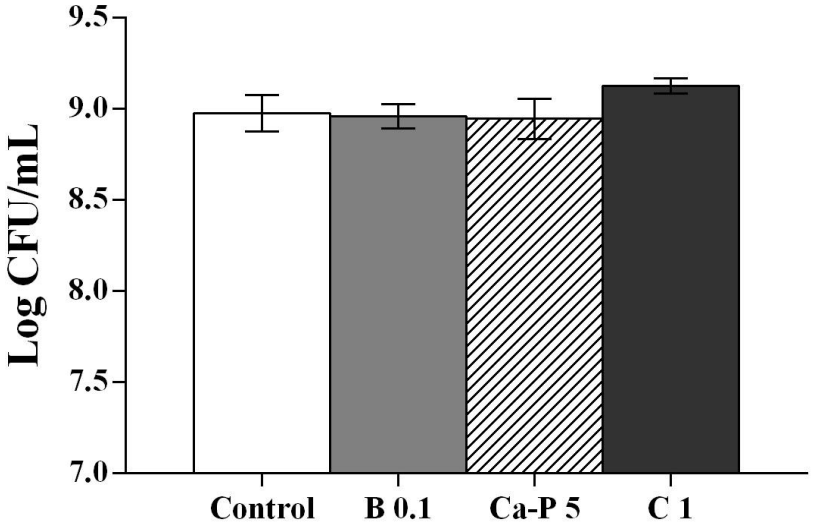

Figure 2.13 Number (Log CFU/mL) of *E. coli* O157:H7 after 3 h treatment with biotin (0.1 mg/mL, B 0.1), calcium pantothenate (5 mg/mL; Ca-P 5), cobalamin (1 mg/mL; C 1), or control (double distilled water) at 37°C, 5% CO_2, static. Bars indicate means of three biological replicates and vertical lines standard deviations (±SD). Repeated measures one-way ANOVA with Holm-Sidak's multiple-comparison test revealed no significant (p>0.05) differences between numbers (Log CFU/mL) of vitamin and control treated *E. coli* O157:H7.

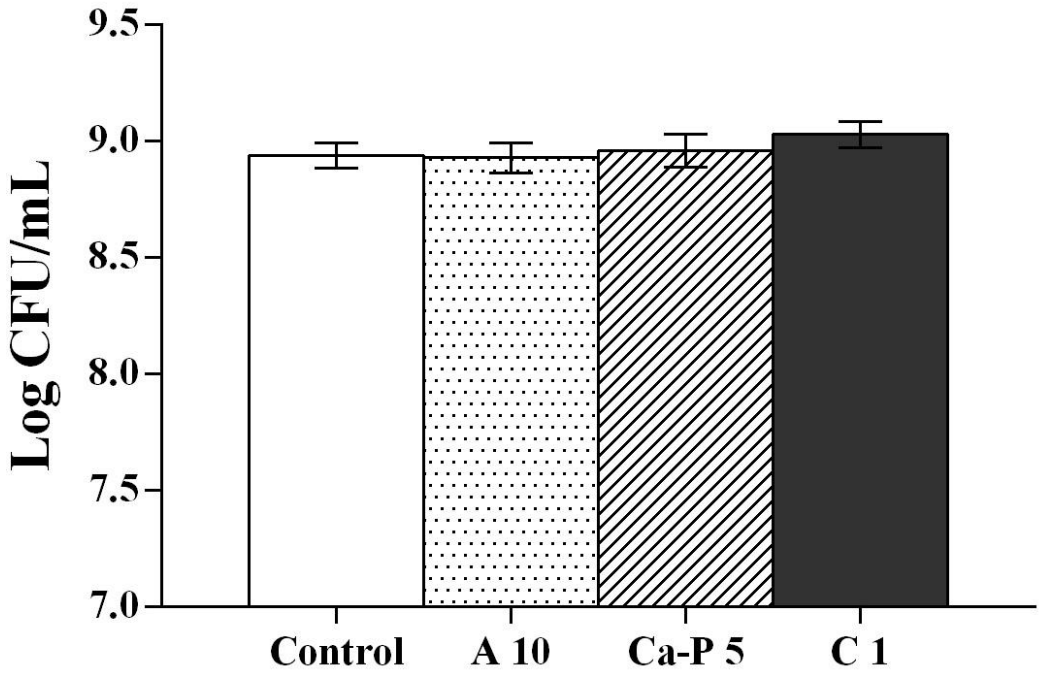

Figure 2.14 Number (Log CFU/mL) of *E. coli* O157:H7 after 3 h treatment with ascorbic acid (10 mg/mL, A 10), calcium pantothenate (5 mg/mL; Ca-P 5), cobalamin (1 mg/mL; C 1), or control (double distilled water) at 37°C, static. Bars indicate means of three biological replicates and vertical lines standard deviations (±SD). Repeated measures one-way ANOVA with Holm-Sidak's multiple-comparison test revealed no significant (p>0.05) differences between numbers (Log CFU/mL) of vitamin and control treated *E. coli* O157:H7.

Chapter 3: Influence of vitamin exposure on *Escherichia coli* O157:H7 stress response gene expression using *lux*-bioluminescence assays and quantitative real-time polymerase chain reaction

3.1 Introduction

Escherichia coli O157:H7 is a foodborne pathogen capable of causing diarrhea, hemorrhagic colitis and hemolytic uremic syndrome in humans (Karmali et al., 2010). In order to survive food processing interventions and cause disease, *E. coli* O157:H7 has to be capable of withstanding stress (Abee & Wouters, 1999). Generally, stress can be defined as any deviation which alters optimal conditions and results in reduced growth rate of bacteria (Storz & Hengge-Aronis, 2000). The general stress response regulator, RpoS (RNA polymerase sigma factor), controls the expression of more than 50 stress response genes, one of which is *osmC* which encodes high osmolarity stress response protein (Bouvier et al., 1998; Hengge-Aronis, 2000). Levels of RpoS may also be influenced by the heat shock protein, DnaK, which inhibits its proteolytic degradation (Hengge-Aronis, 2000). It is well-known that RpoS is expressed upon bacterial entry into stationary phase, but also under varied environmental conditions, such as starvation (Hengge-Aronis, 2002b), osmotic shock (Hengge-Aronis, 1996), cold/heat shock (Muffler et al., 1997; Sledjeski et al., 1996), and acid stress (Lee et al., 1995). It is not known whether vitamins naturally occurring in food or produced by our enteric flora such as biotin, cobalamin, folate, menaquinone, pantothenate, and riboflavin influence stress response in *E. coli* O157:H7 (Guarner & Malagelada, 2003; Hill, 1997; Suttie, 1995). The goal of this research was to determine if food-related or enterically-produced vitamins provide environmental cues for the expression of the general stress response gene in *E. coli* O157:H7 by using *lux*-promoter

constructs. Additionally, quantitative real-time polymerase chain reaction (qPCR) was used to examine the expression of general and specific (acidic and oxidative) stress response genes in *E. coli* O157:H7 cells treated with certain vitamins.

3.2 Materials and methods

3.2.1 Bacterial strain, culture and growth conditions

Escherichia coli O157:H7 str. EDL 933 carrying stress-related promoter fusions *rpoS-lux*CDABE *(rpoS-lux),* *osmC-lux*CDABE *(osmC-lux),* or *dnaK-lux*CDABE *(dnaK-lux),* on plasmids pKA1, pKA4-1, and pKA6 respectively (Dr. Kevin Allen bacterial collection, donated by Dr. Lili R. Mesak; stress response promoter fragments were cloned into pCS26, so the promoter regulates *lux* genes) were streaked on Luria Bertani (LB) agar (Fisher Scientific) containing 50 µg/mL kanamycin mono sulphate (Fisher Scientific) and incubated at 37°C for 24 h. A colony was picked and inoculated in 3 mL M9 minimal medium (M9MM) (BD) supplemented with 0.4% (w/v) D-glucose, 100 µM calcium chloride, 2 mM magnesium sulfate (Fisher Scientific), and kanamycin mono sulphate (50 µg/mL). Cells were grown with shaking (170 rpm) at 37°C. After 16 h, 1 mL culture was transferred in 100 mL M9MM with kanamycin (50 µg/mL), and grown until late exponential phase was reached [optical density at 600 nm (OD_{600}) of 0.50 ± 0.03] (Spectronic 20, Bausch & Lomb), at which time treatments were applied.

3.2.2 Vitamins treatments and bioluminescence measurements

Vitamins were prepared as previously described (section 2.2.2). Late exponential phase *E. coli* O157:H7 (180 µL; OD_{600} of 0.50 ± 0.03) carrying stress-related promoter fusions (section 3.2.1) were exposed to 20 µl of α-tocopherol (0.1, 1, 10 mg/mL), ascorbic acid (1, 10, 100 mg/mL), biotin (0.01, 0.1, 1 mg/mL), calcium pantothenate (5, 50, 500 mg/mL), cobalamin (0.1, 1, 10 mg/mL), folic acid (0.01, 0.1, 1 mg/mL), menaquinone (0.005, 0.05, 0.5 mg/mL),

riboflavin (0.001, 0.01, 0.1 mg/mL), or control (Table 2.1) in a Nunc™ microwell™ 96-well optical-bottom plates with polymer base (Thermo Scientific, Thermo Fisher Scientific Inc., USA). Optical density at 595 nm (OD_{595}) and bioluminescence at 490 nm (indicated as relative luminescence units, RLU) were monitored at 37°C (after shaking) with readings every half an hour over a period of 3 h using a Wallac Victor 1420 Multi-label Counter (Perkin Elmer Life Sciences, Turku, Finland). The experiment was repeated in three biological and three technical replicates. To allow for a comparison between promoter activities of *dnaK*, *osmC*, and *rpoS* in vitamin and control treated cells, relative luminescence units (RLU) were normalized by the respective mean OD_{595} values at each time point (RLU/OD) as described by Bresolin et al. (2006).

3.2.3 Evaluation of stress response gene expression in vitamin treated *E. coli* O157:H7 using qPCR

Escherichia coli O157:H7 EDL 933 was grown and treated with ascorbic acid (10 mg/mL), calcium pantothenate (5 mg/mL), cobalamin (1 mg/mL) or control (double distilled water) as described in sections 2.2.1 and 2.2.13, under conditions applied in Shiga toxin quantification assay (37°C, static). Following 3 h treatment, qPCR was used to evaluate the expression of general, acidic, and oxidative stress response genes on a CFX96 Touch™ real-time PCR thermal cycler (Bio-Rad, Mississauga, ON) using a two-step strategy as described in section 2.2.13. Oligonucleotides (n=7; Table 3.1.) used in qPCR were based on *E. coli* O157:H7 str. Sakai sequence and previously published by Allen (2007), or on *E. coli* O157:H7 str. EDL 933 sequence (AE005174.2, NCBI gene bank). Regarding the latter, oligonucleotides were designed using PrimerQuest® (http://www.idtdna.com/Scitools) (IDT Coralville, IA, USA). All oligonucleotides were assessed for efficiency from cDNA reactions using five-fold dilution

series in nuclease-free water (Qiagen) over a minimum of five points. Oligonucleotide pairs were determined to be acceptable if they had a quantification (or threshold) cycle number (C_t) >15 and <38, efficiencies >85 but <110%, a single amplicon as revealed by melting curve analysis, yielded a single band on a 2.5% agarose gel, and had a standard curve coefficient of determination (R^2) ≥0.985 (Taylor et al., 2010), as determined by CFX Manager™ Software, Version 3.0 (Bio-Rad). Real-time polymerase chain reaction was performed to quantify mRNA transcript levels for a selected gene in vitamin treated *E. coli* O157:H7, relative to the expression of the gene in the control treated cells. Reactions were carried out in a final volume of 20 µL, containing 1 µL cDNA template, 10 µL SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), and 0.25 µM forward and reverse oligonucleotides. Reaction mixtures were first denatured at 95°C for 3 min, and subsequently cycled 39 times as follows: 95°C for 10 sec, annealing at 58°C for 3 sec, and extension at 72°C, 12 sec. Melting curves were generated by increasing temperature from 65°C to 95°C in 0.5°C increments each for 5 sec. Relative changes in the expression levels for genes of interest were normalized against two housekeeping genes (*rpoA*, encoding α-subunit of RNA polymerase, and *tufA*, encoding elongation factor Tu; with the acceptable gene stability value (M) <0.5 and coefficient of variation (CV) <0.25) (Hellemans et al., 2007), and were analyzed by the normalized expression ($\Delta\Delta C_t$) mode in CFX Manager™ Software, Version 3.0 (Bio-Rad). Experiments were run in three biological and two technical replicates. Threshold cycle number standard deviation (SD) for all genes and treatments was ≤0.3. Run-to-run variation within a series of qPCR measurements for a given gene was avoided by using sample maximization strategy (Hellemans et al., 2007), which requires all samples (or as many as possible) to be analysed for a given gene in the same run (Figure 2.3). In this

strategy, no inter-run calibration was required and there was no need to repeat the measurement of the reference gene in each run (Hellemans et al., 2007).

In addition to evaluation of relative gene expression using qPCR, number of *E. coli* O157:H7 at 3 h vitamin or control treatment was assessed by serial dilution in 1X PBS and surface plating on LB agar in three biological and three technical replicates (as described in section 2.2.13). Repeated measures one-way ANOVA with Holm-Sidak's multiple-comparison test revealed no significant (p<0.05) differences in the CFU/ml of *E. coli* O157:H7 between vitamin and control treatments using Prism6 (GraphPad Software, Inc., La Jolla, CA, USA) (results reported in Figure 2.14).

3.2.4 pH

Measurement of pH for M9MM after addition (10:1 ratio) of ascorbic acid (1, 10, 100 mg/mL), calcium pantothenate (5, 50, 500 mg/mL), or cobalamin (0.1, 1, 10 mg/mL), were carried out using an Accumet® Basic AB15 pH Meter (Fisher Scientific).

3.3 Results

3.3.1 Influence of vitamins on *rpoS*, *osmC*, and *dnaK* expression in *E. coli* O157:H7 using *lux*-bioluminescence assay

Optical density at 595 nm of *E. coli* O157:H7 EDL 933 carrying *rpoS-lux*, *osmC-lux*, or *dnaK-lux* promoter constructs was monitored during 3 h treatment with ascorbic acid (0.1, 1, 10 mg/mL), biotin (0.001. 0.01, 0.1 mg/mL), riboflavin (0.0001, 0.001, 0.01 mg/mL), folic acid (0.001, 0.01, 0.1 mg/mL) (Figures 3.1, 3.5, and 3.9), cobalamin (0.01, 0.1, 1 mg/mL), calcium pantothenate (0.5, 5, 50 mg/mL), α-tocopherol (0.01, 0.1, 1 mg/mL), menaquinone (0.0005, 0.005, 0.05 mg/mL) (Figures 3.2, 3.6, and 3.10), or respective controls. Majority of the treatments, including ascorbic acid (0.1, 1, 10 mg/mL), biotin (0.001. 0.01, 0.1 mg/mL),

riboflavin (0.0001, 0.001, 0.01 mg/mL), folic acid (0.001, 0.01, 0.1 mg/mL), cobalamin (0.01 mg/mL), calcium pantothenate (0.5 mg/mL), α-tocopherol (0.01 mg/mL), and menaquinone (0.0005, 0.005 mg/mL) resulted in growth curves similar to the control during 3 h. Ascorbic acid (10 mg/mL) treatment yielded the lowest OD_{595} values of *E. coli* O157:H7 during this time (Figures 3.1, 3.5, and 3.9). While OD_{595} of *E. coli* O157:H7 cells treated with water soluble vitamins increased, treatment with fat soluble vitamins resulted in no change in OD_{595} during 3 h. However, OD_{595} of *E. coli* O157:H7 carrying *rpoS-lux, osmC-lux* or *dnaK-lux* promoter constructs at 10 min (0.2 h) treatment with cobalamin (0. 1, 1 mg/mL), calcium pantothenate (5, 50 mg/mL), α-tocopherol (0.1, 1 mg/mL), or menaquinone (0.05 mg/mL), differed >5.5% compared to the controls. This could be due to insolubility or precipitation of vitamin treatments rather than changes in bacterial cell concentrations. For example, upon addition of calcium pantothenate (≥5 mg/mL; w:v final concentration) to bacterial cells grown in M9MM supplemented with kanamycin (50 µg/mL), a precipitate formed due to reaction of calcium with phosphate buffer system present in M9MM, which could affect the absorbance. In addition, α-tocopherol is not soluble in water at >0.1 mg/mL. Also, cobalamin is intensively red at ≥0.1 mg/mL, which could affect the absorbance as the instrument was not blanked against M9MM with cobalamin prior to the measurements. Since certain vitamins affected OD_{595} at 10 min (0.2 h) treatment, their relative luminescence values could not be normalized against OD_{595}, and therefore were excluded from further analysis. However, if this experiment was to be repeated, with unlimited time and resources, it would be suggested to use plate counts to determine CFU/mL instead, and avoid the OD problem.

Normalized relative luminescence (RLU/OD) of *E. coli* O157:H7 EDL 933 carrying *rpoS-lux, osmC-lux* or *dnaK-lux* promoter constructs was monitored during 3 h treatments with

vitamins that did not affect OD_{595} at 0.2 h for more than 5.5% compared to the controls (Figures 3.3, 3.4, 3.7, 3.8, 3.11, and 3.12). At 3 h, >2.0-fold down regulation in the promoter activities was observed only in *E. coli* O157:H7 EDL 933 carrying *rpoS-lux* (3.9-fold) and *osmC-lux* (5.7-fold) for ascorbic acid (10 mg/mL) treatment compared to control (Figures 3.3 and 3.7). More than 2.0-fold down regulations of *dnaK-lux* noticed in ascorbic acid (10 mg/mL) treatment compared to the control at 1, 1.5, and 2 h were diminished by 3 h (Figure 3.11). At 3 h, all other vitamin treatments resulted with <2.0-fold change in the promoter activities of *E. coli* O157:H7 carrying *rpos-lux*, *osmC-lux* and *dnaK-lux* prompter constructs compared to the controls (Figures 3.3, 3.4, 3.7, 3.8, 3.11, and 3.12). At 0.2 h, average light emissions of *E. coli* O157:H7 EDL 933 wild strain (negative control) in wells containing *E. coli* O157:H7 with *rpoS-lux*, *osmC-lux*, or *dnaK-lux* promoter constructs were 700, 50, and 300 RLU (data not presented). The difference in luminescence levels of the negative control in different wells was probably due to difference in the up-regulated light production in the neighbouring wells.

3.3.2 Evaluation of stress response genes expression in vitamin treated *E. coli* O157:H7 using qPCR

Treatment with ascorbic acid (10 mg/mL; 3 h) strongly induced acid stress response genes *gadA* (9.5-fold) and *gadX* (5.6-fold), and particularly a regulator of superoxide anion stress response *soxS* (11.2-fold), while no effect on regulators of general stress response (*rpoS*) and H_2O_2-inducible genes (*oxyR*) was observed (Table 3.2). In contrast, 3 h exposure to calcium pantothenate (5 mg/mL) reduced the expression of *gadA* (1.7-fold), *rpoS* (2.3-fold), and *soxS* (2.4-fold). In the same time, cobalamin (1 mg/mL) treatment slightly induced only glutamate decarboxylase A gene (*gadA*, 1.6-fold), and had no effect on transcription of oxidative stress

response regulators of H_2O_2 and superoxide anion (*oxyR* and *soxS*), nor regulator of general stress response (*rpoS*).

3.3.3 pH

Ascorbic acid (10 mg/mL, pH 4.9) and calcium pantothenate (50 mg/mL, pH 5.2) treatments had the lowest pH values compared to M9MM (pH 6.9) (Table 3.3). pH of the other vitamin treatments, including ascorbic acid (0.1, 1 mg/mL), calcium pantothenate (0.5, 5 mg/mL), and cobalamin (0.01, 0.1, 1 mg/mL), did not differ much from M9MM (pH difference was <0.1).

3.4 Discussion

The promoter fusions with bioluminescent *lux*CDABE genes can be used to create light emitting-sensors in prokaryotes, which allow for detection of different chemical and physiological processes in bacteria (Meighen, 1993). These bioluminescence reporters were particularly convenient because of their sensitivity, ability to provide results quickly (Meighen, 1993), and monitor gene expression non-destructively and in a real-time (Shimizu et al., 2011). The *lux*CDABE system contains *lux*AB genes which encode luciferase, as well as *lux*CDE genes which encode fatty acid reductase polypeptides responsible for aldehyde substrate synthesis required for the reaction (Meighen, 1993):

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} FMN + RCOOH + H_2O + \text{light (490 nm)}$$

The system is particularly convenient as it does not require supplementation of aldehyde, nor $FMNH_2$ present in bacteria (Meighen, 1993). In the present study, promoter fusions *rpos*-, *osmC*-, or *dnaK*-*lux*CDABE were used to evaluate vitamin influence on the expression of genes

encoding general stress response (*rpoS*), osmolarity stress response (*osmC*), or heat shock (*dnaK*) proteins by monitoring luminescence emission over a period of 3 h.

Based on the luminescence data, transcription of *rpoS* following 3 h treatment with α-tocopherol (0.01 mg/mL), ascorbic acid (0.1, 1 mg/mL), biotin (0.001. 0.01, 0.1 mg/mL), calcium pantothenate (0.5 mg/mL), cobalamin (0.01 mg/mL), folic acid (0.001, 0.01, 0.1 mg/mL), menaquinone (0.0005, 0.005 mg/mL), or riboflavin (0.0001, 0.001, 0.01 mg/mL) was not significantly different than control (Figures 3.3 and 3.4). This was not surprising, as much of RpoS regulation is done by translational and post-translational, rather than transcriptional mechanisms (Hengge-Aronis, 2002b). In the fact, transcription of *rpoS* is known to be triggered only by conditions of gradual decrease of growth rate during bacterial entry into stationary phase (Hengge-Aronis, 2000; Hengge-Aronis, 2002b). In contrast, translation of *rpoS* mRNA to RpoS is stimulated with downshift in temperature (Sledjeski et al., 1996), low pH (Hengge-Aronis, 2002b), increased cell density (Lange & Hengge-Aronis, 1994a), or high osmolarity (Muffler et al., 1996b), while post-translational regulation is known to be triggered by starvation (Lange & Hengge-Aronis, 1994a), low pH (Bearson et al., 1996), upshift in growth temperature (Muffler et al., 1997), and hyperosmolarity (Muffler et al., 1996b) (reviewed in section 1.7.1.1). To the best of the author's knowledge none of the vitamin treatments have been previously assessed as potential environmental cues for *rpoS* expression. Due to the importance of RpoS as a global stress response regulator, we felt it was important to evaluate if any of the vitamins might be environmental triggers for *rpoS* expression. In addition, luminescence data suggest that neither *osmC* nor *dnaK* expression were significantly different after 3 h treatments with selected vitamins compared to control (Figures 3.7, 3.8, 3.11, and 3.12).

Even though ascorbic acid (10 mg/mL) treatment resulted in 3.9-fold down-regulation in the promoter activity in *E. coli* O157:H7 carrying *rpos-lux* (Figure 3.3), a similar effect in *rpoS* expression was not seen using qPCR (Table 3.2). If *rpoS-lux*, *osmC-lux*, and *dnaK-lux* experiment was to be repeated with ascorbic acid (10 mg/mL) treatment, use of plate counts to determine CFU/mL and using it to normalize RLU is recommended, instead of use of OD_{595}.

It is important to note that luminescence results of calcium pantothenate (50 mg/mL, pH 5.2) and cobalamin (1 mg/mL) treatments that were previously shown to impact *E. coli* O157:H7 virulence (Chapter 2; Figures 2.7, 2.8, and 2.12), were excluded from the analysis because OD_{595} was impaired. However, influence of 3 h treatment with calcium pantothenate (5 mg/mL), and cobalamin (1 mg/mL) on the relative expression of general stress response (*rpoS*), as well as certain acidic and oxidative stress response genes in *E. coli* O157:H7 was evaluated using qPCR. The treatment with the highest concentration of calcium pantothenate (50 mg/mL) was not assessed, because high quality RNA could not be obtained (likely due to the calcium phosphate precipitate).

As expected, 3 h treatment with ascorbic acid (10 mg/mL, pH 4.9) strongly induced acid stress response gene *gadA* (9.5-fold, Table 3.2), which encodes glutamate decarboxylase A that uses up H^+ ions to convert glutamate to CO_2 and GABA (Foster, 2004). Additionally, the ascorbic acid treatment induced acid stress response gene *gadX* (5.6-fold; Table 3.2) which encodes GadX, a direct activator of gene *gadE* (which encodes GadE, a key activator of *gadA*), thus indirectly enhancing transcription of *gadA* (Sayed et al., 2007). This was in line with the low pH 4.9 of the treatment (Table 3.3). Furthermore, ascorbic acid (10 mg/mL) treatment particularly induced gene *soxS* (11.2-fold, Table 3.2), which encodes a regulator of superoxide anion stress response (SoxS). That data suggest that following ascorbic acid (10 mg/mL, pH 4.9)

treatment, *E. coli* O157:H7 experienced both acid and oxidative stress. This was not surprising as previous pro-oxidant activity of ascorbic acid-iron system has been reported (Rietjens et al., 2001). Specifically, ascorbic acid converts Fe^{3+} to Fe^{2+}, which subsequently reacts with molecular oxygen to produce superoxide anion (Rietjens et al., 2001). Although iron was not present in M9 minimal medium nor in ascorbic acid used in this study (Tables 2.1 and 2.2), trace amounts of iron were possible in reagents due to impurity. For example, ≥99% pure ascorbic acid (Sigma-Aldrich, catalogue number A5960-100G) may contain traces of iron (≤0.0005%). If iron was present in ascorbic acid powder in these minute amounts, this could contribute to c.a. 0.85 μM Fe in ascorbic acid (10 mg/mL) treatment that could potentially lead to the oxidative stress.

In contrast, 3 h exposure to calcium pantothenate (5 mg/mL) reduced the expression of *gadA* (1.7-fold), *rpoS* (2.3-fold), and *soxS* (2.4-fold), suggesting that *E. coli* O157:H7 might experience "lower stress" in the presence of calcium pantothenate compared to the control. Cobalamin (1 mg/mL) treatment only slightly induced glutamate decarboxylase A gene (*gadA*, 1.6-fold), and had no effect on transcription of oxidative stress response regulators of H_2O_2 and superoxide anion (*oxyR* and *soxS*), nor regulator of general stress response (*rpoS*). The neutral pH 7.0 of the treatment however does not suggest acidic conditions were applied.

Further studies are needed to better understand mechanisms behind ascorbic acid, calcium pantothenate, and cobalamin treatments. It might be worth examining the levels of RpoS or microarray profiling of *E. coli* O157:H7 following the treatments with these vitamins.

3.5 Conclusion

In summary, none of the vitamin treatments affected *rpoS*, *osmC*, or *dnaK* as measured by *lux*-bioluminescence assay, with the exception of ascorbic acid (10 mg/mL), which down-

regulated *rpoS* and *osmC* more than 2.0-folds at 3 h. However, qPCR results did not confirm the previously seen down-regulation of *rpoS*, suggesting that *lux*-luminescence data might have been impaired. In the future *lux*-bioluminescence experiments, use of plate counts to determine CFU/mL and using it to normalize RLU is recommended, instead of use of OD_{595}.

Escherichia coli O157:H7 experienced both acid and oxidative stresses after 3 h treatment with ascorbic acid (10 mg/mL, pH 4.9, 3 h). Specifically, acid stress response genes *gadA* and *gadX* were strongly induced (9.5- and 5.6-fold), as wells as superoxide anion stress response gene *soxS* (11.2-fold). Trace amounts of iron (up to c.a. 0.85 μM) could contribute to the pro-oxidative activity of ascorbic acid-iron system in this treatment.

While 3 h exposure to calcium pantothenate (5 mg/mL) reduced the expression of *gadA*, *rpoS*, and *soxS* (1.7-, 2.3-fold, and 2.4-fold) suggesting that *E. coli* O157:H7 might experience "lower stress" compared to the control, cobalamin (1 mg/mL) only slightly induced *gadA* (1.6-fold), and had no effect on transcription of *oxyR*, *soxS*, and *rpoS*. The neutral pH 7.0 of the cobalamin treatment however does not suggest acidic conditions were applied.

Further studies examining mechanisms behind ascorbic acid, calcium pantothenate, and cobalamin treatments are necessary. These may include determination of RpoS level or microarray profiling of *E. coli* O157:H7 following the treatments with these vitamins.

Table 3.1 Oligonucleotides (n=7) used in qPCR to assess stress response gene expression in ascorbic acid, calcium pantothenate, or cobalamin treated *E. coli* O157:H7 EDL 933 under conditions used in Shiga ELISA assay (37°C, 3h, static).

Gene	Primer	Sequence (5'→3')	Length (bp)	Tm (°C)	Physiological function	Reference
gadA	1	GCGTTGCCATCAAGATATAA	20	50.5	Glutamate decarboxylase A	This study
gadA	2	ATGGACCAGAAGCTGTTA	18	50.4		
gadX	1	CAGACTTGGACTCATCAAC	19	50.3	AraC-type activator protein	This study
gadX	2	TCTTCGCTATGCAGAAATG	19	50.1		
oxyR	1	TTCGTAAGCTGGAAGATGAG	20	51.9	Positive regulator of H_2O_2-inducible genes	Allen (2007)
oxyR	2	CAGTAACTGGTGGGTCTGTG	20	55.2		
rpoA	1	GTCAATTCCAGATCGTCAAC	20	51.2	α-subunit of RNA polymerase	Allen (2007)
rpoA	2	TTCATTCGGAAGAAGATGAG	20	49.6		
rpoS	1	GCTTCATATCGTCATCTTGC	20	51.4	Regulator of the general stress response (σ^S)	Allen (2007)
rpoS	2	AACCAAACCCGTACTATTCG	20	52.4		
soxS	1	GATAATCGCTGGGAGTGC	18	53.1	Regulator of superoxide anion stress response	Allen (2007)
soxS	2	ATGGATTGACGAGCATATTG	20	50.3		
tufA	1	ATCGTGTTCCTGAACAAATG	20	50.9	Elongation factor Tu	Allen (2007)
tufA	2	TCTACACGACCGGTAACAAC	20	54.2		

Table 3.2 List of *E. coli* O157:H7 EDL 933 genes, relatively up- or down-regulated to control following the treatment with ascorbic acid, calcium pantothenate, or cobalamin under conditions used in Stx ELISA assay (37°C, 3 h, static).

Functional category and gene		**Physiological function**	**Fold change for the treatment***		
			Ascorbic acid, 10 mg/mL	**Calcium pantothenate, 5 mg/mL**	**Cobalamin, 1 mg/mL**
Acid					
	gadA	Glutamate decarboxylase A	**9.5**	**-1.7**	**1.6**
	gadX	AraC-type activator protein of *gadE*	**5.6**	1.2	1.3
General stress response					
	rpoS	Regulator of the general stress response (σ^S)	1.3	**-2.3**	-1.1
Oxidative					
	oxyR	Positive regulator of H_2O_2-inducible genes	1.3	-1.1	-1.4
	soxS	Regulator of superoxide anion stress response	**11.2**	**-2.4**	-1.0

* Relative changes in the expression levels for genes of interest were normalized against two housekeeping genes: *rpoA*, encoding α-subunit of RNA polymerase, and *tufA*, encoding elongation factor Tu. Housekeeping genes had acceptable gene stability value (M) <0.5 and coefficient of variation (CV) <0.25. Bold are results >1.5-fold up- ("+") or down-("-") regulated. Ct standard deviation for all genes and treatments was ≤0.3.

Table 3.3 pH of vitamin stock solutions diluted in M9 minimal medium (pH 6.9).

Vitamin	Concentration, mg/mL*	pH
Ascorbic acid		
	0.1	6.9
	1	6.8
	10	**4.9**
Ca-pantothenate		
	0.5	6.9
	5	6.8
	50	**5.2**
Cobalamin		
	0.01	6.9
	0.1	7.0
	1	7.0

*Stock solutions of ascorbic acid (1, 10, 100 mg/mL), calcium pantothenate (5, 50, 500 mg/mL), or cobalamin (0.1, 1, 10 mg/mL) were diluted in M9 minimal medium (M9MM) in 1:10 ratio. pH was measured using Accumet® Basic AB15 pH Meter (Fisher Scientific). Bold are treatments with pH lower than M9MM by >0.1.

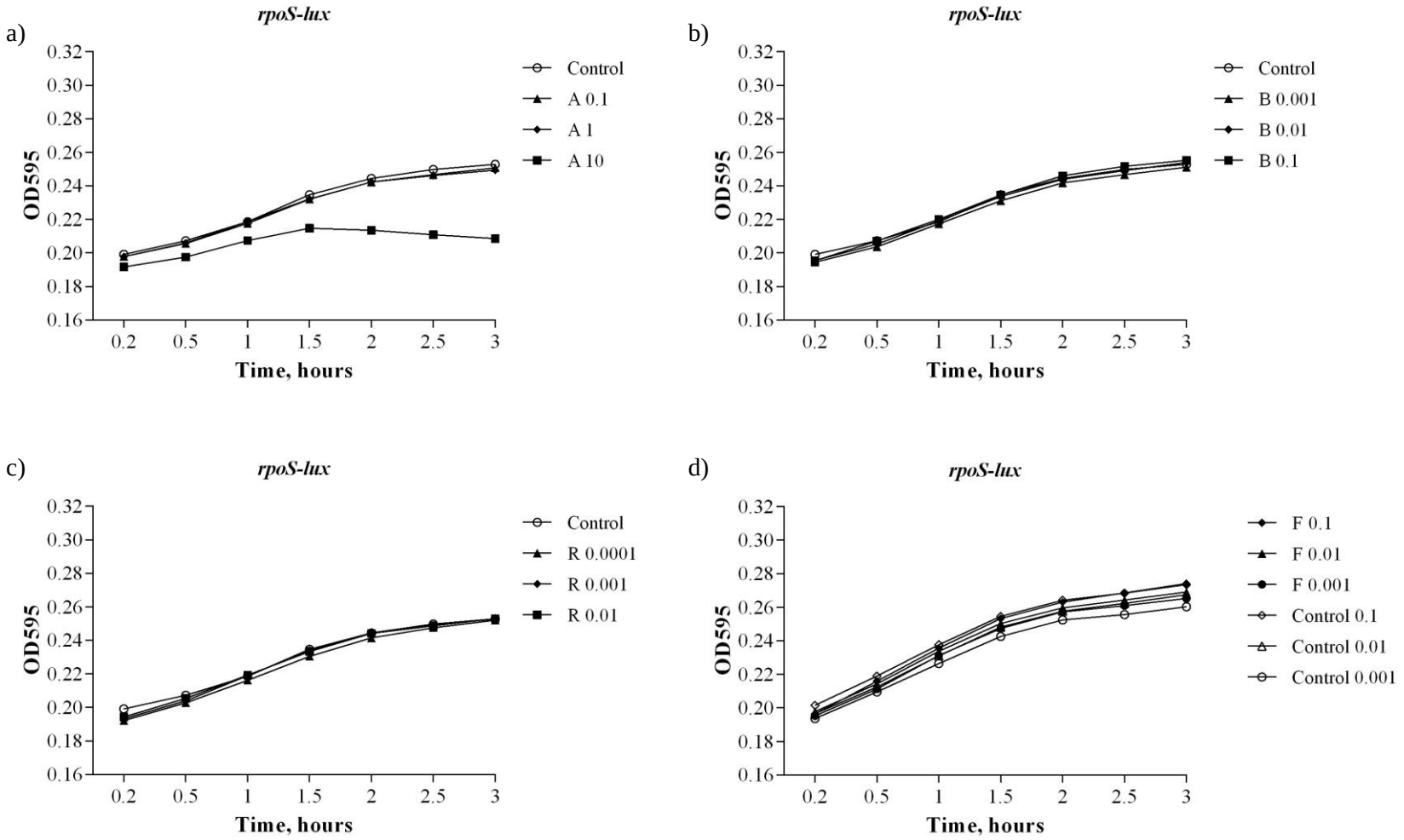

Figure 3.1 Mean optical density at 595 nm (OD_{595}) of *E. coli* O157:H7 EDL 933 (n=3) carrying *rpoS-lux* promoter constructs during 3 h treatment with a) ascorbic acid (A), b) biotin (B), c) riboflavin (R), and d) folic acid (F), or respective control a), b), and c) double distilled water, d) potassium hydroxide (pH 7.4; adjusted with hydrochloric acid). Vitamin concentration (mg/mL) follows the initial of the vitamin name. Standard deviation ranged from 0.001 to 0.008 with the highest level in riboflavin (0.001 mg/mL) at 0.5 h.

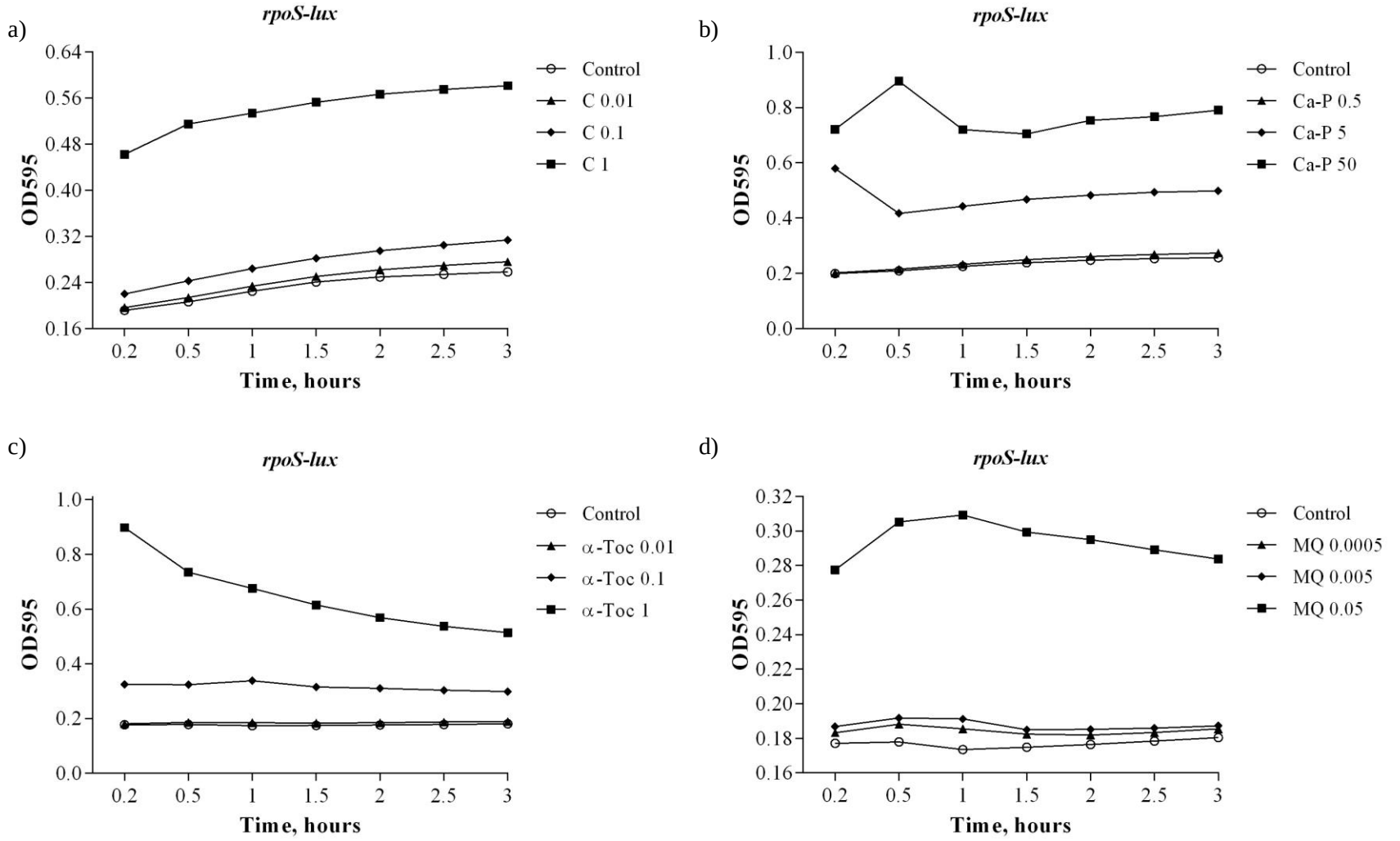

Figure 3.2 Mean optical density (OD_{595}) of *E. coli* O157:H7 EDL 933 (n=3) carrying *rpoS-lux* promoter constructs during 3 h treatment with a) cobalamin (C), b) calcium pantothenate (Ca-P), c) α-tocopherol (α-Toc), and d) menaquinone (MQ), or respective control a) and b) double distilled water, c) and d) 8% ethanol. Vitamin concentration (mg/mL) follows the initial of the vitamin name. Standard deviation ranged from 0.001 to 0.05 with the highest level in cobalamin (1 mg/mL) at 0.5 h.

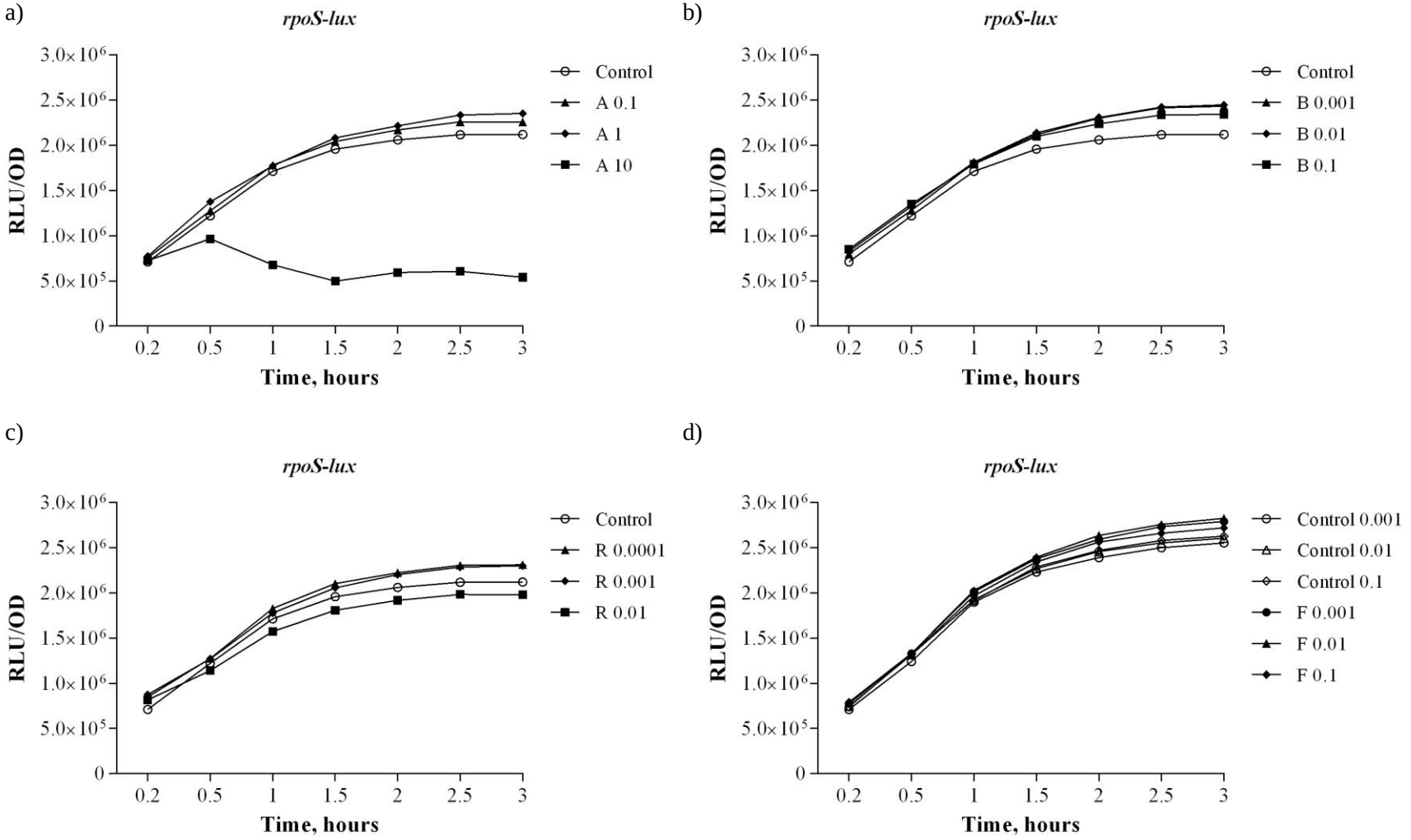

Figure 3.3 Normalized mean relative luminescence (RLU/OD) of *E. coli* O157:H7 EDL 933 (n=3) carrying *rpoS-lux* promoter constructs during 3 h treatment with a) ascorbic acid (A), b) biotin (B), c) riboflavin (R), and d) folic acid (F), or respective control a), b), and c) double distilled water, d) potassium hydroxide (pH 7.4; adjusted with hydrochloric acid). Vitamin concentration (mg/mL) follows the initial of the vitamin name. Normalized relative luminescence (RLU/OD) was obtained by dividing measured relative luminescence data with respective mean OD_{595} values at each time point. Standard deviation ranged from $0.008x10^6$ to $0.2x10^6$, with the highest level in folic acid (0.001 mg/mL) at 2 h.

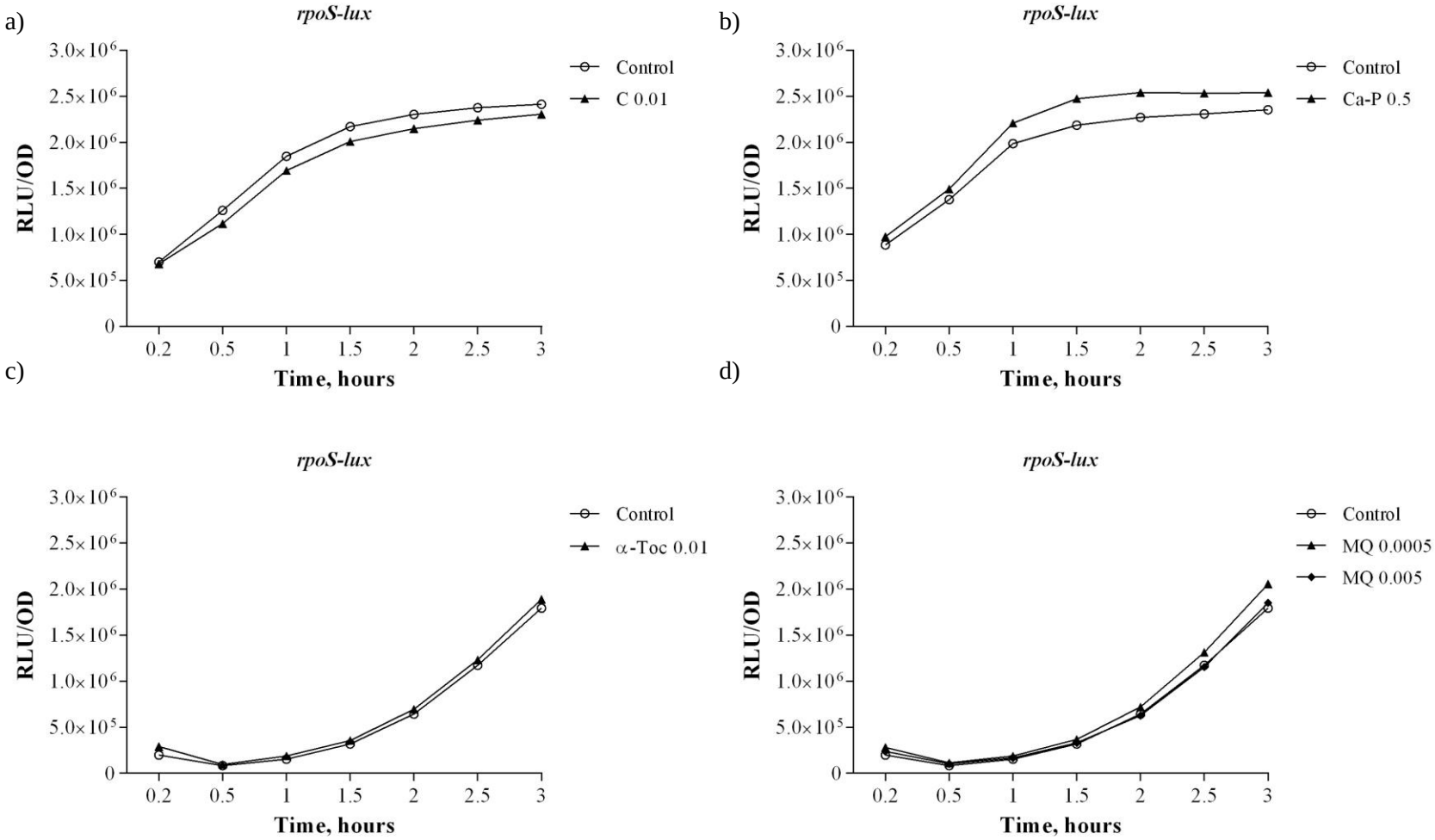

Figure 3.4 Normalized mean relative luminescence (RLU/OD) of *E. coli* O157:H7 EDL 933 (n=3) carrying *rpoS-lux* promoter constructs during 3 h treatment with a) cobalamin (C), b) calcium pantothenate (Ca-P), c) α-tocopherol (α-Toc), and d) menaquinone (MQ), or respective control a) and b) double distilled water, c) and d) 8% ethanol. Vitamin concentration (mg/mL) follows the initial of the vitamin name. Normalized relative luminescence (RLU/OD) was obtained by dividing measured relative luminescence data with respective mean OD_{595} values at each time point. Standard deviation ranged from $0.04x10^5$ to $0.3x10^6$ with the highest level in menaquinone (0.0005 mg/mL) at 3 h.

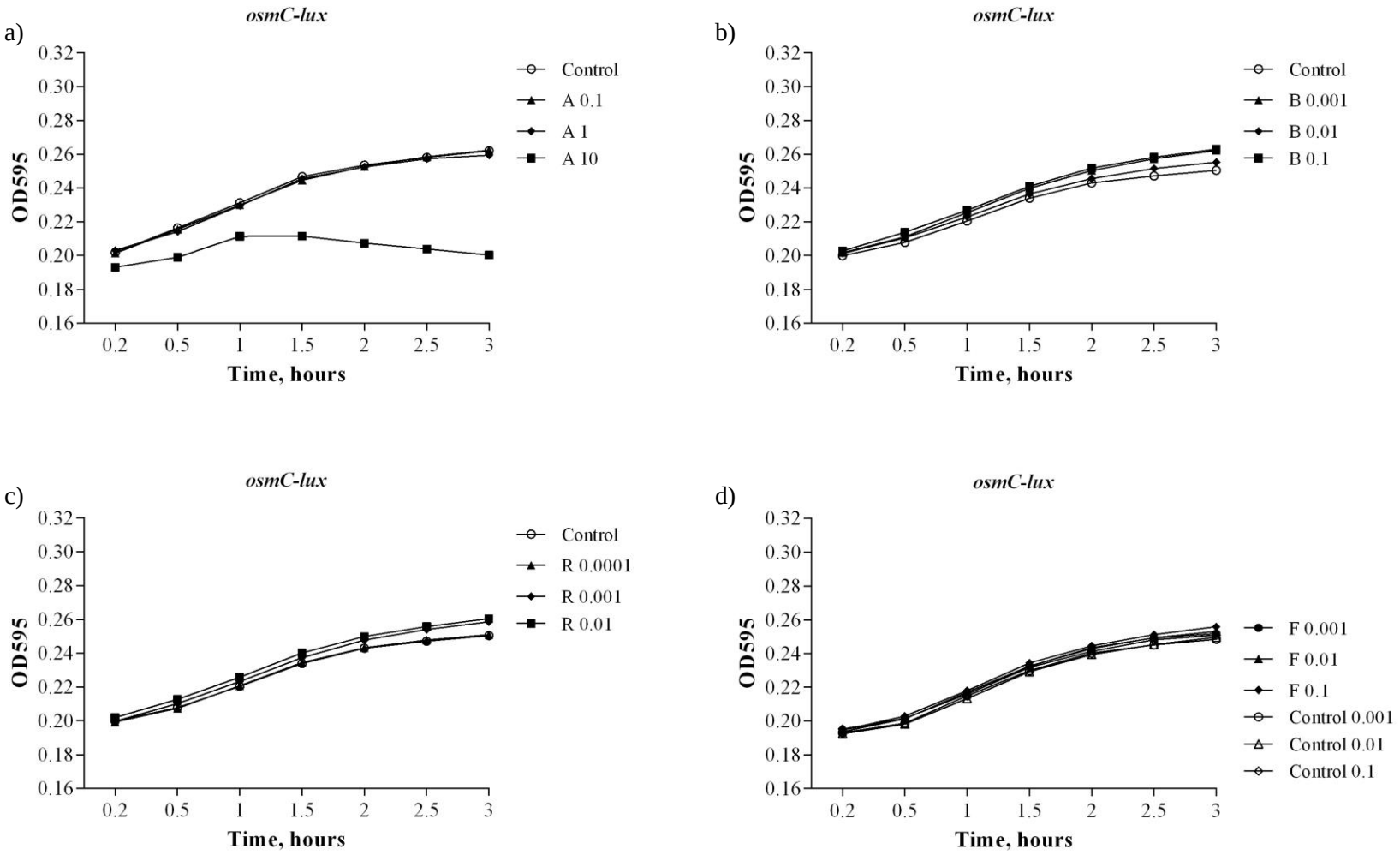

Figure 3.5 Mean optical density at 595 nm (OD_{595}) of *E. coli* O157:H7 EDL 933 (n=3) carrying *osmC-lux* promoter constructs during 3 h treatment with a) ascorbic acid (A), b) biotin (B), c) riboflavin (R), and d) folic acid (F), or respective control a), b), and c) double distilled water, d) potassium hydroxide (pH 7.4; adjusted with hydrochloric acid). Vitamin concentration (mg/mL) follows the initial of the vitamin name. Standard deviation ranged from 0.0004 to 0.03, with the highest level in biotin (0.1 mg/mL) at 3 h.

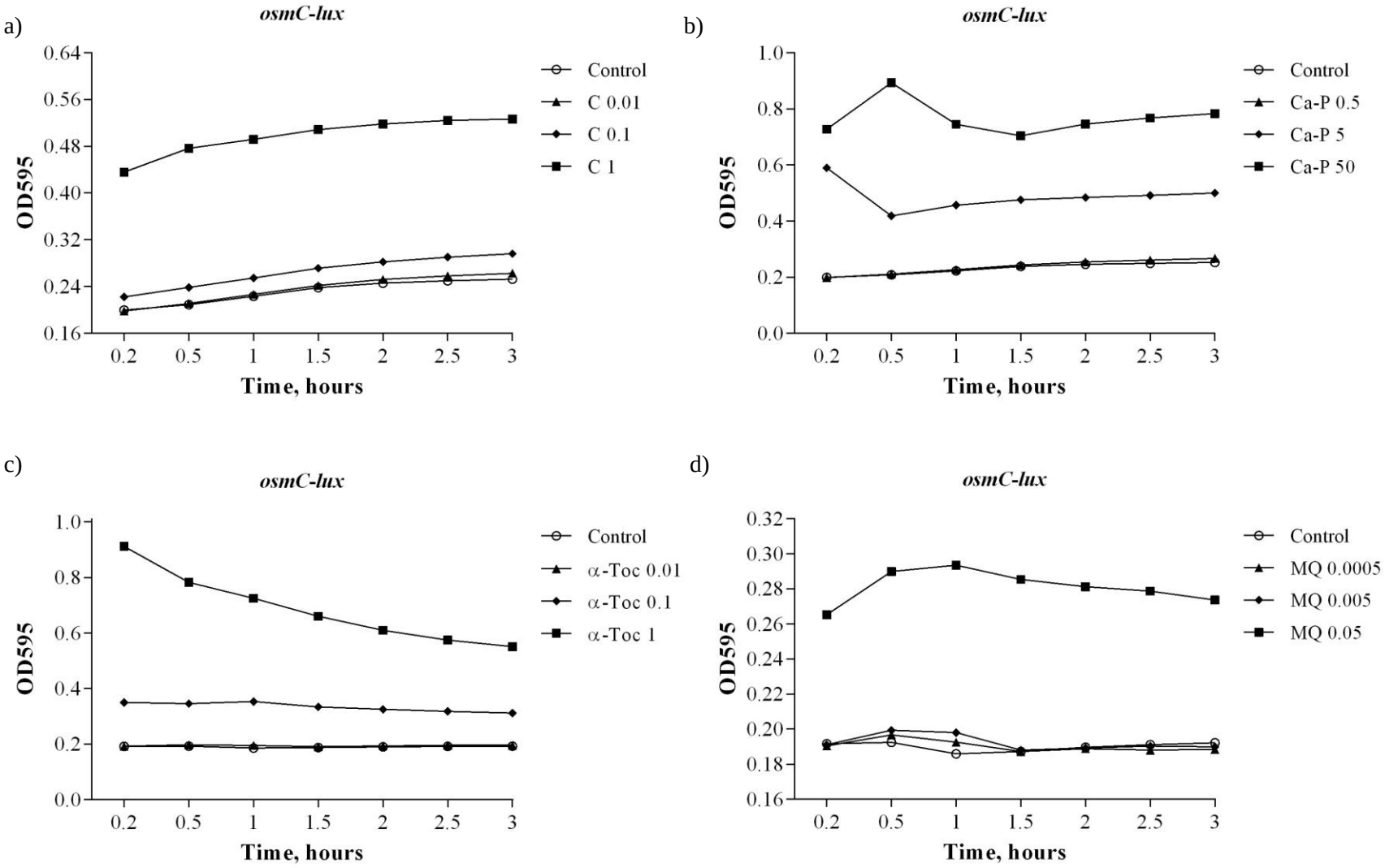

Figure 3.6 Mean optical density (OD_{595}) of *E. coli* O157:H7 EDL 933 (n=3) carrying *osmC-lux* promoter constructs during 3 h treatment with a) cobalamin (C), b) calcium pantothenate (Ca-P), c) α-tocopherol (α-Toc), and d) menaquinone (MQ), or respective control a) and b) double distilled water, c) and d) 8% ethanol. Vitamin concentration (mg/mL) follows the initial of the vitamin name. Standard deviation ranged from 0.003 to 0.07 with the highest level in cobalamin (1 mg/mL) at 3 h.

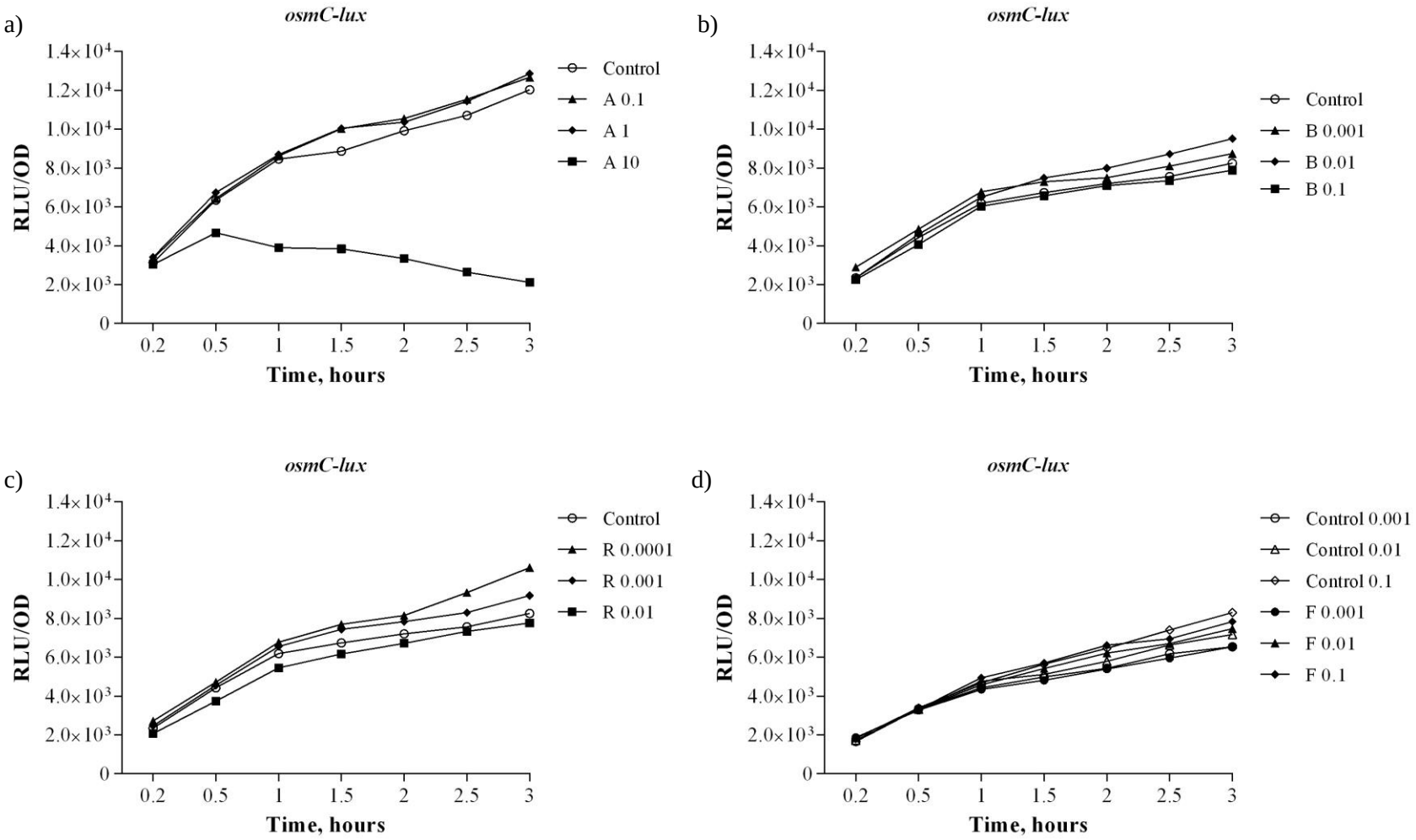

Figure 3.7 Normalized mean relative luminescence (RLU/OD) of *E. coli* O157:H7 EDL 933 (n=3) carrying *osmC-lux* promoter constructs during 3 h treatment with a) ascorbic acid (A), b) biotin (B), c) riboflavin (R), and d) folic acid (F), or respective control a), b), and c) double distilled water, d) potassium hydroxide (pH 7.4; adjusted with hydrochloric acid). Vitamin concentration (mg/mL) follows the initial of the vitamin name. Normalized relative luminescence (RLU/OD) was obtained by dividing measured relative luminescence data with respective mean OD_{595} values at each time point. Standard deviation ranged from $0.008x10^4$ to $0.4x10^4$ with the highest level in riboflavin (0.0001 mg/mL) at 3 h.

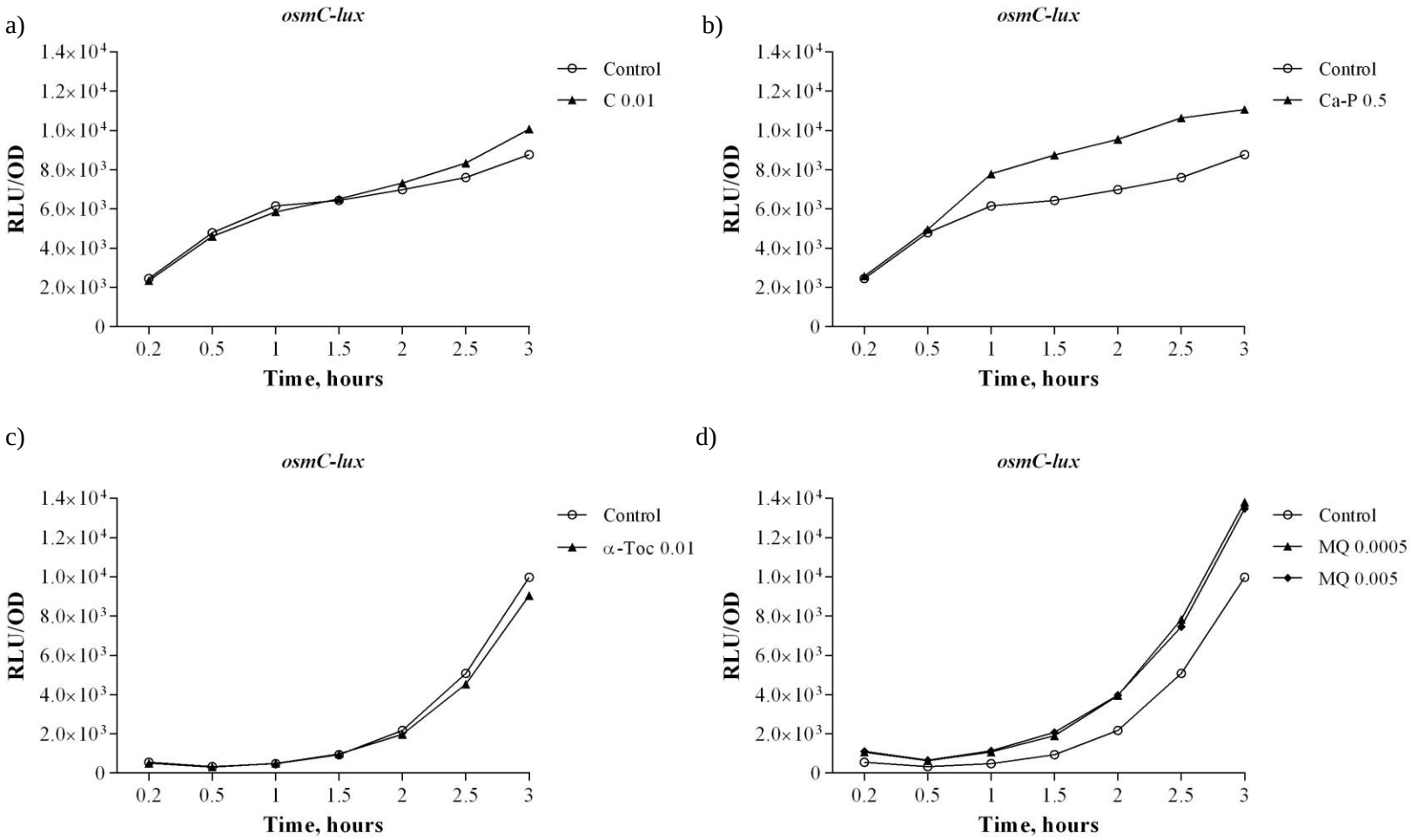

Figure 3.8 Normalized mean relative luminescence (RLU/OD) of *E. coli* O157:H7 EDL 933 (n=3) carrying *osmC-lux* promoter constructs during 3 h treatment with a) cobalamin (C), b) calcium pantothenate (Ca-P), c) α-tocopherol (α-Toc), and d) menaquinone (MQ), or respective control a) and b) double distilled water, c) and d) 8% ethanol. Vitamin concentration (mg/mL) follows the initial of the vitamin name. Normalized relative luminescence (RLU/OD) was obtained by dividing measured relative luminescence data with respective mean OD_{595} values at each time point. Standard deviation ranged from 0.0009 $x10^4$ to 0.7$x10^4$ with the highest level in menaquinone (0.005 mg/mL) at 3 h.

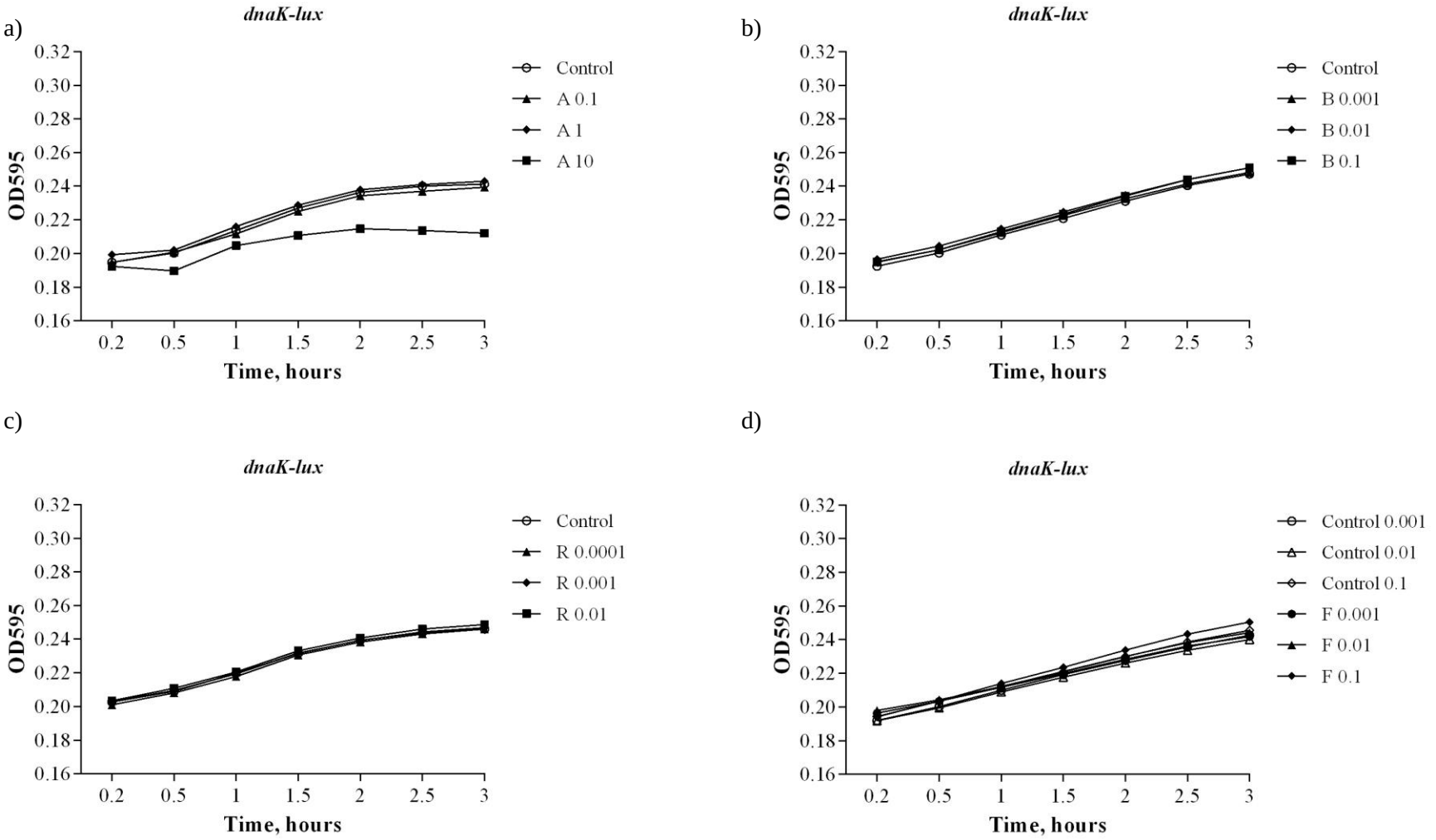

Figure 3.9 Mean optical density at 595 nm (OD_{595}) of *E. coli* O157:H7 EDL 933 (n=3) carrying *dnaK-lux* promoter constructs during 3 h treatment with a) ascorbic acid (A), b) biotin (B), c) riboflavin (R), and d) folic acid (F), or respective control a), b), and c) double distilled water, d) potassium hydroxide (pH 7.4; adjusted with hydrochloric acid). Vitamin concentration (mg/mL) follows the initial of the vitamin name. Standard deviation ranged from 0.0006 to 0.01 with the highest level in biotin (0.1 mg/mL) at 3 h.

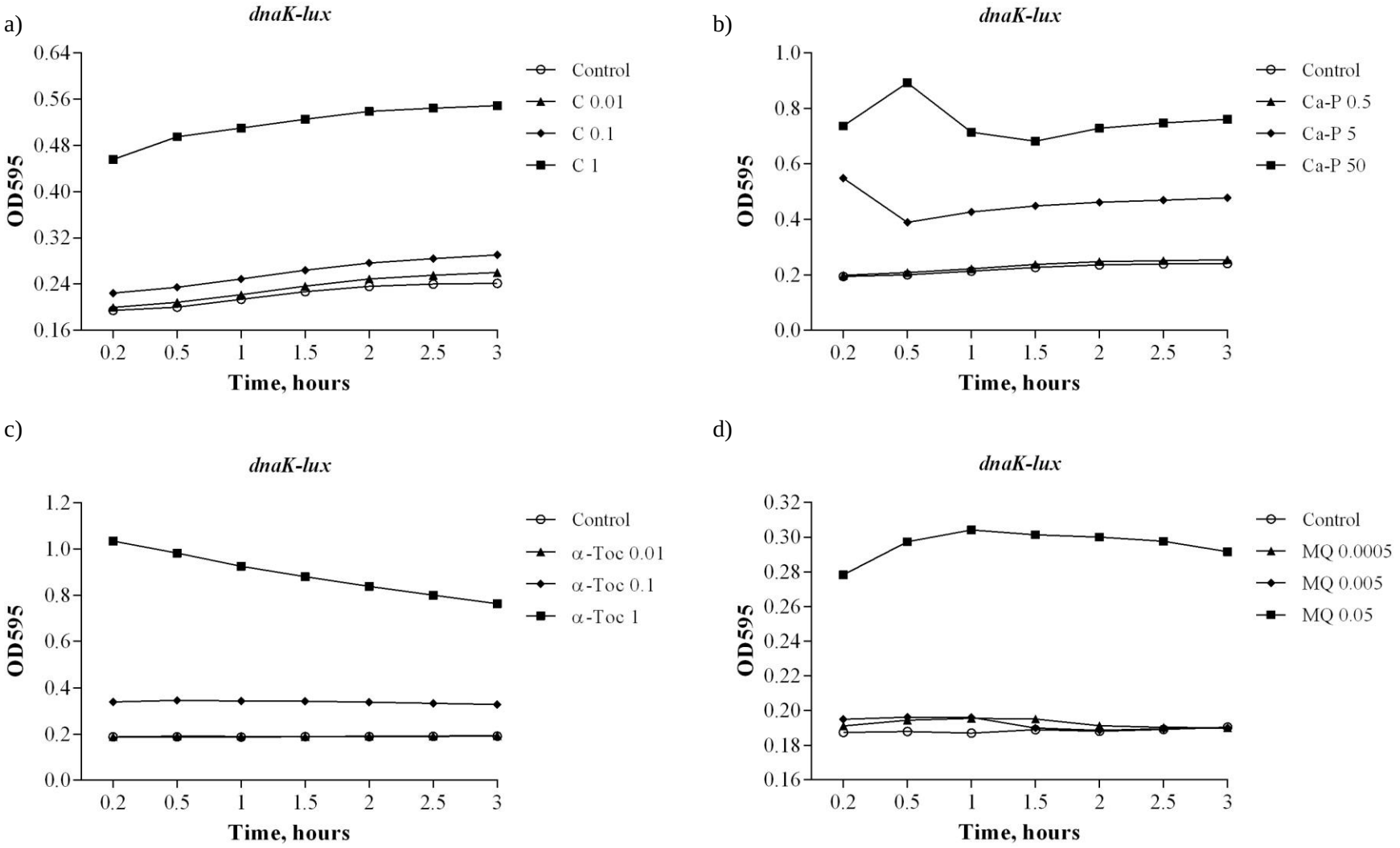

Figure 3.10 Mean optical density (OD_{595}) of *E. coli* O157:H7 EDL 933 (n=3) carrying *dnaK-lux* promoter constructs during 3 h treatment with a) cobalamin (C), b) calcium pantothenate (Ca-P), c) α-tocopherol (α-Toc), and d) menaquinone (MQ), or respective control a) and b) double distilled water, c) and d) 8% ethanol. Vitamin concentration (mg/mL) follows the initial of the vitamin name. Standard deviation ranged from 0.0006 to 0.05 with the highest level in α-tocopherol (1 mg/mL) at 3 h.

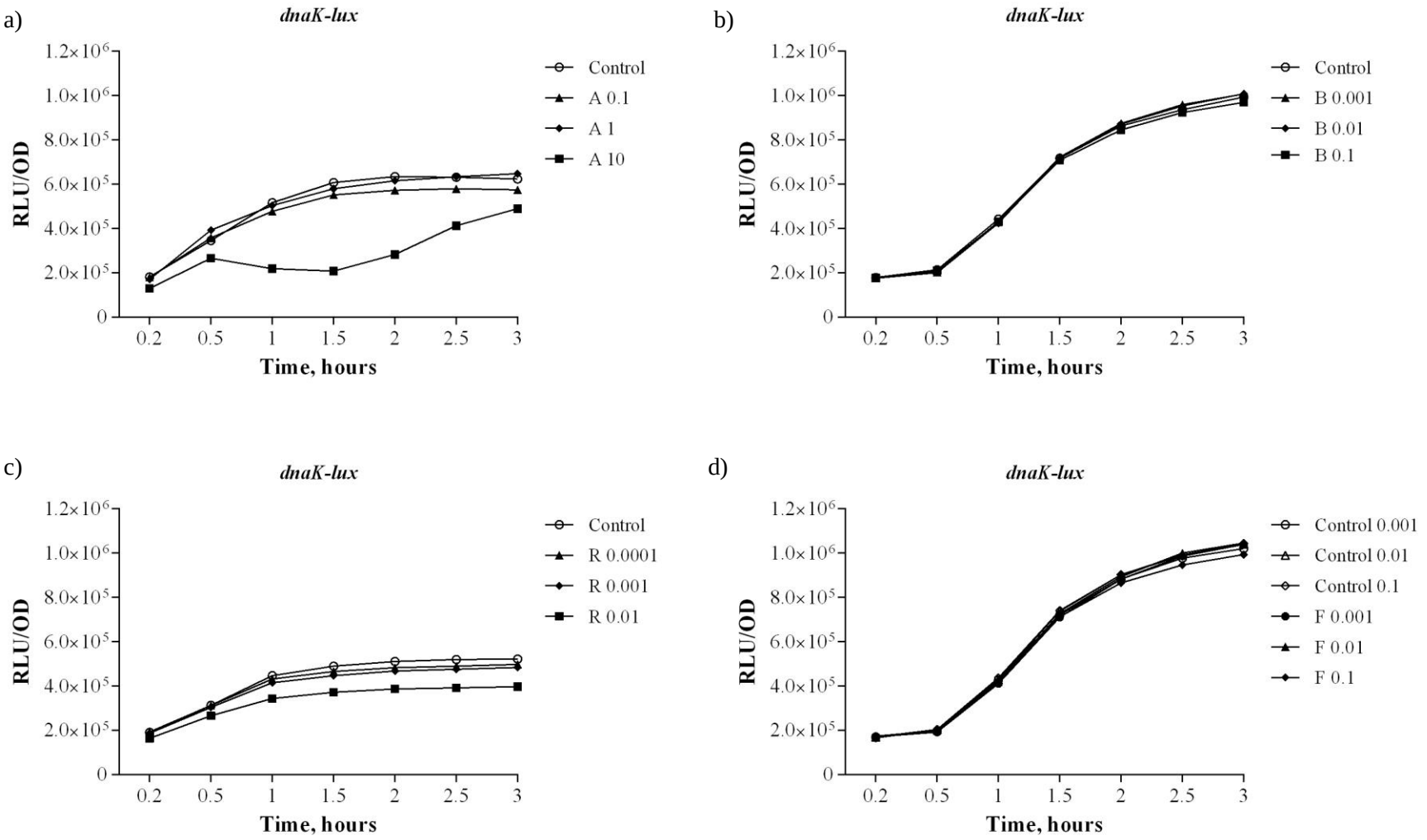

Figure 3.11 Normalized mean relative luminescence (RLU/OD) of *E. coli* O157:H7 EDL 933 (n=3) carrying *dnaK-lux* promoter constructs during 3 h treatment with a) ascorbic acid (A), b) biotin (B), c) riboflavin (R), and d) folic acid (F), or respective control a), b), and c) double distilled water, d) potassium hydroxide (pH 7.4; adjusted with hydrochloric acid). Vitamin concentration (mg/mL) follows the initial of the vitamin name. Normalized relative luminescence (RLU/OD) was obtained by dividing measured relative luminescence data with respective mean OD_{595} values at each time point. Standard deviation ranged from 0.08 $x10^5$ to $2x10^5$ with the highest level in control (double distilled water, c) at 3 h.

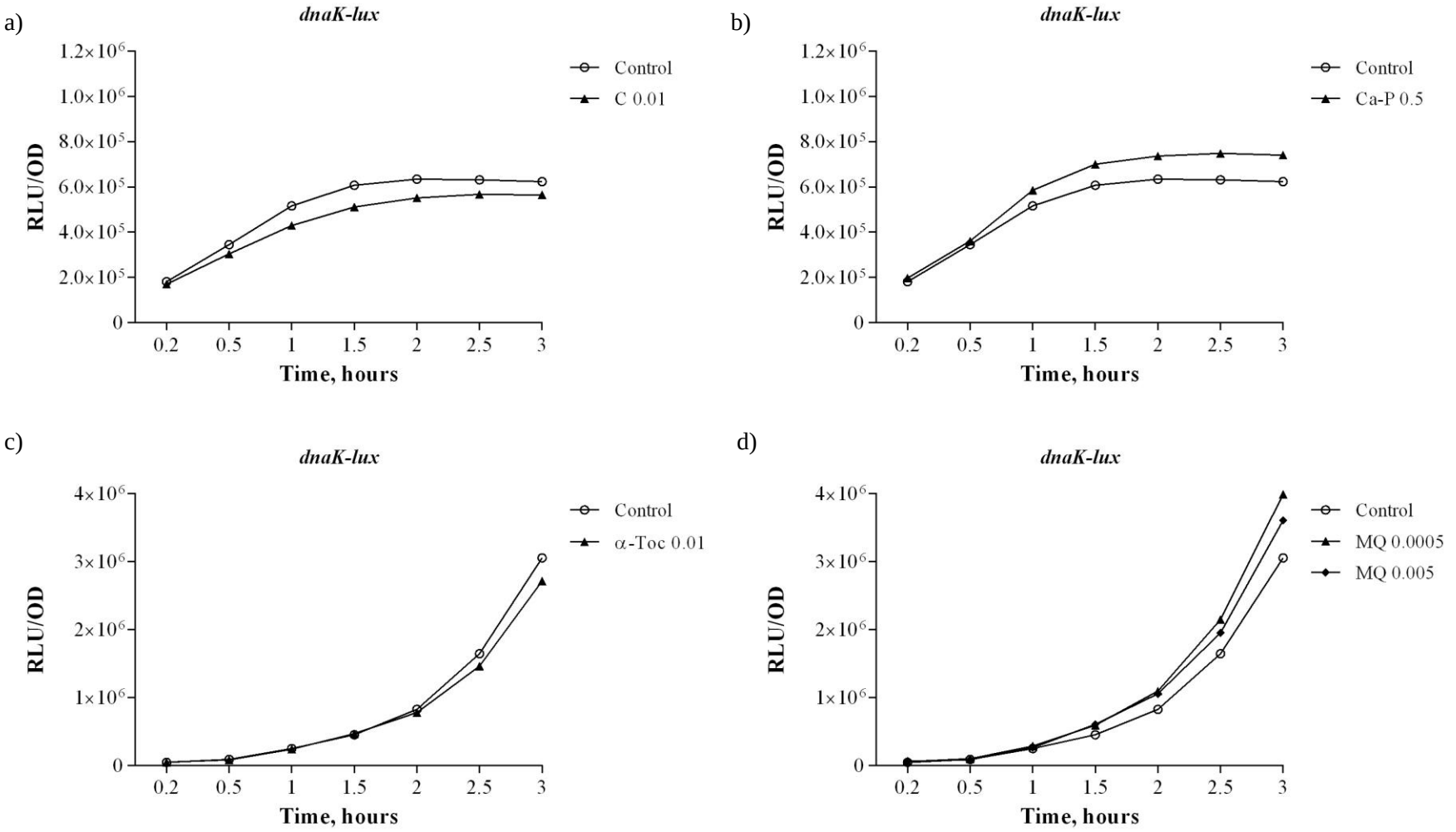

Figure 3.12 Normalized mean relative luminescence (RLU/OD) of *E. coli* O157:H7 EDL 933 (n=3) carrying *dnaK-lux* promoter constructs during 3 h treatment with a) cobalamin (C), b) calcium pantothenate (Ca-P), c) α-tocopherol (α-Toc), and d) menaquinone (MQ), or respective control a) and b) double distilled water, c) and d) 8% ethanol. Vitamin concentration (mg/mL) follows the initial of the vitamin name. Normalized relative luminescence (RLU/OD) was obtained by dividing measured relative luminescence data with respective mean OD_{595} values at each time point. Standard deviation ranged from 0.008 $x10^6$ to $0.8x10^6$ with the highest level in menaquinone (0.0005 mg/mL) at 3 h.

Chapter 4: Influence of vitamin exposure on *Escherichia coli* O157:H7 attachment to spinach leaves

4.1 Introduction

Over the last two decades, numerous produce outbreaks and recalls have been associated with *Escherichia coli* O157:H7 contamination of fresh, minimally processed leafy green vegetables, primarily lettuce and spinach (CDC, 2013; Rangel et al., 2005). The most eminent one is the multi-state spinach outbreak in 2006, with 199 infections and three deaths (CDC, 2006). Although plants can become contaminated with *E. coli* O157:H7 at any point along the production continuum, the most significant risks of transmission are believed to occur in the field from manure-contaminated soil or irrigation water (Solomon et al., 2002). Studies demonstrated *E. coli* O157:H7 can attach to damaged and intact leaf surfaces (Seo & Frank, 1999), or enter the root system and eventually reach edible parts of plants (Solomon et al., 2002). During harvesting, processing, and packaging, produce may be damaged resulting in vitamin release through injured plant cells. In the previous study on vitamin influence on *E. coli* O157:H7 virulence (Chapter 2), it was demonstrated that vitamins, particularly calcium pantothenate and cobalamin, increase adherence to HeLa cells (Figures 2.7 and 2.8) and trigger virulence gene expression (up-regulate *eae* encoding intimin and *espA* encoding translocator protein EspA). It could be possible that vitamins can influence the ability of *E. coli* O157:H7 to attach to spinach leaves, potentially impacting its long-term survival and persistence on the leaf surface. Ultimately, this would enable transmission through the food supply to humans. The purpose of this study was to determine whether vitamin exposure influences the attachment of *E. coli* O157:H7 to the surface of spinach leaves.

4.2 Materials and methods

4.2.1 Spinach preparation

Ready-to-eat spinach (Fresh Express, Salinas, CA, USA) was obtained from a local retailer, stored at 4°C, and used before the stated best before date. Prior to experiments, four 2.25 cm^2 sections were cut from a single spinach leaf using a sterilized scalpel with care taken to avoid major veins. Using double-sided Seal-It tape (Conros Corporation, Toronto, ON), the abaxial (lower) leaf surface was fixed to the bottom surface of a 12-well plate (Celltreat, Ottawa, ON) (Figure 4.1). Only spinach leaves with undamaged surface were used in the experiment.

4.2.2 Bacterial strain and growth conditions

Escherichia coli O157:H7 str. EDL 933 was grown in vitamin free M9 minimal medium (M9MM) as described in section 2.2.1. Once an optical density at 600 nm (OD_{600}) of 0.50±0.03 (Spectronic 20, Bausch & Lomb; c.a. 2.4 x 10^8 CFU/mL) was reached, 50 mL of late exponential phase cells was centrifuged at 10,400 x g at room temperature using a Sorval RC 5B Plus centrifuge (Mandel Scientific Co. Ltd., Guelph, ON) for 15 min. To wash away remaining M9MM, bacterial pellets were re-suspended in 50 mL 1 X phosphate buffered saline (PBS, Fisher Scientific) by vortexing for 15 sec and centrifuged at 8,000 rpm for 15 min. The OD_{600} of bacterial culture was adjusted to 0.50±0.03 with 1X PBS and vitamin treatments applied.

4.2.3 Vitamin treatments

Three biological replicates of *E. coli* O157:H7 EDL 933 (900 µL) were treated with 100 µL α-tocopherol (10 mg/mL), ascorbic acid (1, 10, 100 mg/mL), biotin (0.1, 1 mg/mL), calcium pantothenate (50, 500 mg/mL), cobalamin (0.1, 1, 10 mg/mL), riboflavin (0.001,

0.01, 0.1 mg/mL), or respective controls (Table 2.1) in a 1.5 mL microfuge tube (Eppendorf, Mississauga, ON) at 37°C for 3 h, static. All vitamins selected for this study had previously been shown to induce a significantly increased adherence of *E. coli* O157:H7 to HeLa cells after 3 h treatment in M9 minimal medium at 37°C (5% CO_2, static), except for ascorbic acid and riboflavin that resulted in no significant difference (Chapter 2; Figures 2.7 and 2.8). Influence of 3 h treatment with the vitamin or control on the numbers of *E. coli* O157:H7 in 1X PBS was not assessed.

4.2.4 Attachment to spinach leaves

Attachment to spinach leaves was performed as previously described by Deng et al. (2011) with some modifications. Briefly, three 10 µL volumes of vitamin treated bacteria (section 4.2.3) were spotted on the adaxial (upper) surface of spinach leaf cuts fixed to the bottom of a 12-well plate (section 4.2.1), with a care being taken to avoid placing the drops near cut edges. Each plate was covered with a lid, placed in a sealed 16.5 x 14.9 cm bag (Ziploc, S.C. Johnson and Son Limited, Brantford, ON, Canada) to maintain humidity, and incubated at 25°C for 0, 0.3, 1, 3, and 24 h. In 24 h attachment assays, one empty well per 12-well plate was filled with 3 mL of double distilled water, to aid in maintaining leaf quality. At the indicated time points, inoculated spinach surfaces were washed four times with 3 mL of 1X PBS per well to remove loosely adhered cells. Immediately after washing, each spinach cut was transferred into a 50 mL conical tube (Corning Incorporated, Corning, NY) containing 5 mL of 1X PBS and five 3 mm sterile glass beads (Fisher Scientific) using sterilized tweezers. Attached bacteria were recovered by vortexing vigorously for 20 sec, serially diluted in 1X PBS, and surface plated in triplicate on MacConkey agar with sorbitol (Neogen, Lansing, MI) supplemented with cefixime and tellurite (Mast Diagnostics,

Merseyside, U.K.). Typical *E. coli* O157:H7 sorbitol negative, colorless colony forming units were counted after 24 h incubation at 37°C. The experiment was repeated in three biological replicates.

4.2.5 pH

Measurement of pH for 1X PBS after addition (10:1 ratio) of ascorbic acid (1, 10, 100 mg/mL) or calcium pantothenate (50, 500 mg/mL) was carried out using an Accumet® Basic AB15 pH Meter (Fisher Scientific).

4.3 Results

4.3.1 Attachment of *E. coli* O157:H7 to spinach leaves

Influence of 3 h treatment with α-tocopherol (1 mg/mL), ascorbic acid (0.1, 1, 10 mg/mL), biotin (0.01, 0.1 mg/mL), calcium pantothenate (5, 50 mg/mL), cobalamin (0.01, 0.1, 1 mg/mL), or riboflavin (0.0001, 0.001, 0.01 mg/mL) on *E. coli* O157:H7 attachment to spinach leaves was assessed over a period of 24 h. In the control and vitamin treatments, *E. coli* O157:H7 attached to spinach leaves immediately after spotting (Figures 4.2. to 4.7). The CFU/mL of attached *E. coli* O157:H7 increased over 24 h for all vitamin and control treatments except for ascorbic acid (10 mg/mL; Figure 4.3), which resulted in a decline.

During the first 3 h of attachment, the increase in the number of attached *E. coli* O157:H7 ranged from 0.3 log CFU/mL in ascorbic acid (10 mg/mL) to 1.5 log CFU/mL in control (double distilled water) treatment (Figure 4.3). Over the next 21 h, increase in the CFU/mL of attached *E. coli* O157:H7 was not as pronounced (≤0.5 log CFU/mL) in all vitamin and control treatments except α-tocopherol (1mg/mL), which resulted in 0.9 log CFU/mL increase (Figure 4.2), and ascorbic acid (0.1, 10 mg/mL), which led to decrease in the number of attached *E. coli* O157:H7 by 0.1 and 0.6 log CFU/mL (Figure 4.3).

Among all vitamin treatments, ascorbic acid (10 mg/mL) was particularly effective in reducing the CFU/mL of attached *E. coli* O157:H7 to spinach leaves over a period of 24 h compared to the control. The difference in the number of attached *E. coli* O157:H7 between ascorbic acid (10 mg/mL) and control treatments increased over time from 0.8 log CFU/mL (0.33 h), 1.3 log CFU/mL (1 and 3 h) to 1.9 log CFU/mL at 24 h.

4.3.2 pH

Ascorbic acid (10 mg/mL, pH 3.6) and calcium pantothenate (50 mg/mL, pH 5.5) treatments had the lowest pH values compared to 1X PBS (pH 7.2) (Table 4.1). While ascorbic acid (1 mg/mL, pH 6.5) and calcium pantothenate (5 mg/mL, pH 6.1) had lower pH, pH of the lowest concentration of ascorbic acid (0.1 mg/mL, pH 7.2) did not differ from 1X PBS.

4.4 Discussion

Generally vitamin concentrations used in this study exceeded the concentrations naturally found in spinach (Chapter 1, Table 1.3) for approximately 50-fold for α-tocopherol, 3- to 30-fold for ascorbic acid (1, 10 mg/mL), 330- to 3,300-fold for biotin (0.01, 0.1 mg/mL), 500- to 5,000-fold for calcium pantothenate (5, 50 mg/mL), and 100- to 10,000 for riboflavin (0.0001, 0.01 mg/mL), while cobalamin is not naturally detected in spinach. Only ascorbic acid (0.1 mg/mL) was of similar concentration to the one naturally occurring in spinach (0.3 mg/g; Table 1.3). Vitamins were used at concentrations that were above physiologically relevant levels, in order to better notice the influence of the vitamins on attachment of *E. coli* O157:H7. The majority of vitamins tested have also been previously shown to induce a significantly increased adherence of *E. coli* O157:H7 to HeLa cells (Chapter 2; Figures 2.7 and 2.8).

Attachment is the initial step in bacterial colonization on the leaf surface (Brandl, 2006). It has been previously demonstrated that *E. coli* O157:H7 can attach to damaged and intact leaf surfaces (Seo & Frank, 1999). In all vitamin or control treatments *E. coli* O157:H7 attached to spinach leaves immediately after spotting (Figures 4.2. to 4.7). The increase in the Log CFU/mL of attached *E. coli* O157:H7 during the first 3 h could be because more bacteria came into contact with the leaf surface or the cells established themselves more tightly on the surface of the spinach leaf (Figures 4.2 to 4.7). Interestingly, a long term effect on *E. coli* O157:H7 attachment to spinach leaf surface was seen only in treatments with much lower pH than of PBS (7.2), such as ascorbic acid (10 mg/mL, w:v, pH 3.6; Figure 4.3) and calcium pantothenate (50 mg/mL, w:v, pH 5.5, Figure 4.5), which resulted in 1.9 and 0.6 log CFU/mL fewer numbers of attaching *E. coli* O157:H7 at 24 h compared to the control. Calcium pantothenate (5 mg/mL, w:v, pH 6.1; Figure 4.5) had less pronounced pH difference compared to the control and resulted in fewer numbers of *E. coli* O157:H7 attaching to spinach leaves only in a short (≤3 h) period of time. It would be worth investigating if the fewer numbers of *E. coli* O157:H7 attaching to spinach leaf surface over a longer period of time are a result of low pH of the treatments or direct physiological changes associated with the vitamin exposure. In case of calcium pantothenate treatment, influence of Ca^{2+} on the attachment of *E. coli* O157:H7 to spinach leaves should be assessed.

Interestingly, among all vitamin treatments only ascorbic acid (10 mg/mL, w:v, pH 3.6) was effective in reducing the numbers of attaching *E. coli* O157:H7 at 24 h below the initial inoculum level. This was not surprising as destructive effects of ascorbic acid (2.8%, pH 2.6, 10 min) on *E. coli* O157:H7 growth has been previously reported (Derrickson-Tharrington et al., 2005). In recent years, research was done looking into the

potential use of ascorbic acid as an antioxidant and/or reducing agent in order to delay lettuce deterioration at the post-harvesting stage. Rivera et al. (2006) reported that immersion of lettuce in ascorbic acid (1%, w/v, pH 3.0) at 5°C for 2 min increased total ascorbate content for up to 7 days and helped in maintaining relative water content in lettuce during storage at 5°C for 21 days. This suggests application of ascorbic acid may be a beneficial treatment in improving both quality and safety of leafy green vegetables. However, further research is needed to determine the extent to which ascorbic acid may serve as novel pathogen mitigation strategy for fresh produce. For example, it may be worth investigating which concentration, temperature, or duration of ascorbic acid treatment would be the most effective against reducing numbers of *E. coli* O157:H7 attaching on the leaf surface, and whether the effect of ascorbic acid was due to the effect of ascorbic acid on the bacteria, the effect of the low pH only or the effect of both low pH and ascorbic acid. Additionally, it might be interesting to determine a potential use of ascorbic acid as a more "natural" alternative or a pre-treatment to the existing antimicrobial treatments used to minimize or reduce contamination of fresh produce at post-harvesting stage, such as treatment with 100-150 ppm chlorine in wash water for 5 min or peracetic acid (CFIA, 2014).

4.5 Conclusion

Influence of vitamin exposure on *E. coli* O157:H7 attachment to spinach leaves has not been previously investigated. Majority of the vitamin treatments had no long term effect on *E. coli* O157:H7 attachment to the adaxial (upper) surface of the spinach leaf cuts. The only exceptions were ascorbic acid (10 mg/mL, pH 3.6, Figure 4.3) and calcium pantothenate (50 mg/mL, pH 5.5, Figure 4.5) treatments, which resulted in fewer numbers (1.9 and 0.6 Log CFU/mL) of *E. coli* O157:H7 at 24 h compared to the control. These effects could be

due to low pH or direct physiological changes associated with the vitamin exposure; which could be a focus of investigation of the future research.

Additionally, ascorbic acid (10 mg/mL) was the only treatment that reduced numbers of attached *E. coli* O157:H7 at 24 h below the initial inoculum level. This was in line with the findings of Derrickson-Tharrington et al. (2005), who reported destructive effects of ascorbic acid (2.8%, pH 2.6, 10 min) on growth of *E. coli* O157:H7 inoculated on apple slices. Finally, considering that hydrocooling of leaf lettuce by immersion or spraying of 1% ascorbic acid improved its quality (Rivera et al., 2006), it might be worth investigating a potential of ascorbic acid treatment as a strategy for improving both quality and safety of leafy green vegetables.

Table 4.1 pH of vitamin stock solutions diluted in phosphate-buffered saline (pH 7.2).

Vitamin	Concentration, mg/mL*	PH
Ascorbic acid		
	0.1	7.2
	1	**6.5**
	10	**3.6**
Ca-pantothenate		
	5	**6.1**
	50	**5.5**

*Stock solutions of ascorbic acid (1, 10, 100 mg/mL) or calcium pantothenate (50, 500 mg/mL) were diluted in 1X phosphate-buffered saline (PBS) in 1:10 ratio. Measurements were performed using Accumet® Basic AB15 pH Meter (Fisher Scientific). Bold are treatments with pH lower than 1X PBS by >0.1.

Figure 4.1 Spinach leaves after being cut into 2.25 cm^2 and fixed in a 12-well plate.

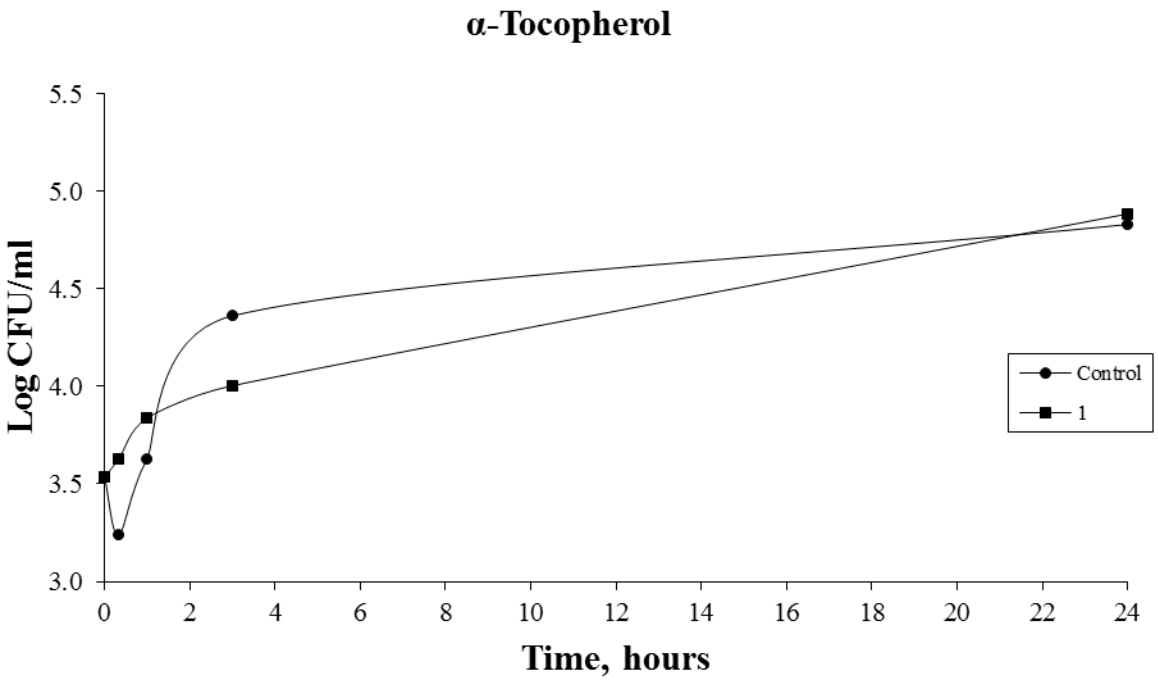

Figure 4.2 Number of attached *E. coli* O157:H7 (Log CFU/mL) to spinach leaves following 3 h treatment with α-tocopherol (1 mg/mL) or control (8% ethanol) at 37°C, static. *E. coli* O157:H7 was plated on MacConkey agar with sorbitol supplemented with cefixime and tellurite at 0, 0.3, 1, 3, and 24 h. Mean of three biological replicates are presented with points. Standard deviation ranged from 0.1 to 0.5 with the highest level in control treatment at 1 and 3 h.

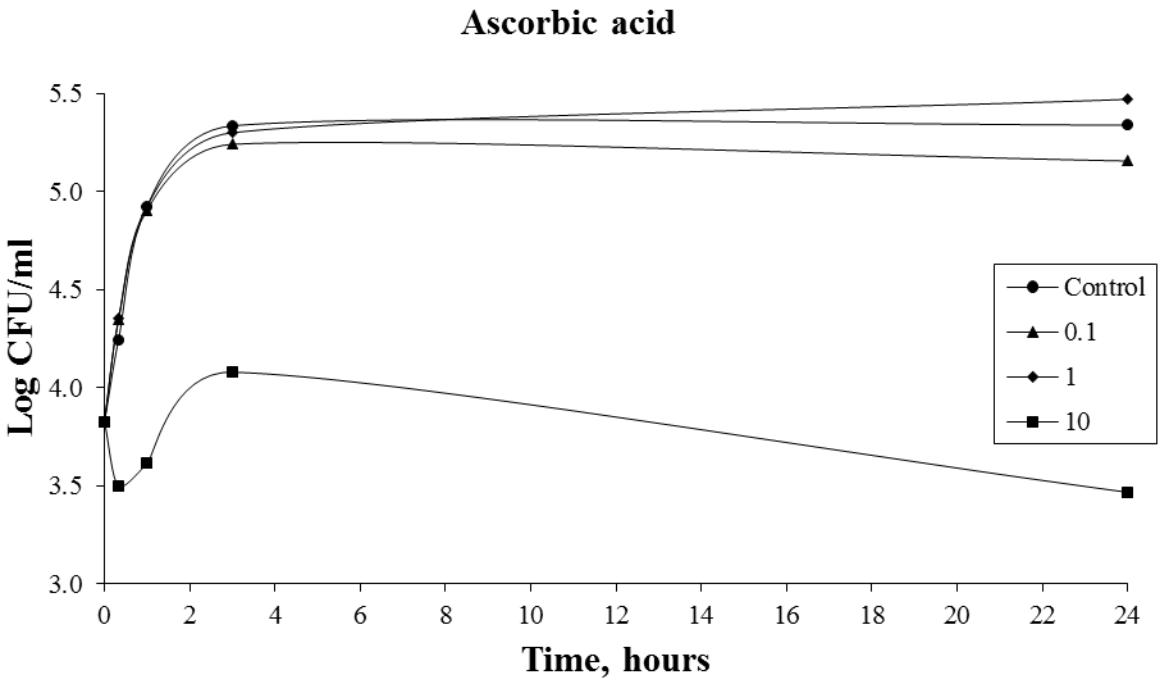

Figure 4.3 Number of attached *E. coli* O157:H7 (Log CFU/mL) to spinach leaves following 3 h treatment with ascorbic acid (0.1, 1, 10 mg/mL) or control (double distilled water) at 37°C, static. *E. coli* O157:H7 was plated on MacConkey agar with sorbitol supplemented with cefixime and tellurite at 0, 0.3, 1, 3, and 24 h. Mean of three biological replicates are presented with points. Standard deviation (SD) ranged from 0.1 to 0.4 for all treatments except ascorbic acid (10 mg/mL) in which SD was from 0.1 to 1.3 with the highest level at 24 h.

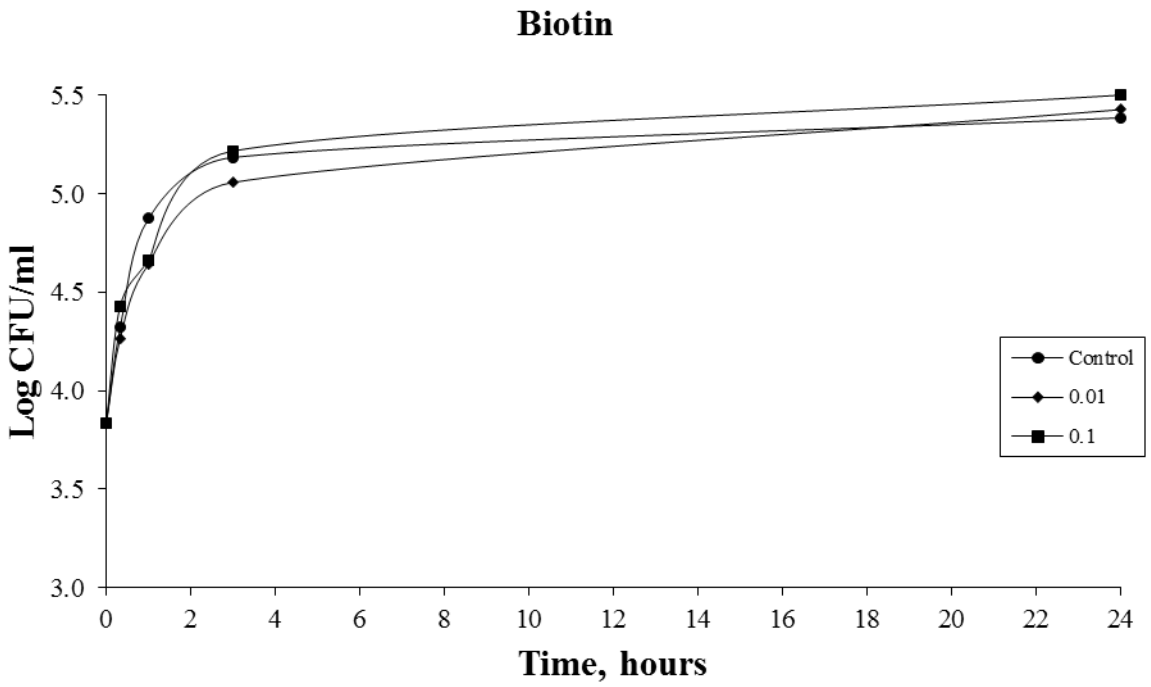

Figure 4.4 Number of attached *E. coli* O157:H7 (Log CFU/mL) to spinach leaves following 3 h treatment with biotin (0.01, 0.1 mg/mL) or control (double distilled water) at 37°C, static. *E. coli* O157:H7 was plated on MacConkey agar with sorbitol supplemented with cefixime and tellurite at 0, 0.3, 1, 3, and 24 h. Mean of three biological replicates are presented with points. Standard deviation ranged from 0.1 to 0.4 with the highest level in control treatment at 3 h.

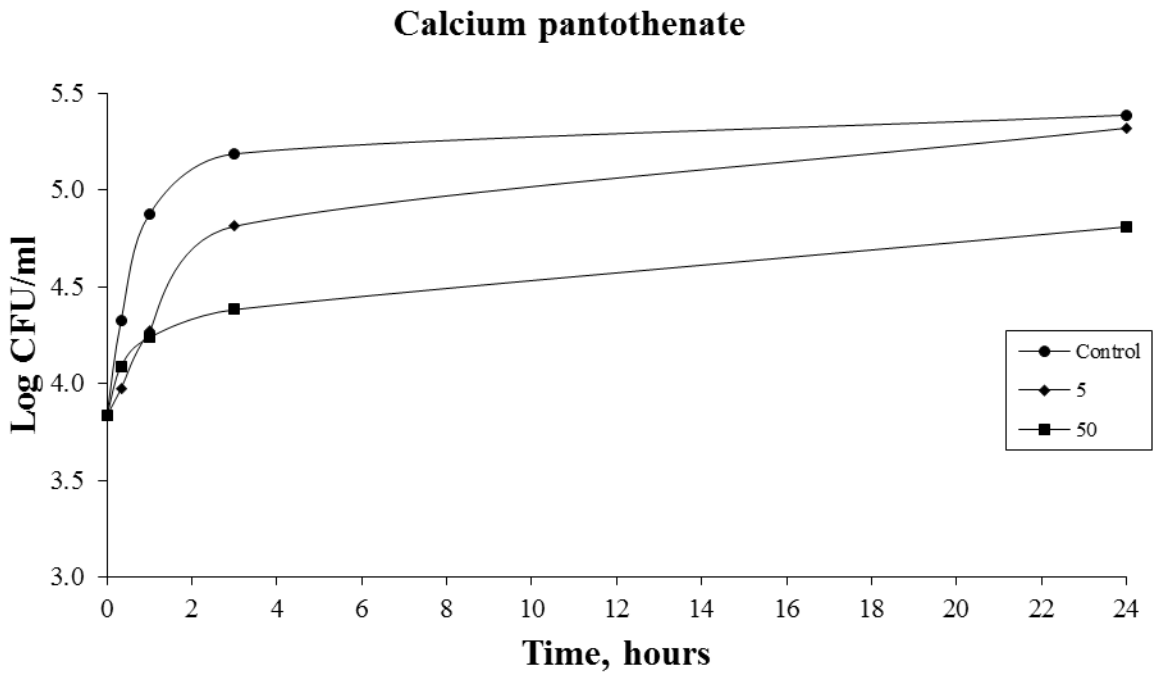

Figure 4.5 Number of attached *E. coli* O157:H7 (Log CFU/mL) to spinach leaves following 3 h treatment with calcium pantothenate (5, 50 mg/mL) or control (double distilled water) at 37°C, static. *E. coli* O157:H7 was plated on MacConkey agar with sorbitol supplemented with cefixime and tellurite at 0, 0.3, 1, 3, and 24 h. Mean of three biological replicates are presented with points. Standard deviation ranged from 0.1 to 0.4 with the highest level in control (3 h), calcium pantothenate (5 mg/mL, 3 h; 50 mg/mL, 0.3 and 1 h).

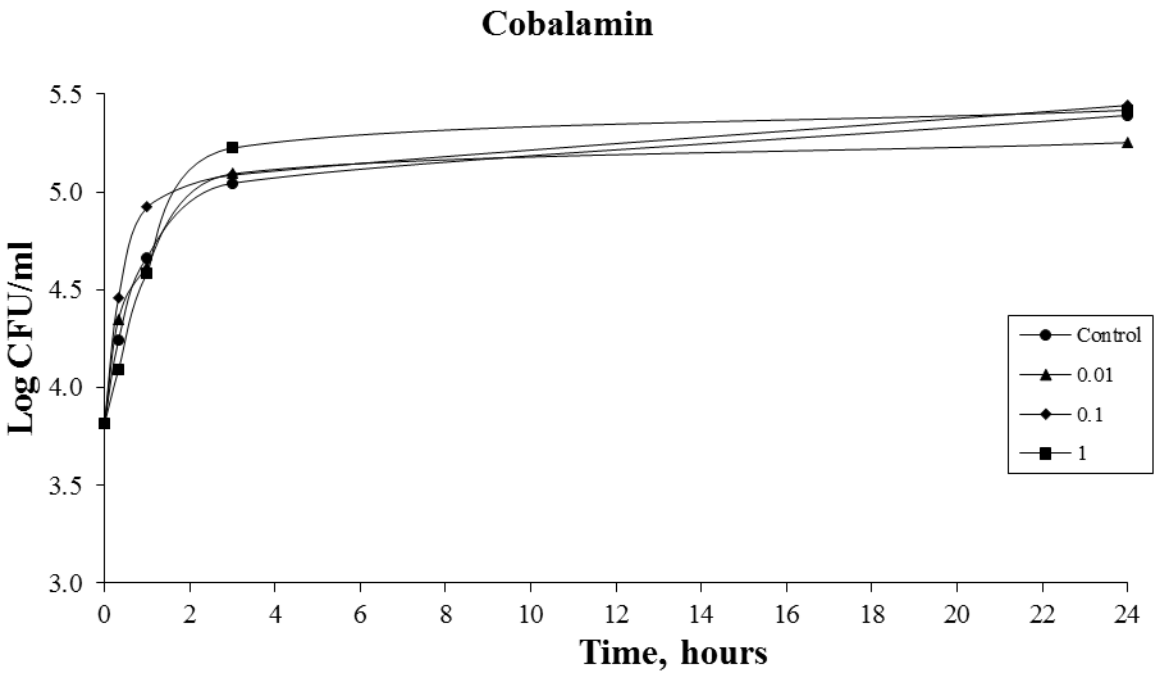

Figure 4.6 Number of attached *E. coli* O157:H7 (Log CFU/mL) to spinach leaves following 3 h treatment with cobalamin (0.01, 0.1, 1 mg/mL) or control (double distilled water) at 37°C, static. *E. coli* O157:H7 was plated on MacConkey agar with sorbitol, supplemented with cefixime and tellurite at 0, 0.3, 1, 3, and 24 h. Mean of three biological replicates are presented with points. Standard deviation ranged from 0.0 to 0.4 with the highest level in cobalamin (0.01, 0.1 mg/mL) treatment at 1 h.

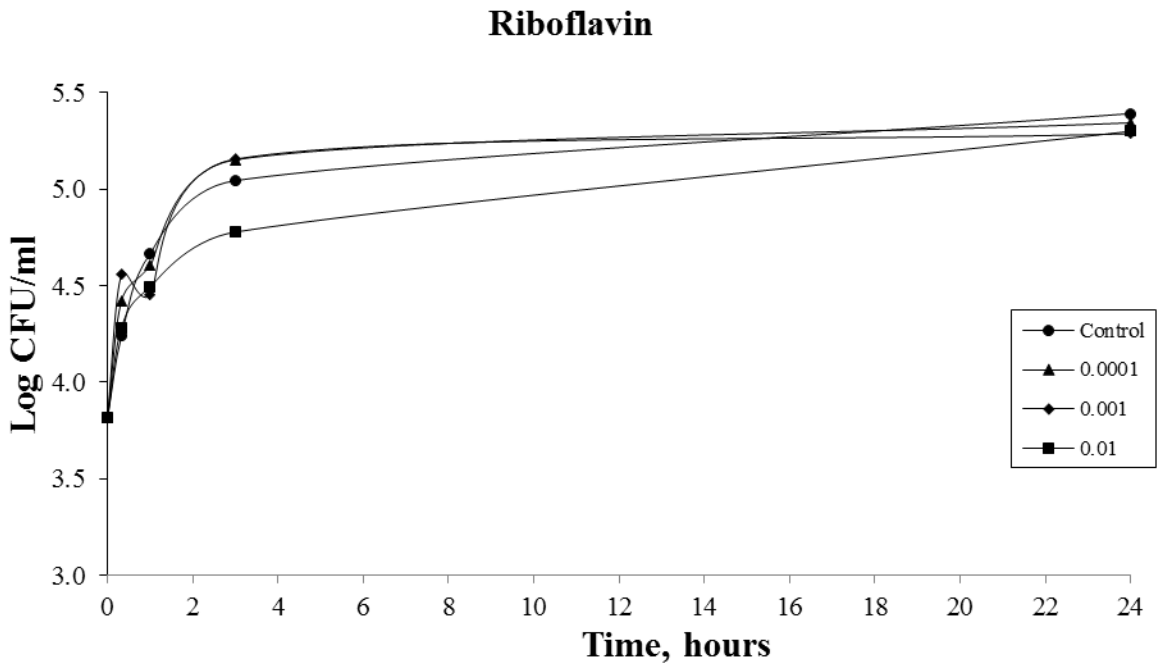

Figure 4.7 Number of attached *E. coli* O157:H7 (Log CFU/mL) to spinach leaves following 3 h treatment with riboflavin (0.0001, 0.001, 0.01 mg/mL) or control (double distilled water) at 37°C, static. *E. coli* O157:H7 was plated on MacConkey agar with sorbitol, supplemented with cefixime and tellurite at 0, 0.3, 1, 3, and 24 h. Mean of three biological replicates are presented with points. Standard deviation ranged from 0.0 to 0.4 with the highest level in riboflavin (0.0001, 0.01 mg/mL) treatment at 1 h.

Chapter 5: Conclusions and future direction

Fresh produce is a natural source of vitamins in our diet. Additionally, our enteric flora produces several vitamins, including biotin, cobalamin, folate, menaquinone (MQ), pantothenate, and riboflavin (Guarner, 2006; Hill, 1997). The goal of this study was to determine whether enterically-produced (biotin, cobalamin, folate, MQ, pantothenate, and riboflavin) or food-related vitamins (ascorbic acid and α-tocopherol) may increase attachment of *Escherichia coli* O157:H7 str. EDL933 to spinach leaves, and trigger expression of key stress and virulence genes, thereby enabling its gastrointestinal survival.

Exposure of *E. coli* O157:H7 to α-tocopherol (1 mg/mL), biotin (0.01, 0.1 mg/mL), calcium pantothenate (5, 50 mg/mL; due to Ca^{2+}), and cobalamin (0.01, 0.1, 1 mg/mL) increased the capacity of the bacteria to adhere to HeLa cells. Accordingly, the hypothesis that enterically produced or food-related vitamins promote adhesion of in *E. coli* O157:H7 to enterocytes is accepted. Furthermore, calcium pantothenate (5 mg/mL) and cobalamin (1 mg/mL) triggered the expression of *eae* and *espA*, which encode intimin and translocator EspA respectively, while biotin (0.1 mg/mL) slightly down-regulated *eae*. Therefore, the hypothesis that exposure of *E. coli* O157:H7 to enterically-produced biotin, cobalamin, and pantothenate results in enhanced transcription of *LEE* virulence genes is accepted for calcium pantothenate and cobalamin, and rejected for biotin treatment.

While exposure to cobalamin (1 mg/mL, 3 h) resulted in 3.7- and 4-fold increased cell associated Stx1 and secreted Stx2 by *E. coli* O157:H7, treatments with calcium pantothenate (5 mg/mL) and ascorbic acid (10 mg/mL) led to lower Stx2 levels compared to the control. Consequently, the hypothesis that cobalamin, pantothenate or ascorbic acid stimulates

production of Stx1 and Stx2 in *E. coli* O157:H7 is accepted for cobalamin and rejected for ascorbic acid and calcium pantothenate treatments. Ascorbic acid (10 mg/mL) was the only treatment that led only to increased stx_{2a} gene, thus the hypothesis that exposure to cobalamin, pantothenate, or ascorbic acid results in enhanced transcription of Shiga toxin virulence genes is rejected.

None of the vitamin treatments stimulated the expression of *rpoS* in *E. coli* O157:H7. Therefore the hypothesis that enterically produced or food-related vitamins stimulate transcription of general stress response gene in *E. coli* O157:H7 is rejected.

While ascorbic acid (10 mg/mL) treatment stimulated expression of acid (*gadA*, *gadX*) and oxidative (*soxS*) stress response genes in *E. coli* O157:H7, cobalamin (1 mg/mL) slightly induced *gadA*, and calcium pantothenate (5 mg/mL) down-regulated the expression of *gadA*, *rpoS*, and *soxS*. Therefore, the hypothesis that exposure of *E. coli* O157:H7 to cobalamin, pantothenate or ascorbic acid induces transcription of selected acid and oxidative stress response genes is accepted for ascorbic acid and cobalamin, and rejected for calcium pantothenate.

Finally, none of the vitamins (α-tocopherol, ascorbic acid, biotin, calcium pantothenate, cobalamin, and riboflavin) enhanced the attachment of *E. coli* O157:H7 to spinach leaves. Interestingly, ascorbic acid (10 mg/mL) was the only treatment which reduced numbers of attached *E. coli* O157:H7 at 24 h below the initial inoculum level. As such, the hypothesis that exposure of *E. coli* O157:H7 to α-tocopherol, ascorbic acid, biotin, calcium pantothenate, cobalamin, and riboflavin results in enhanced attachment to leafy green produce surface is rejected.

5.1 Limitations and future direction

This study was an initial screening study in which influence of various food-related or enterically-produced vitamins at different concentrations on *E. coli* O157:H7 virulence, stress response, and attachment to leafy green produce was examined. Considering the novel nature of this work, collected data have potential to provide focus for future research.

In order to detect vitamin influence on *E. coli* O157:H7 easily, vitamins were prepared at the highest possible concentration. In addition, two 10-fold dilutions were applied to ensure a wide range of concentrations were covered. However, in gastro intestinal environment or in food, it is very unlikely that *E. coli* O157:H7 will be exposed to such high vitamin concentrations. Therefore, future studies should examine if the similar effects of vitamins on *E. coli* O157:H7 can be observed at physiological vitamin concentrations. For example, it would be interesting to examine if the virulence gene expression can be triggered by calcium pantothenate and cobalamin at concentrations naturally found in a human gut, and if cobalamin at those concentrations may increase Stx1 and Stx2 production. In line with this, examination of kinetic profiles of Stx1 and Stx2 production, and stx_1 and stx_2 expression over a longer period of time may contribute to better understanding of cobalamin influence on *E. coli* O157:H7 virulence. Also, it might be worth investigating mechanism by which cobalamin stimulates production of both Stx1 and Stx2.

Another limitation was inability to use OD_{595} values for some of the vitamin treatments (due to insolubility or precipitate) to normalize relative luminescence data in *lux*-bioluminescence assay. If this experiment was to be repeated, the use of plate counts instead of OD_{595} is recommended. Further studies examining influence of ascorbic acid, calcium

pantothenate, or cobalamin treatment on *E. coli* O157:H7 stress response may focus on determination of RpoS level or microarray profiling.

Finally, the effects of ascorbic acid on *E. coli* O157:H7 attachment to spinach leaves could be due to low pH or direct physiological changes associated with the vitamin exposure; that could be a focus of investigation of the future research. It may be worth investigating a potential of ascorbic acid treatment as a strategy for improving both quality and safety of leafy green vegetables.

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