Antimicrobial Activity and Mechanism of Allyl Isothiocyanate Action against Bacteria

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Food Science

University of Manitoba

Winnipeg

ACKNOWLEDGEMENTS

I would first like to express my sincerest gratitude to my supervisor, Dr. Richard A. Holley, for his guidance, patience, and motivation throughout my study. His immense knowledge and experience was my greatest resource. I continue to be both grateful and humbled by his willingness to guide me through these past years. Under his supervision, I was able to develop my skills as a scientist in an encouraging environment, and I aspire to follow his example by applying all I've learned to my future endeavours. In addition to my advisor, I would like to thank my committee members, Dr. Claudia Narvaez and Dr. Richard Sparling, for their support, suggestions, and input for my research project. Special thanks to Dr. Ayush Kumar for allowing me to use his strains of wild-type and mutant *Ps. aeruginosa* and *E. coli* K-12.

I am sincerely grateful to God for granting me a multitude of gifts, including the ability and fortitude to complete this project. To my parents, Mun Keong Yee and Yuk Choi Yee, who raised me and loved me, thank you for your unwavering support. You instilled in me my love of learning and encouraged and fostered my curiosity from a young age. For this, and everything, I am extremely grateful. To my siblings, May-Sann ("Gatz"), May-Ting ("Getz"), and Randy ("Silo"), thanks for the advice, the laughs, and the Scrabble sessions. I look up to each of you and am constantly inspired by all you have accomplished. To my extended family, thank you for your encouragement and for keeping in touch despite the distance and time differences.

I would also like to thank the staff, students, and my classmates at the department of Food Science for their help and friendship. Special thanks to Roniele, who prepared me for my project

by devoting many hours of her time to guiding me through the lab work and theory required for my own research project, as well as always taking time to consider my questions. Your help was invaluable to me and I am so grateful for your patience and kindness. To Musarrat, Amin, VJ, and Dust, thank you all for being there to listen, to offer advice, and to answer my questions. Many of your suggestions benefited my research and I was very lucky to have you all as my sounding point. Thank you to Pat, Allison, Yang, Jennifer, and Carola for your help in familiarizing me with the lab equipment and for facilitating all administrative details during my time in the department.

To all my dear friends who offered their help, support, encouragement, or their ears, thank you for being there for me before, during, and undoubtedly after my study. There are too many of you to name, but know that I feel extremely lucky to have such a caring support system.

DEDICATION

To my parents.

ORGANIZATION OF THESIS

This thesis is organized into 5 chapters standardized for their presentation in this thesis. Chapter 1 offers an introduction to the subjects of this study, including the dangers of the food pathogen *E. coli* O157:H7 and the use of glucosinolates and isothiocyanates to control this organism, amongst others. The objectives of this study are detailed in this chapter.

Chapter 2 is a comprehensive literature review describing the background behind glucosinolate and isothiocyanate use in dry cured sausages, as well as the research to date describing the antibacterial mechanisms of isothiocyanate action. Additionally, the mechanisms of antibiotic action and the mechanisms of resistance towards these antibiotics are discussed.

Chapter 3 describes the first part of this study, where the correlations between the MIC profiles of 13 antibiotics and allyl isothiocyanate action against 12 strains of bacteria were determined.

Chapter 4 describes the second half of this study. Twelve bacteria were examined for their ability to exhibit myrosinase-like activity, and *E. coli* O157:H7 was monitored for its sinigrin degradation activity in an environment containing different concentrations of glucose or sorbitol, galactose, mannose, or ribose.

Chapter 5 presents the general integrated discussion from both parts of the study and contains suggestions for future research.

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ABSTRACT

Work was undertaken to examine the antibacterial mechanism of allyl isothiocyanate (AITC), as well as explore the hydrolysis of sinigrin by a range of bacteria and sinigrin metabolism in *Escherichia coli* O157:H7. Glucosinolates are sulfur- and nitrogen-containing compounds found in plants of the family *Brassicaceae* that react with the plant enzyme myrosinase to form antimicrobial compounds called isothiocyanates (ITCs). ITCs have been studied for their ability to mitigate the growth of pathogens in foods and food products and are attractive for this use due to their natural origin. However, the mechanism behind the bactericidal action of ITCs is not well understood. During inhibition experiments using a group of 12 bacteria including foodborne pathogens and starter cultures, it was found that AITC exhibited a minimum inhibitory concentration (MIC) profile against bacteria most similar to that of ciprofloxacin and polymyxin B. Ciprofloxacin is known to affect bacteria by inhibiting the enzymes topoisomerase IV and DNA gyrase involved in DNA synthesis. Similarly, AITC has been shown to affect enzymes and interfere with amino acids required for both DNA and RNA synthesis due to its ability to react readily with hydroxyls, amines, and thiols. Polymyxin B disrupts bacterial cell membrane integrity by attaching to lipopolysaccharides and acting as a surfactant, and also inhibits enzymes important in bacterial respiration. Likewise, AITC has been shown to disturb membrane structure and cause leakage of metabolites, as well as alter cellular respiration processes. Previous research studied the breakdown of glucosinolates by bacteria in myrosinase-deactivated mustard flours. The sinigrin degradation capacity of a range of bacteria was examined in the present work. *Escherichia (E.) coli* O157:H7, *E. coli* K-12, *Staphylococcus (S.) carnosus*, *S. aureus*,

Pseudomonas (Ps.) aeruginosa PAO1, *Ps. aeruginosa* PAO75, *Ps. fluorescens*, *Pediococcus (P.) pentosaceus* UM 121P, *P. acidilactici* UM 119P, and *Lactobacillus (L.) plantarum* UM 134L were all found to be able to hydrolyse sinigrin to some extent, whereas *P. acidilactici* UM 129P and *L. plantarum* UM 131L were unable. The effect of glucose in combination with each of sorbitol, galactose, maltose, or ribose on the sinigrin degradation capacity of *E. coli* O157:H7 was examined. No statistically significant differences in sinigrin utilisation were observed. The pH of the environment during these experiments showed that *E. coli* O157:H7 metabolised sinigrin to form acidic metabolites in the absence of additional glucose before utilising the amino acids present in the growth medium. The growth rate of *E. coli* O157:H7 during these experiments did not indicate a shift in carbon source, indicating that the organism likely hydrolysed sinigrin constitutively.

CHAPTER 1

1. Introduction

Escherichia coli serotype O157:H7 is a pathogenic organism whose emergence in the food industry began in 1982 after causing two American outbreaks of haemorrhagic colitis (Riley et al., 1983; Wells et al., 1983). Since then, foodborne illness outbreaks attributed to this pathogen have occurred and are occurring with increasing frequency in more than 30 countries (Luciano et al., 2010). These outbreaks have often been associated with the consumption of undercooked meat. Dry fermented sausages, which are food products traditionally consumed raw, pose a particularly large threat as they are manufactured without thermal processing. Commonly, various hurdles are established in such products to prevent the growth of foodborne pathogens, such as: reduction of water activity and/or pH, the addition of antimicrobial compounds such as salt, lactic acid, nitrite, and spices (Rode et al., 2012). However, strains of *E. coli* O157:H7 have been known to be salt and pH tolerant and easily remain viable in the finished food product (Lin et al., 1996). The pathogen also often goes undetected as it can exist in food products without altering the sensory characteristics of the food item (Arnold and Kaspar, 1995; Chacon et al., 2006). This ability, combined with its low infectious dose of approximately 50 cells, contributes to a high number of human infections (Tuttle et al., 1999; Trachtman and Christen, 1999). As a result, several outbreaks involving *E. coli* O157:H7 in dry fermented sausages have been reported, which have led to the implementation of regulations for the manufacture of fermented meat products in Canada and the U.S, requiring the process to reduce the numbers of *E. coli* O157:H7 by at least 5 log CFU/g (CFIA, 1999; Reed, 1995).

Glucosinolates are glucose- and amino acid-derived secondary metabolites commonly found in plants belonging to the family *Brassicaceae*. Upon structural damage to the plant, the enzyme myrosinase (EC 3.2.1.147) is released and interacts with the glucosinolates to form isothiocyanates (ITCs), a system which acts as a defense mechanism against herbivorous pests including insects, fungi, and bacteria (Brader et al., 2006). When myrosinase is introduced to the system, the thioglucose bond of the glucosinolate is cleaved and results in formation of several degradation products, including ITCs, thiocyanates, nitriles, epithonitriles, oxazolidine-thiones, and elementary sulphur (Mithen, 2006). ITCs, of which allyl isothiocyanate (AITC) is most well-researched, possess an established antimicrobial ability and have been added to food products to inhibit and even kill bacteria (Nadarajah et al., 2005). This addition of ITCs is regarded favourably due to consumers' growing interest in natural food products and additives; however, these compounds are difficult to incorporate into foods due to their pungency (Chacon et al., 2006). Interestingly, mustard powder that has undergone heat treatment to inactivate myrosinase is capable of causing the required reduction in the numbers of *E. coli* O157:H7 during the ripening process of dry fermented sausages (Graumann and Holley, 2008). This bactericidal activity is thought to be due to the bacteria intracellularly transforming the glucosinolates into ITCs, although the exact mechanism is not known (Luciano et al., 2011). Many bacterial species have been observed to possess this "myrosinase-like" activity, including lactic acid bacteria and foodborne pathogens alike (Ekanayake et al., 2006; Herzallah et al., 2011; Lara-Lledó et al., 2012; Luciano and Holley, 2010).

The mechanism behind ITCs' bactericidal action is unknown, although it has been extensively investigated. Researchers have studied the effect of ITCs on a variety of bacterial components.

For example, in conjunction with the inhibition of sulfhydryl enzymes in bacteria, ITCs are thought to interact with the thiol-containing molecules in these enzymes such as thioglycollate and cysteine (Zsolnai, 1966). Luciano and Holley (2009) tested the ability of AITC to inhibit thioredoxin reductase and acetate kinase, two enzymes that play a significant role in the metabolism of *E. coli*, and found that levels 50-fold lower than the MIC of AITC were able to competitively inhibit thioredoxin reductase levels. As well, researchers have focused on the effect of ITCs on bacterial cell membranes and leakage of intracellular components, including ATP and cellular metabolites (Lin et al., 2000a). Despite the attempts to identify the mode of ITC action, no distinct mechanism has been established. It is hypothesized that ITCs exert their antibacterial action via a multitude of inhibitory mechanisms (Luciano and Holley, 2009). Additionally, the ability of bacteria to convert glucosinolates to ITCs is of particular interest. Little information is available describing the process of sinigrin metabolism, although it is thought that the organisms will utilise glucosinolates as an alternative energy source (Cordeiro et al., 2015; Lara- Lledó et al., 2012). It has also been noted that in the presence of low concentrations of glucose, the organism has been observed to increase its hydrolysis of sinigrin, whereas higher concentrations of glucose seem to suppress this activity (Olaimat et al., 2014; Cordeiro et al., 2015).

The main objective of this research was to identify the similarities in bacterial resistance profiles between antibiotics of several classes and AITC as well as to explore aspects of bacterial myrosinase-like activity in a range of bacteria. Firstly, by correlating antibiotic and AITC minimum inhibitory concentrations, similarities in bacterial resistance profiles can be identified and parallels can be drawn between mechanisms of action. Secondly, identifying the sinigrin

degradation abilities of a range of bacteria may also help in understanding the mechanism behind bacterial myrosinase-like activity. An attempt was made to correlate the ability to degrade sinigrin with antibiotic resistance profiles in order to determine whether a relationship existed. Thirdly, observing the consumption of sinigrin by *E. coli* O157:H7 in the presence of different concentrations of glucose and a range of monosaccharides may help in understanding the system utilised in the metabolism of the glucosinolate.

CHAPTER 2

2. Literature Review

2.1 Structure and transformation of glucosinolates

Glucosinolates, glucose- and amino acid-derived secondary metabolites, are found in the vacuoles of all cruciferous vegetables in the family *Brassicaceae* [Fig. 2.1] (Bjerg and Sorensen, 1987). In combination with the enzyme myrosinase (EC 3.2.1.147), these two phytochemicals, which are released and meet upon tissue or cell injury, are thought to act as a defense mechanism in members of the *Brassicaceae* family by forming pungent metabolites to protect the plant from insect infestation, microbial infection, as well as damage from other predators (Brader et al., 2006). The two compounds are stored separately in the plant: glucosinolates are stored in the plant vacuoles while myrosinase is stored in the cytoplasm of cruciferous plant cells (Radojčić Redovniković et al., 2008). Upon structural damage to the plant, both compounds are released and interact with each other in the presence of water. This interaction cleaves the thioglucose bond of the glucosinolate, resulting in the formation of glucose and a highly unstable intermediate aglycone compound, thiohydroximate-*O*-sulphonate (Choubdar et al., 2010). Then, via Lossen rearrangement, the unstable aglycone spontaneously rearranges to produce several of the following compounds: isothiocyanates (ITCs), thiocyanates, nitriles, epithonitriles, oxazolidine-thiones, and elementary sulphur [Fig. 2.2] (Mithen, 2006). The ratio at which these degradation products are produced relies on the pH of the environment at the time of breakdown (Bones and Rossiter, 1996). This activation of glucosinolates by myrosinase, known as the

"glucosinolate-myrosinase system", is a well-studied herbivorous defense system (Kliebenstein et al., 2005).

Glucosinolates have been found to arise from amino acids through chain elongation, oxidation, desaturation and hydroxylation processes (Fenwick et al., 1983; Hansen et al., 1995). Glucosinolate structure varies mainly at the R-group side chain, which could be indolic, aromatic, heteroaromatic, or aliphatic, allowing for a multitude of biological activities, including anticarcinogenic properties, anti-nutritional effects, and pest-repelling abilities (Bones and Rossiter, 1996; Hirani et al., 2012; Mithen et al., 2000). Chain elongations of amino acids before glucosinolate formation, as well as secondary modifications to the side chain, are responsible for structural diversity in glucosinolates (Wittstock and Halkier, 2002). To date, over 120 different glucosinolates from various plant sources have been isolated (Fahey et al., 2001).

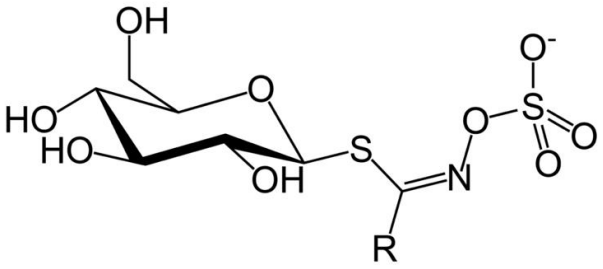

Figure 2.1: General structure of a glucosinolate.

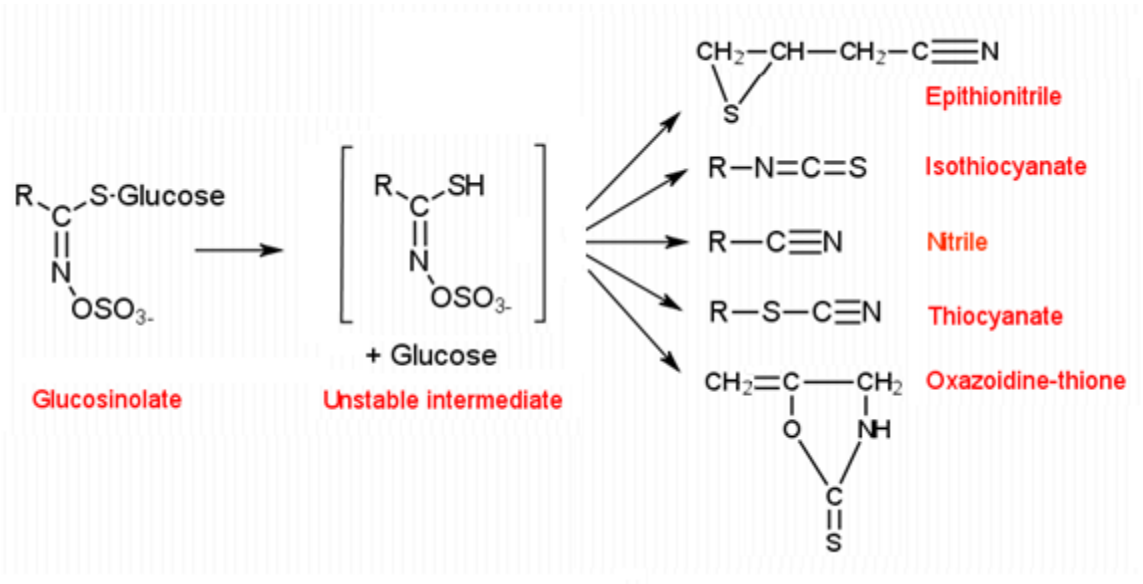

Figure 2.2: The enzymatic degradation of a glucosinolate to an unstable intermediate and subsequent conversion to other degradation products (adapted from Mithen, 2006).

Of the members of the *Brassicaceae* family, mustard is known to have a high concentration of glucosinolates and possesses significant myrosinase activity (Clarke, 2010; Cui and Eskin, 1998; Sang et al., 1984; Zrybko et al., 1997). In particular, commercial mustard plants *Sinapis alba* (white or yellow mustard) and *Brassica juncea* (brown and Oriental mustards) contain high levels of the glucosinolates sinalbin and sinigrin, respectively (Raney and Rakow, 2007). These cultivars have also been reported to contain high myrosinase activity. *Sinapis alba* crude extract was found to contain 14 different myrosinase isoenzymes whose activity was 10-fold higher than the myrosinase activity of other mustard plants (Bones, 1990; Buchwaldt et al., 1986). When studying the ITC-forming properties of these two cultivars, Cui and Eskin (1998) found that yellow mustard was able to form up to 2.5% of its weight of ρ-hydroxybenzyl isothiocyanate (ρHBITC), while Oriental mustard was capable of forming up to 0.8% of AITC.

2.2 Food safety in dry fermented meat products

2.2.1 Starter cultures in dry fermented meat products

Dry fermented uncooked sausages are cured, ready-to-eat, shelf-stable products that have been consumed for centuries. These sausages, although uncooked, have been preserved by a multitude of methods. These methods involve hurdles frequently in the form of a reduction of water activity, pH reduction, and the addition of antimicrobial compounds such as salt, lactic acid, nitrite, nitrate, and spices (Rode et al., 2012). Most importantly, the addition of microorganisms that produce lactic acid (lactic acid bacteria, or LAB) is the method of preservation that characterizes these sausages. Adding LAB starter cultures reduces the pH of the meat matrix to create an unfavourable environment for the survival of pathogenic and spoilage bacteria while also contributing to the sensory quality of the sausage (Getty et al., 2000).

Currently, starter culture mixtures are commercially available for the production of dry fermented sausages and generally include a mixture of LAB and a Gram-positive, catalase positive, coagulase-negative coccus (Aymerich et al., 2003). Commonly, the LAB used in commercial fermented sausage culture mixtures include organisms from several species, including *Lactobacillus casei, Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus sakei, Pediococcus pentosaceus* and *Pediococcus acidilactici* (Hammes and Hertel, 1998; Hugas and Monfort, 1997). These bacteria are chosen for their ability to produce a rapid pH drop in the meat batter. The Gram-positive, catalase-positive, coagulase-negative cocci most commonly used include *Staphylococcus carnosus*, *Staphylococcus equorum*, *Staphylococcus saprophyticus*, *Staphylococcus succinus*, and *Staphylocuccus xylosus* (Kaban and Kaya, 2008). In fermented

meat products, the coagulase-negative staphylococci are responsible for the reduction of hydrogen peroxide produced by the LAB, thus preventing off-odours (Rosenstein et al., 2008). In addition, these staphylococci reduce nitrate to form nitrite, resulting in the combination of nitrite with myoglobin, forming nitrosomyoglobin, which promotes the appealing red colour in the product (Rosenstein et al., 2008).

2.2.2 Survival of Escherichia coli *O157:H7 in dry fermented sausages*

Despite the various hurdles that are put in place to prevent the growth of foodborne pathogens such as *Staphylococcus aureus, Bacillus cereus* and *Clostridium botulinum* in food products, strains of the enterohemorrhagic pathogen *Escherichia coli* O157:H7 have been known to overcome these barriers and remain viable in the finished dry sausage (Hinkens et al., 1996). As a result, dry fermented sausages have been implicated in several outbreaks of *E. coli* O157:H7 infection over the past several decades (Tilden et al., 1996; Williams et al., 2000; MacDonald et al., 2004; Sartz et al., 2008). Due to the threat of *E. coli* O157:H7 surviving the dry fermented salami manufacturing process, Canada and the United States have imposed a mandatory ≥5 log CFU/log *E. coli* O157:H7 reduction in finished dry cured sausages (CFIA, 1999; Getty, 2000). However, because dry fermented sausages are uncooked meat products, alternative methods of preservation that do not alter the taste and texture of the final product are required. Several methods are currently in development for use in the food industry, including utilisation of strong acid- and bacteriocin-producing LAB strains, addition of diacetyl and lactoferrin, and adjusting the hurdle ratios, preparation parameters and storage conditions of the product (Kang and Fung, 1999; Al-Nabulsi and Holley, 2007; Sartz et al., 2008; Simpson et al., 2008; Ruíz et al., 2012).

Nevertheless, these methods are not able to produce consistently effective results in the dry fermented sausage product (Sartz et al., 2008; Holck et al., 2011).

2.2.3 Myrosinase-like activity in microorganisms

In the 1990s, Canadian-based UFL Foods introduced a thermal process for treating yellow mustard seeds, rendering the myrosinase inactive (Luciano and Holley, 2011). The resulting mustard powder not only lacked the intense hot flavour normally caused by ρ-HBITC, but also possessed remarkable emulsifying, binding, stabilizing and thickening properties (Luciano and Holley, 2011). By incorporating this "deodorised" mustard flour into cured sausages, Graumann and Holley (2008) discovered that this ingredient was capable of bringing about a significant reduction in the numbers of *E. coli* O157:H7 in the product while minimally affecting sausage texture.

Research has indicated that the use of deheated mustard flour can cause a substantial reduction (≥5 log CFU/g) in the numbers of *E. coli* O157:H7 in dry fermented meat products during the ripening process (Graumann and Holley, 2008; Luciano et al., 2008; Luciano et al., 2010; Nilson and Holley, 2012). This bactericidal activity is due to the ability of the bacteria to intracellularly transform glucosinolates present into ITCs, effectively inhibiting the producing organism (Luciano et al., 2011). Myrosinase is not exclusive to plants; enzymes exhibiting "myrosinase-like" activity have also been identified in fungi and bacteria, and have been extracted from various fungal species (Ohtsuru et al., 1969; Palop et al., 1995; Rakariyatham et al., 2002). Bacterial myrosinase-like activity has been observed in a variety of organisms, including LAB

and foodborne pathogens such as *E. coli, Salmonella, Campylobacter*, and *Listeria monocytogenes* (Ekanayake et al., 2006; Herzallah et al., 2011; Lara-Lledó et al., 2012; Luciano and Holley, 2010; Olaimat et al., 2014). Graumann and Holley (2008) demonstrated that a 4% (w/w) deodorised mustard powder content in dry fermented sausages successfully reduced the required ≥5log CFU/g of *E. coli* O157:H7 within a ripening period of 30 days. However, later research on the sensory characteristics of these fermented meat products indicated that dry sausages containing greater than 2% (w/w) deodorised mustard flour was less acceptable to consumers (Li et al., 2013).

The mechanism responsible for the unexpected reduction in viability of *E. coli* O157:H7 in dry fermented sausage was investigated extensively. Luciano and Holley (2010) discovered that *E. coli* O157:H7 possesses "myrosinase-like" activity which can hydrolyse glucosinolates to form ITCs in the absence of plant myrosinase. The pathogen, in the absence of an energy source, will hydrolyse the glucosinolate and utilize the glucose produced, inadvertently forming the lethal ITC by-product (Luciano and Holley 2010). In addition to the formation of ITC, it is likely that phenolic acids formed from the autoclave deodorising of mustard exert some microbial control over *E. coli* O157:H7 (Luciano et al., 2011). Further investigation determined that other bacteria in addition to *E. coli* O157:H7 will hydrolyse glucosinolates in a similar fashion, including foodborne pathogens and meat starter cultures alike (Herzallah et al., 2011; Luciano and Holley, 2010). Herzallah and colleagues (2011) determined that *E. coli* O157:H7, *P. pentosaceus*, *S. carnosus, Salmonella* Typhimurium, *S. aureus* and *Enterococcus faecalis* were all capable of degrading sinigrin but *Pseudomonas fluorescens* possessed little ability to cause its hydrolysis. Luciano and Holley (2011) investigated the ability of 25 LAB and staphylococci strains to

hydrolyse sinalbin and found that while most starter cultures were able to degrade the glucosinolate, there were several that did not. Additionally, it was determined that *E. coli* O157:H7 yielded the greatest degradation of sinalbin when compared to the meat starter cultures *S. carnosus* and *P. pentosaceus* (Luciano and Holley, 2010).

2.2.4 Bacterial degradation of glucosinolates and carbon catabolite repression

In the presence of high (>0.025 M) concentration of a preferred bacterial energy source (i.e. glucose), the ability of a bacterium to synthesize myrosinase-like enzymes and metabolically utilize glucosinolates is suppressed, supporting the idea that the organism will utilise glucosinolates as an alternative energy source (Cordeiro, unpublished; Lara-Lledó et al., 2012). Carbon catabolite repression (CCR), most commonly exemplified in *E. coli* strains, is the mechanism that is observed when, in the presence of a preferred carbon source in the growth medium, gene expression and/or protein activity are inhibited (Hogema et al., 1998). A well-studied example is the repression of the *lac* operon in *E. coli* in the presence of glucose plus lactose. In the presence of both lactose and glucose, the synthesis of the β-galactosidase enzyme required for hydrolysis of lactose will be suppressed until all the glucose has been utilised (Loomis and Magasanik, 1965). Therefore, the bacterium has conserved energy and resources by suppressing the action of the *lac* operon and it has also consumed the nutrient source that allows for the fastest growth rate first (Pérez-Alfaro et al., 2014).

The mechanisms behind carbon catabolite repression have been investigated and several have been identified.

1) In the presence of glucose and other phosphotransferase system (PTS) sugars, unphosphorylated glucose-specific permeases (Enzyme II A^{Glc}, or $EIIA^{Glc}$) will bind to the resting state of non-PTS sugar transporters, stabilizing the structure and subsequently preventing transport of alternative carbon sources into the cell (Jones-Mortimer and Kornberg, 1974; Stülke and Hillen, 1999). This process, denoted "inducer exclusion", has been witnessed with a number of non-phosphotransferase system carbon sources and is believed to be the main mechanism behind CCR (Hogema et al., 1998).

2) The catabolic operons of sugars are subject to repression by at least one specific regulator under normal circumstances (Jacob and Monod, 1961). Upon the binding of a corresponding sugar, the complex is removed from the operator zone, allowing the operon to activate and initiate gene transcription (Sellitti et al., 1987). This local transcriptional regulation of CCR allows only the specific operons to function in the presence of the corresponding sugar.

3) Global transcriptional regulation of CCR describes the effect of the presence of glucose on the transcription of sugar catabolic genes. Researchers have hypothesized that a breakdown product of glucose inhibits the formation of a catabolite activator protein (CAP) and cyclic adenosine monophosphate (cAMP) complex (CAP-cAMP). This CAP-cAMP complex recruits RNA polymerase to attach to the promotion site of catabolic operons, therefore, when this complex is not formed, transcription from these promoters does not occur (Griffiths et al., 2000). If glucose is not present, high levels of cAMP are available and readily interact with CAP to form the CAP-cAMP complex, allowing the transcription of sugar catabolic genes (Pérez-Alfaro et al., 2014).

4) Small RNAs (sRNAs) regulate gene expression in bacteria (Wright et al., 2013). A known sRNA that globally regulates catabolite repression is Spot 42, a multi-target sRNA that inhibits the translation of 14 genes, some of which are related to the cellular use of non-PTS sugars. To

ensure the use of preferred sugars, Spot 42 is repressed by the CAP-cAMP complex, and together both form a multi-output feed-forward loop to avoid the use of non-PTS sugars (Beisel and Storz, 2011).

Glucose, as the preferred carbon source for *E. coli*, will be consumed by the organism before other carbon sources that may be present in the media (Monod, 1942). In previous work, it seemed that a concentration of 0.025 M glucose in the growth medium enhanced the overall degradation of glucosinolate by *E. coli* O157:H7 (Cordeiro, unpublished). In comparison to an environment with no glucose present where the degradation rate was 458 ppm (1152.3 μM) in 13 d, in the presence of >0.05 M of glucose, only 298 ppm (749.8 μM) was degraded in 13 d. Cordeiro found that a 0.025 M concentration of glucose increased the amount of sinigrin degraded by up to 37% (627 ppm or 1577.5 μM degraded). This suggested that not only did the organism prefer glucose as an energy source to the glucosinolate, but also it seemed that the initial consumption of glucose stimulated glucosinolate hydrolysis.

Aside from lactose, other sugars have also been found to be subject to repressive genetic behaviour in *E. coli*, and a hierarchy of sugar preferences has been investigated. Desai and Rao (2010) determined that, similarly to lactose, *E. coli* cells will first consume glucose before moving on to arabinose and then xylose. The authors also found that the genes in the xylose metabolic pathway are repressed if the organism is in an environment containing arabinose. Recently, Pérez-Alfaro and colleagues (2014) determined the hierarchical order of sugar preference for several other sugars: *E. coli* preferred first glucose, arabinose, sorbitol and then galactose. Therefore, it seems that an understanding of the mechanism of bacterial myrosinase-

like activity and subsequent glucosinolate degradation can be gained by observing the genetic response to the presence of glucosinolates.

2.3 Isothiocyanates

2.3.1 Structure and stability of isothiocyanates

Isothiocyanates formed upon the hydrolysis of glucosinolates through the enzymatic action of myrosinase are known to be particularly volatile compounds that are easily evaporated (Wu et al., 2009). There is a wide variety of ITCs due to the spectrum of R-side groups on the ITC component [Fig. 2.3] (Delaquis and Mazza, 1995). These compounds' reactivity can be attributed to their molecular arrangement where the electron-deficient central carbon atom is prone to reactions with nucleophiles including amines, amino acids, alcohols, water, and sulfites in both food processing and physiological conditions (Drobnica et al., 1997; Cejpek et al., 1998; Cejpek et al., 2000).

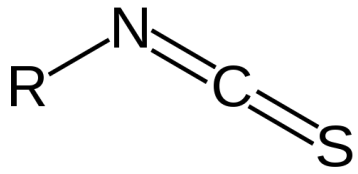

Figure 2.3: General structure of an isothiocyanate.

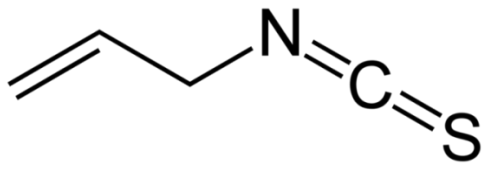

Figure 2.4: Allyl isothiocyanate.

Allyl isothiocyanate [Fig. 2.4], the major degradation product of sinigrin, is particularly unstable in aqueous solution where it is rapidly decomposed to form allyl dithiocarbamate, which further degrades to form diallyl tetra- and pentasulfide, as well as other degradation products (Kawakishi and Namiki, 1969). Being a strong electrophilic reagent, AITC is decomposed in aqueous environments by a nucleophilic attack of water against its ITC group, which weakens its antimicrobial activity (Chen and Ho, 1998). It has also been determined that the pH and temperature of the environment affects the rate of AITC degradation. In acidic solutions and at lower temperatures, the degradation of AITC is reduced (Ohta et al., 1995; Chen and Ho, 1998). Cordeiro et al. (2014) found that AITC in Mueller Hinton broth was more stable at pH 5 and 25°C. At a similar pH, it has also been identified that AITC antibacterial action is most effective, requiring a 20-fold lower inhibitory concentration against *E. coli* O157:H7 at pH 4.5 than at pH 8.5 (Luciano and Holley, 2011).

2.3.2 Antibacterial activity of isothiocyanates

ITCs have been commonly used in home remedies to treat a number of ailments and these compounds' antibacterial properties have been well known for decades (McKay et al., 1959). Against a wide variety of bacterial and fungal organisms, ITCs have been demonstrated to exert strong antimicrobial effects both in liquid and vapour form.

Over the past several decades, there have been many studies investigating the sensitivity of various microorganisms to ITCs. Bacteria as a whole are sensitive to these compounds, but seem less sensitive in comparison to fungi (Delaquis and Mazza, 1995). A number of ITCs have been

studied for both bacteriostatic and bactericidal properties, with AITC having been investigated most extensively for its broad-spectrum antimicrobial effects which are bacteriostatic, bactericidal, fungistatic, and fungicidal (Ahn et al., 2001; Uda, et al., 2000). However, the results of these studies describe a contradiction over which bacterial Gram-type is more sensitive, leading to the conclusion that sensitivity to the effect of ITCs seems to be dependent on strain, not Gram-type (Delaquis and Mazza, 1995).

In early research, Foter and Golick (1938) reported that the Gram-positive *Bacillus subtilis* was inhibited by horseradish vapours, whereas Gram-negative *Escherichia coli* was resistant. Foter (1940) went on to test the effects of AITC, methyl isothiocyanate (MITC), and ethyl isothiocyanate (EITC) against several strains. He found that *E. coli* and *S. aureus* were the most resistant whereas *B. subtilis*, *Bacillus mycoides* and *Serratia marcescens* were most sensitive, and also indicated that these ITCs were capable of inducing both bacteriostatic and bactericidal effects. In McKay and colleagues' (1959) comprehensive review of ITCs, the relationship between ITC carbon chain length and antibacterial ability was correlated. It was shown that long-chain ITCs were more effective against Gram-positive organisms than short-chain homologues, which were in turn more effective against Gram-negative organisms. This theory about ITCs may help further describe the varied resistances among bacterial strains.

Later, Virtanen (1965) determined that benzyl isothiocyanate (BITC), β-phenylethyl (PEIT), m-methoxybenzyl, and p-methoxybenzyl ITCs were able to inhibit *S. aureus*, while Zsolnai (1966) conversely reported that *Streptococcus pyogenes* and *S. aureus* were not affected by concentrations of AITC and phenyl isothiocyanate (PITC) that had previously been proven to

inhibit yeasts and fungi. Isshiki and colleagues (1992) detailed a dichotomy in resistance by demonstrating that Gram-positive bacteria such as *B. cereus, B subtilis, S. aureus* and *Staphyloccoccus epidermis* were more resistant to AITC than Gram-negative organisms *Ps. aeruginosa*, *Vibrio parahaemolyticus* and *E. coli*, yet *Salmonella* Enteritidis, a Gram-negative bacterium, was as resistant as the Gram-positive organisms. Further research by Uda et al. (1993) described the ability of an ITC derived from Japanese radish (4-methylthio-3-butanyl) to effectively inhibit the growth of *B. cereus* and *S. aureus*, which contrasts with the inhibitory activity of AITC. In an attempt to better quantify and characterize bacterial inhibition by ITCs, Delaquis and Mazza (1995) studied the effects of AITC on three foodborne pathogens: *Salmonella* Typhimurium, *E. coli* and *L. monocytogenes*. The authors determined that growth of *S.* Typhimurium and *E. coli* was inhibited at 1000 μg L^{-1} and above, while bactericidal effects were observed at concentrations of >1500 μg L^{-1}.

In 1997, Delaquis and Sholberg demonstrated that Gram-negative bacteria were more sensitive to gaseous AITC than Gram-positive bacteria. Tests examined its effect against several foodborne pathogens and spoilage bacteria and it was found that concentrations as low as 1500 μg L^{-1} were able to inhibit the growth of *S.* Typhimurium, *L. monocytogenes*, and *E. coli*. The authors also observed that the bactericidal effect of ITCs against bacteria varied between strains and increased with time of exposure and AITC concentration. Similarly, Lin and colleagues (1999) found that *L. monocytogenes* was more resistant to gaseous AITC than *E. coli* O157:H7 and *Salmonella* Montevideo (8 log reduction by 400 μl of AITC at 2 and 4 d, respectively), whereas AITC was more effective against *L. monocytogenes* than *E. coli* O157:H7 and *S.* Montevideo (Lin et al., 2000a). Ahn et al. (2001) demonstrated the bactericidal effect of AITC

on *L. monocytogenes* and described the reduction of intracellular ATP levels in response to the ITC.

Further evidence indicating that sensitivity to ITCs is dependent on bacterial species was collected in a study by Wilson and colleagues (2013), where the authors examined the antimicrobial activities of 10 ITCs against 14 strains of bacteria and found that, overall, Gram-negative bacteria were more sensitive to ITCs than Gram-positive organisms. However, the results of the study indicated that considerable variations in ITC sensitivity occurred among bacteria within the same Gram-type, suggesting instead that the species of a bacterium plays a significant role in determining ITC sensitivity. Similarly, it is known that a dichotomy exists in the resistance of starter culture bacteria and pathogenic bacteria to ITCs. Chacon et al. (2006) and Ward et al. (1998) found that *E. coli* O157:H7 was more susceptible to AITC than meat starter cultures *Pediococcus pentosaceus, Lactobacillus sakei* and *S. carnosus*. Furthermore, differences in the resistance of and reactions to AITC exist even within these two groups of organisms. Several meat starter cultures were more resistant to ITCs than others, though this resistance did not seem to be based on Gram-type. Chacon and associates (2006) described the sensitivity of meat starter culture *S. carnosus* to AITC, where a ≤2 log CFU/g reduction was observed at 500 ppm, and the contrasting resistance of starter culture *P. pentosaceus* to the ITC, where levels of 500-1000 ppm did not significantly affect survival of the latter organism. Additionally, the Gram-positive starter culture *Lactobacillus plantarum* has also been found to resist the effects of AITC (Pérez-Díaz and McFeeters, 2010).

Although attempts have been made to categorize the effect of ITCs on bacteria, no clear and consistent pattern of bacterial inhibition is evident in the experimental data available. Past research has implied that Gram-positive bacteria and strict aerobes are more frequently reported as sensitive to the effects of various ITCs, but more recent reports suggest that Gram-negative organisms are more sensitive. Additionally, bacterial pathogenicity does not seem to affect ITC sensitivity; both pathogens and starter cultures alike can be susceptible or resistant. However, despite these contradictions, it can be concluded that strain specific resistance and/or sensitivity to ITCs exists.

2.3.3 Use of isothiocyanates in food

There has been much interest in incorporating ITCs into foods as natural antimicrobials based on consumers' perceived safety of food additives of natural origin as well as the demand for fresh and natural food products (Evans et al., 2010). Because it is able to effectively inhibit many pathogenic organisms, AITC has been studied in various food matrices for its potential use as a food antimicrobial. Previously, AITC has been shown to exert its antimicrobial activity against pathogenic and spoilage organisms in food, including *Vibrio parahaemolyticus* in tuna meat (Hasegawa et al., 1999), spoilage organisms in cooked rice (Kim et al., 2002), *E. coli* O157:H7 in ground beef (Chacon et al., 2006a; Nadarajah et al., 2005), *E. coli* O157:H7 in fermented dry sausages (Chacon et al., 2006b), pathogens *E. coli*, *Salmonella* Enteritidis, *S. aureus* and *L. monocytogenes* on Chinese cabbage (Inatsu et al., 2006), brown rot-causing fungus *Monilinia laxa* on nectarines and peaches (Mari et al., 2008), yeast and moulds in cottage cheese (Conceição Gonçalves et al., 2009), *Salmonella* and *E. coli* O157:H7 on fresh sliced and whole

tomatoes (Obaidat and Frank, 2009a), *E. coli* O157:H7 on dry-cured ham (Graumann and Holley, 2009), *E. coli* O157:H7 on lettuce and spinach leaves (Obaidat and Frank, 2009b; Seo et al., 2012), spoilage organisms on blueberries (Wang et al., 2010), *Zygosaccharomyces globiformis* in cucumbers (Pérez-Díaz and McFeeters, 2010), *L. monocytogenes, S.* Typhimurium, *Salmonella* Choleraesuis and *Campylobacter* species in chicken breast and meat (Dias et al., 2013; Olaimat et al., 2014; Shin et al., 2010b), microorganisms in tofu (Shin et al., 2010a), *Salmonella enterica* in liquid egg albumen (Jin and Gurtler, 2011), *Penicillium expansum* and *Botrytis cinerea* on apples (Wu et al., 2011), *Salmonella* strains on fresh cantaloupe (Chen et al., 2012), spoilage organisms in kimchi (Ko et al., 2012), *L. monocytogenes* on fresh cut onions (Piercey et al., 2012), *Listeria innocua* on frozen ready-to-eat shrimp (Guo et al., 2013), *Fusarium* molds in bread (Azaiez et al., 2013), *Ps. aeruginosa* on catfish fillets (Pang et al., 2013), and *Aspergillus flavus* and *Aspergillus parasiticus* in peanuts (Hontanaya et al., 2014; Otoni et al., 2014).

Although AITC has been proven to be a potent antimicrobial in food matrices, its use is limited due to the characteristic pungent odour and flavour associated with ITCs. At higher concentrations, the organoleptic qualities of a food product containing ITCs are not acceptable to consumers. For example, dry fermented sausages containing a concentration of microencapsulated AITC greater than the effective concentration of 750 ppm were considered unacceptable to sensory panellists (Chacon et al., 2006). Thus, flavour and odour changes limit the use of ITCs in foods for the control of pathogens and spoilage organisms.

2.4 Inhibitory mechanisms of isothiocyanates

Although ITCs are well known for their antimicrobial properties against bacterial pathogens, the mechanism behind the microbiocidal action of ITCs is unclear. Previous research has indicated that the compounds can exert both bacteriostatic and bactericidal effects (Delaquis and Mazza, 1995). Several studies have investigated various interactions between ITCs and bacteria in order to determine characteristics of ITCs that may play a significant role in its microbial antagonism. Despite these studies, a full understanding of the specific antimicrobial mechanism has not yet been achieved.

2.4.1 Reaction of isothiocyanates with thiol groups and enzymes

ITCs are particularly reactive compounds and are subject to nucleophilic attack at its electron-lacking central carbon atom (Wu et al., 2009). In the presence of thiol molecules, this carbon atom is attacked and dithiocarbamates are formed (Shibata et al., 2011). Initial research suggested that the antimicrobial activity of ITCs may be linked to the inhibition of sulfhydryl enzymes in bacteria given that thiol-containing molecules such as thioglycollate and cysteine are able to interact with ITCs and diminish the antibacterial activity (Zsolnai, 1966). It has been found that the inhibition of sulfhydryl enzymes may play a role in the antimicrobial action of ITCs (Zsolnai, 1966; Luciano and Holley, 2009).

A report by Delaquis and Mazza (1995) indicated that ITCs are more effective against strictly aerobic organisms than facultative anaerobes, which can be used to explain the antimicrobial

mechanism of ITCs. Few studies have verified the interactions between ITCs and enzymatic systems. A report by Kojima and Ogawa (1971) examined the levels of oxygen uptake in yeast when allyl, methyl, phenyl and beta-phenylethyl ITCs were applied. The authors observed a reduction in oxygen uptake in the presence of these compounds as well as significant inhibition of cytochrome c oxidase by AITC. However, the ITC levels required to achieve enzymatic and oxygen uptake inhibition were 100 times greater than the minimum inhibitory concentration of AITC for those organisms (Kojima and Ogawa, 1971; Luciano and Holley, 2009). From these data, it was concluded that AITC was not a strong respiratory inhibitor and that its role in inhibiting oxygen intake was not a major factor behind its antimicrobial function.

Luciano and Holley (2009) followed up with research involving ITC interactions with enzyme systems. The authors tested the ability of AITC to inhibit thioredoxin reductase and acetate kinase, two enzymes important in the metabolism of *E. coli*. The research described the dose-dependent inhibitory activity of AITC against *E.* coli thioredoxin reductase and indicated that even levels 50-fold lower than the MIC of AITC were able to competitively inhibit thioredoxin reductase by levels ≥58%. AITC was also able to non-competitively inhibit acetate kinase activities at levels ≥10 μL L^{-1}. Together, these results suggest that the antimicrobial activity of AITC against *E. coli* O157:H7 may be attributed to the action of more than one inhibitory mechanism.

2.4.2 Reaction of isothiocyanates with proteins and amino acids

The electron-deficient central carbon of ITCs is also susceptible to attack from amino groups, forming thiourea derivatives (Wu et al., 2009). In studying reactions between ITCs and proteins, Kawakishi and Kaneko (1985, 1987) observed the electrophilic action of AITC. The ability of AITC to initiate disulfide bond oxidative cleavage in cysteine moieties and to react with free amino groups of lysine and arginine was identified; however, the authors did not relate these results to microbial proteins, therefore it is unclear if the same effect would occur *in vivo*. Additionally, the experiments were performed using temperatures over 37°C with intensive stirring, both conditions which expedite ITC-protein interactions (Kawakishi and Kaneko, 1985; 1987).

2.4.3 Interactions of isothiocyanates and bacterial cellular membranes

Some previous work has shown that Gram-negative bacteria were more sensitive to inhibition by AITC than Gram-positive bacteria (Isshiki et al., 1992; Lin et al., 2000b). In *Escherichia coli* K-12, Lin et al. (2000) indicated that AITC caused damage to the cell membrane, resulting in leakage of cellular metabolites. Further, it was found that AITC inhibited microbes in a similar manner as the antibiotic polymyxin B, which is more effective against Gram-negative bacteria than Gram-positive due to its ability to bind to LPS in the outer cell membrane, a structure which Gram-positive bacteria lack. AITC showed potent bactericidal activity against bacteria in the exponential phase and affected Gram-negative bacteria more severely than Gram-positives (Lin et al., 2000a). However, upon further research investigating the effect of AITC on the cell membrane, Ahn et al. (2001) showed no damage to the cell wall or leakage of ATP in response to AITC by *Listeria monocytogenes*; nevertheless, the internal levels of ATP were reduced.

Furthermore, the researchers observed a change in the internal structure of AITC-treated cells in comparison to non-treated bacteria when analyzed by transmission electron microscopy, indicating an intracellular effect caused by AITC (Ahn et al., 2001).

Work by Sofrata et al. (2011) indicated that the bacterial membrane undergoes physical changes in the presence of benzyl isothiocyanate (BITC), the ITC common in the shrub *Salvadora persica*. The authors observed that 2.8 μmol BITC was able to cause protrusions in the cell membranes of Gram-negative *Aggregibacter actinomycemcomitans*. It was postulated that these changes in cell physiology may have been caused by the infiltration of BITC through the outer bacterial membrane and subsequent interference with the bacterial redox system, affecting the ability of the bacterium to maintain its membrane potential (Sofrata et al., 2011). BITC has also been observed to alter the mitochondrial transmembrane potential, as well as modify other mitochondrial processes, including interfere with respiration, initiate mitochondrial swelling, and induce the release of cytochrome c in eukaryotic cells (Nakamura et al., 2002).

2.4.4 Effect of isothiocyanates at different growth stages

In a report by Lin et al. (2000), the authors observed a dose- and time-related inhibitory effect of AITC against the Gram-negative bacteria *E. coli* O157:H7 and *Salmonella* Montevideo. The work showed that these bacteria were more susceptible to AITC during the early and late exponential growth stages in comparison to the lag and stationary phases. However, in the Gram-positive *L. monocytogenes*, the authors noted no difference in sensitivity in any of its growth

stages. The study concluded that AITC was able to show effective bactericidal activity against bacteria of both Gram-types at all growth stages (Lin et al., 2000a).

2.5 Genetic response to isothiocyanates

2.5.1 Identification of isothiocyanate resistance genes

ITC resistance genes dubbed "*sax*" (survival in *Arabidopsis* extracts) were identified in both *E. coli* and *Pseudomonas syringae* which allowed the bacteria to resist the effects of sulphorophane (4-metholsulfinylbutyl ITC), the ITC found in *Arabidopsis* plants (Fan et al., 2011). The authors found the *sax* operon to consist of several nonessential genes (*sax*A, B, C, F, D and G), each of which had a distinct role but were all complementary and required in *P. syringae* for resistance to sulphorophane. *SaxF, saxD* and *saxG* genes were identified as likely multidrug resistance efflux genes, which function to expel a variety of substrates, including antibiotics and other foreign molecules (Blair and Piddock, 2009; Fan et al., 2011). These genes, when individually deleted, resulted in slightly increased sensitivity of the organism to sulphorophane; however, when deleted in groups, sensitivity substantially increased. The roles of each gene in the *sax* operon were investigated, but the mechanism of *sax*-mediated resistance of *P. syringae* to ITCs remains unidentified and merits further examination (Fan et al., 2011).

2.5.2 Gene expression in response to isothiocyanates

Quorum sensing, a chemical communication process used in regulation of bacterial behavior, is employed by *Pseudomonas aeruginosa* to elicit virulence and biofilm formation (O'Loughlin et al., 2013). The ITCs sulphorophane and iberin (1-isothiocyanato-3-(methylsulfinyl) propane), an ITC derived from horseradish, have been discovered to inhibit quorum sensing in *Ps. aeruginosa* (Ganin et al., 2013; Jakobsen et al., 2012). Ganin and colleagues (2013) indicated that both sulphorophane and iberin bound to the *Ps. aeruginosa LasR* transcriptional activator protein, effectively reducing the organism's quorum sensing activation ability and virulence. In Jakobsen and colleagues' research (2012), iberin was found to inhibit the expression of the *LasB* quorum sensing gene in *Ps. aeruginosa*, although the growth of the organism was not affected even at high concentrations of the ITC. Further, this research identified the upregulation of three genes in the MexEF-OprN operon, which encode for an efflux pump, suggesting that the efflux of ITCs may be a resistance mechanism. A glutathione S-transferase (*GST*) gene was also upregulated in the presence of iberin; *GST* has been studied for its ability to catalyse the conjugation of isothiocyanates to form glutathione in cyanobacteria (Wiktelius and Stenberg, 2007) as well as in human cells (Kolm et al., 1995). This rapid conversion of toxic ITCs to glutathione may act as another resistance mechanism in the organism. Other highly upregulated genes in *Ps. aeruginosa* included those coding for oxidoreductases, which suggests a response to a disturbance in the organism's redox homeostasis, and those coding for the heat stress proteins GroEL and DnaK, which indicates the presence of misfolded proteins.

Similarly, in an early transcriptomic response to exposure to BITC, *Campylobacter jejuni* was revealed to have a number of genes related to heat shock response highly induced, including the *GroEL* and *DnaK* (Dufour et al., 2013). These genes are required for protein refolding,

suggesting the development of misfolded proteins as a result of exposure to BITC. In addition, the study identified the upregulation of many other genes, including those coding for a number of proteins involved in oxidative stress response, as well as genes related to the formation of iron-sulfur clusters, which are involved in oxidation-reduction reactions of mitochondrial electron transport. Further investigation revealed the expression of genes involved in electron transport and energy metabolism, as well as the induction of genes involved in the tricarboxylic acid cycle and other metabolic pathways. Using this information, the authors hypothesized a mechanism for the antibacterial effect of BITC on *C. jejuni* where the ITC damaged protein structure and function by attacking the thiol groups, resulting in a heat shock-like response that diverted metabolic activity to help maintain energy needed for the stress response and the organism's survival. ITCs also induced an oxidative stress response by disrupting disulfide reductase activity and electron transport, resulting in the production of reactive oxygen species (Dufour et al., 2013).

In *E. coli* there exists a two-component BaeSR regulatory system that plays a large role in the organism's ability to overcome inhibitory concentrations of essential oils (Zoetendal et al., 2008). This system contains a signaling enzyme, histidine kinase (BaeS), and BaeR, a cytoplasmic response regulator (Cordeiro et al., 2014). Deletion of both these two genes (*baeS* and *baeR*) in *E. coli* O157:H7 increased the organism's sensitivity to AITC slightly, limiting the ability of *E. coli* O157:H7 to overcome AITC challenge. However, Cordeiro et al. (2014) speculated that there may be cross-regulation among multiple two-component systems which allowed the Δ*baeSR* mutant to withstand AITC exposure.

2.3.3 Resistance to allyl isothiocyanate

Unlike the response bacteria show to antibiotics, bacteria seem unable to develop resistance to AITC. Cordeiro and colleagues (2014) showed that *E. coli* O157:H7 (strain 02-0304) was able to adapt to withstand high concentrations of AITC from an initial MIC of 51 ppm to 216 ppm after several generations, but interruption of growth (freezing the culture) caused its resistance to return back to its sensitive state upon reactivation. This reversion of sensitivity indicates that the organism responded to AITC as an environmental stress rather than as a drug toxicity (Cordeiro et al., 2014). It seemed that *E. coli* was not able to acquire sustained resistance to AITC, and only exhibited transient, phenotypic resistance.

2.6 Antibiotics

2.6.1 Antibiotic mechanisms of action

Since the discovery of penicillin in 1929, antibiotics have been used extensively in human and veterinary therapy to promote health as well as in animal husbandry as growth promoters (Rodríguez-Rojas et al., 2013). Originally defined as microorganism-produced substances that inhibited the growth of other microorganisms, antibiotics are now described as agents possessing systemic antimicrobial activity and these agents can either be synthetic analogues and derivatives or naturally occurring (Hodges, 2011). Although there have been many new antibiotics developed over the past few decades, the number of antibiotic classes, which are grouped according to their spectrum of activity, remains very small. Against bacteria, antibiotics can exert bacteriostatic or bactericidal effects by interfering with essential metabolic processes.

Commonly, antibiotics cause: 1) inhibition of cell wall synthesis, 2) inhibition of replication and/or transcription of genetic material, 3) inhibition of protein synthesis, and/or 4) inhibition of cell membrane functions (Lancini et al., 1995). It is known that the β-lactams, glycopeptides, and bacitracin interfere with cell wall synthesis; the aminoglycosides, tetracyclines, macrolides, azalides, and chloramphenicol hinder protein biosynthesis; the polymyxins disrupt cell membrane functions; and the sulfonamides and trimethoprim interfere with folate synthesis (Lambert, 2011).

The cell walls of bacteria contain peptidoglycan, a macromolecule composed of glycan chains and short peptide cross-links that is responsible for maintaining the shape and the mechanical strength of the cell. In bacteria, peptidoglycan is a vital component of the cell wall that comprises nearly 50% of the weight of the wall in Gram-positive bacteria and 10-20% in Gram-negative cells (Lambert, 2011). As peptidoglycan is not present in human cells, it is an ideal target for antibiotics because only the infectious microorganism would be affected (Woodin and Morrison, 1994). Antibiotics such as β-lactams and glycopeptides affect the formation of peptidoglycan, resulting in the cell wall becoming distorted due to osmotic pressure and prone to rupture (Gale, 1963). β-lactam drugs such as penicillins, cephalosporins, carbapenems, and monobactams interfere with the final stage of peptidoglycan assembly by mimicking the D-alanyl-D-alanine moiety of peptidoglycan and reacting with the transpeptidase enzymes required for the final stage of cross-linking (Green, 2002). The transpeptidase enzyme is therefore rendered unable to interact with the terminal D-alanine group of the glycan chain, and, as a result, crosslinking between chains does not occur. The glycopeptide antibiotics (vancomycin and teicoplanin), on the other hand, affect peptidoglycan synthesis at an earlier point. Peptidoglycan precursors are

formed in the cytoplasm and travel through the cell membrane into the cell wall by action of the transglycosylase enzyme (Lancini et al., 1995). Glycopeptide antibiotics bind to the D-alanyl-D-alanine residue of the stem peptide, and the bulky size of the molecule prevents the peptide fragment from entering the cell wall (Reynolds, 1989).

Aminoglycosides are common, broad-spectrum antibiotics that are bactericidal towards both Gram-positive and Gram-negative bacteria alike (Kotra et al., 2000). These antibiotics show a binding affinity towards nucleic acids, especially towards certain portions of prokaryotic rRNAs (Fourmy et al., 1996). Aminoglycosides interact with the 16S rRNA of the 30S ribosomal subunit and disrupt the elongation of peptide chains by causing misreading and/or premature termination of translation (Mingeot-Leclercq et al., 1999). Different classes of aminoglycosides, due to their structure, will bind to different sites on the 16S rRNA. Neomycin, paromycin, gentamicin, and kanamycin are thought to bind to the A-site of the rRNA, which disrupts the recognition of tRNA by rRNA during translation (Noller, 1991). Similarly, tetracyclines possess a broad spectrum of antibiotic action and also bind to the 30S subunit of ribosomes. By doing so, the tetracycline molecule blocks the binding of aminoacyl-tRNA to the ribosomal acceptor site (A site) and prevents amino acids from being added to the nascent peptide chain (Chopra and Roberts, 2001).

There are also many antibiotics that interact with the 50S subunit of bacterial ribosomes. Chloramphenicol, a broad-spectrum antibiotic, binds to the 50S ribosomal subunit around the A site of the 23S rRNA, preventing aminoacyl-tRNA from binding to the site and subsequently disallowing peptidyl transferase from forming peptide bonds to the nascent peptide chain

(Lambert, 2011; Pestka, 1969). Macrolides, such as erythromycin, spiramycin, oleandomycin, azithromycin and lincosamides, are a group of antibiotics characterized by a 12-16-membered lactone ring that are primarily effective against Gram-positive organisms. These antibiotics bind at a site on the 23S rRNA very close in proximity to the site to which chloramphenicol binds. However, unlike chloramphenicol, macrolides block the translocation process of protein synthesis, disassociating the peptidyl-tRNA and causing the early release of incomplete polypeptides (Kanoh and Rubin, 2010).

Quinolone antibiotics are known for their activity against *E. coli* and other Gram-negative pathogens, as well as their efficacy against Gram-positive cocci and *Ps. aeruginosa* (Lambert, 2011; Sanders, 1988). This group of antibiotics, including ciprofloxacin, interferes with bacterial nucleic acid synthesis by targeting the type II and IV topoisomerases vital to DNA replication (Anderson et al., 1998). These enzymes are responsible for chaperoning the winding of the DNA strand as well as creating double-stranded breaks in the bacterial chromosome (Aldred et al., 2014). Studies have found that quinolones act by turning these topoisomerases' ability against the bacterium, resulting in fragmenting of the bacterial chromosome (Mitscher, 2005).

Polymyxins are a group of polycation antibiotics that target the bacterial cell membrane. These bactericidal antibiotics are lipopetides in structure and are comprised of a polycationic peptide ring and a tripeptide side chain with a fatty acid tail, allowing them a "detergent-like" mechanism of action and the ability to self-promote its uptake (Hancock, 1997; Zavascki et al., 2007). Polymyxins bind to the lipopolysaccharide (LPS) of Gram-negative bacteria, destabilize the molecule, and distort the outer membrane structure, leading to its destruction (Hancock,

1997). The destruction of this permeability barrier allows the polymyxin molecules' fatty acid side chains to then further bind with the LPS, disrupt cytoplasmic membrane integrity, and release cytoplasmic components (Lambert, 2011). Additionally, these breaches allow a variety of molecules to enter the cell, including the drug itself, leading to cell death (Hermsen, 2003). Polymyxins B and E (a colistin) are frequently used in the treatment of Gram-negative bacterial infections, especially against *Ps. aeruginosa* but have no activity against Gram-positive bacteria due to their lack of LPS (Zavascki et al., 2007).

Lastly, there are groups of antibiotics that target folate synthesis in bacteria. In all living cells, folate is an essential cofactor required for the biosynthesis of many cellular components, including DNA and RNA (Kamen, 1997; Bermingham and Derrick, 2002). Sulfonamide antibiotics are structural analogues of the *p*-aminobenzoic acid (PABA) molecule, which is a precursor required in the bacterial synthesis of folate. The antibiotic competitively inhibits the incorporation of PABA into the dihydropteroic acid molecule, preventing continued folate synthesis (Bermingham and Derrick, 2002). Trimethoprim, on the other hand, inhibits bacterial dihydrofolate reductase, an enzyme responsible for reducing dihydrofolic acid into tetrahydrofolate acid to be used in subsequent synthesis of adenine, guanine, thymine and methionine (Lambert, 2011).

2.6.2 Antibiotic resistance mechanisms

Bacteria possess various mechanisms in order to withstand antibiotic challenge. The major methods of resistance include enzymatic inactivation of the antimicrobial agent, modification of

the antibiotic binding sites, alteration of cell wall permeability towards the agents, or active drug efflux from the cell (Dever and Dermody, 1991). These resistances may be inherent ("natural") or acquired characteristics of the organism (Bockstael and Van Aerschot, 2008). Against β-lactam antibiotics, some organisms produce β-lactamases, which hydrolyze the β-lactam ring and render the antibiotic ineffective (Poole, 2004). Many β-lactamase-producing bacteria have been identified, including *E. coli* and *Salmonella* species, as well as *Campylobacter*, *Enterococcus*, and *Staphylococcus* (Li et al., 2007). Modification of antibiotic target sites is another approach used by some bacteria such as *S. aureus*, where its altered transpeptidase enables the organism to be resistant to methicillin and other β-lactam antibiotics (Lambert, 2005). In lactobacilli, glycopeptide resistance is considered to be inherent; *Lactobacillus* species and *Pediococcus* species that are resistant to vancomycin possess cell wall peptides with a D-Ala-D-lactate modification, preventing the antibiotic from binding (Danielsen et al., 2007; Klein et al., 2000). Alteration of outer membrane permeability preventing access to the cell wall is yet another way bacteria can protect themselves from antibiotics. Organisms such as *E. coli* and *Ps. aeruginosa* have exhibited antibiotic resistance through a decrease or functional change of porin channels, preventing antibiotics from crossing the outer membrane of the organism (Delcour, 2009; Nikaido, 2003). Additionally, efflux channels effectively allow bacteria to eliminate antibiotics from within the cell. For example, *Ps. aeruginosa* is known to be intrinsically resistant to numerous antibiotics due to, in part, the expression of multi-drug efflux systems that work to promote the expulsion of antibiotics from within the cell (Poole et al., 1996; Poole, 2001). Additionally, polymyxin-resistant *S.* Typhimurium and *E. coli* have been identified and only bind 25% of polymyxin bound by the wild type strain (Vaara et al., 1979). These resistant

mutants possess LPS that contains more 4-aminoarabinose and phosphoethanolamine, effectively reducing the negative charge of LPS, which inhibits the binding of polymyxins.

By correlating antibiotic and AITC minimum inhibitory concentrations (MICs) in a wide range of bacteria, similarities in resistance profiles can be identified and parallels can be drawn between mechanisms of action and/or mechanisms of resistance. Identifying the sinigrin utilisation abilities of these bacteria may also aid in the understanding of bacterial myrosinase-like activity, and investigating the effect of the addition of various carbon sources (including glucose, sorbitol, and monosaccharides) on this activity may present a new perspective on the metabolism of sinigrin in *E. coli* O157:H7.

CHAPTER 3

3. Antimicrobial activity and mechanism of action of allyl isothiocyanate against meat starter cultures

3.1 Abstract

Isothiocyanates (ITCs) are pungent compounds used as a defense mechanism by plants in the family *Brassicaceae*. Allyl isothiocyanate (AITC) is the ITC obtained from the glucosinolate sinigrin found in Oriental and brown mustards (*Brassica juncea*). Against a wide range of microorganisms, AITC is regarded as a potent antimicrobial. In food products, the use of this agent to control foodborne pathogens such as *E. coli* O157:H7 is appealing due to its natural origin and its efficacy against various pathogenic and spoilage organisms, which has been documented in the past. However, few studies have successfully identified the mechanism by which AITC exacts its bactericidal effects, although several have described possible mechanisms by which the compound affects bacteria. In the present work, the MIC profile of AITC against 12 organisms positively correlated with the inhibitory action of the antibiotics ciprofloxacin (81.7%) and polymyxin B (76.2%). Ciprofloxacin is a fluoroquinolone antibiotic known to affect DNA gyrase and topoisomerase IV in bacterial cells, halting the synthesis of DNA. Similarly, AITC has been shown to attack proteins and cause DNA damage, as well as inhibit DNA and RNA synthesis. Polymyxin B is an antibiotic that works by attacking the lipopolysaccharides of the cell outer membrane, making this agent more effective against Gram-negative than Gram-positive bacteria. AITC and other ITCs have been shown to damage the outer membrane and

cause cellular leakage in Gram-negative bacteria. Additionally, polymyxin B has been shown to inhibit type-II NADH-quinone oxidoreductases, enzymes involved in the electron transport chain. ITCs are known to affect bacterial respiration processes and have been observed to cause the up-regulation of genes encoding for oxidoreductases, suggesting that they may possess a mechanism of action similar to that of polymyxin B. With this information, future research investigating the mechanism of AITC antimicrobial action can be focused on its effect on enzymes involved in DNA synthesis, its effect on cellular membranes, and its effect on enzymes required for respiration.

3.2 Introduction

In plants belonging to the family *Brassicaceae,* glucose- and amino-acid derived secondary metabolites called glucosinolates are found in the cell vacuoles (Radojčić Redovniković et al., 2008). Upon structural damage to the plant, glucosinolates are released and interact with the enzyme myrosinase, which catalyzes the cleavage of the glucose group from the glucosinolate, forming isothiocyanates (ITCs) and other secondary breakdown products (Delaquis and Mazza, 1995). Allyl isothiocyanate (AITC) is an organosulfur compound derived from breakdown of the glucosinolate sinigrin found in Oriental mustard, *Brassica juncea,* and is responsible for the distinctive, pungent characteristic of horseradish and mustard preparations. AITC, as well as other ITCs, have been studied as effective antimicrobial compounds against a diverse range of microorganisms, including foodborne pathogens. AITC use in food and food products has been researched due to its GRAS status and effective activity against foodborne pathogens such as *E. coli* O157:H7. Although several reports have investigated the mechanism behind the

antimicrobial activity of AITC and other ITCs, its specific inhibitory mechanism remains unknown.

Reports have indicated that AITC is capable of causing cell membrane damage, resulting in leakage of cellular metabolites in Gram-negative bacteria (Lin et al., 2000a). However, in Gram-positive organisms such as *Listeria monocytogenes*, the same phenomenon was not witnessed, although a reduction in intracellular levels of ATP was observed (Ahn et al., 2001). Further, in a comparison between AITC and several antibiotics, AITC was found to be most similar to polymyxin B in its microbiocidal activity due to its effectiveness against bacteria in stationary phase and its potency against Gram-negative over Gram-positive bacteria (Lin et al., 2000a). Similarly, benzyl isothiocyanate (BITC) has also been observed to cause physical membrane damage in Gram-negative *Aggregibacter actinomycemcomitans* (Sofrata, et al., 2011). AITC is known to possess lipophilic and electrophilic properties and these characteristics, shared with BITC, were thought to provide the ITC with the ability to infiltrate into bacterial cells via the outer membrane and interfere with the bacterial redox system, affecting the ability of the cell to maintain its membrane potential (Sofrata et al., 2011; Azimova et al., 2012). A similar effect on membranes was noted in eukaryotic cells: many ITCs have been observed to alter the mitochondrial transmembrane potential as well as inhibit respiration, induce mitochondrial swelling, and release cytochrome c (Nakamura et al., 2002; Tang and Zhang, 2005).

ITCs are prone to nucleophilic attack at their electron-lacking central carbon atom, and thus are particularly reactive compounds (Wu et al., 2009). In the presence of thiol molecules, this carbon is attacked and dithiocarbamates are formed (Shibata et al., 2011). Early research by Zsolnai

(1966) indicated that the microbiocidal activity of ITCs may be linked to the inhibition of sulfhydryl enzymes in bacteria as thiol-containing molecules including thioglycollate and cysteine are able to interact with ITCs and reduce antibacterial activity. To date, research has indicated that the inhibition of sulfhydryl enzymes may be one of several antimicrobial mechanisms of ITCs (Zsolnai, 1966; Luciano and Holley, 2009).

Additionally, the central carbon of ITCs is also susceptible to attack from amino groups, forming thiourea derivatives (Wu et al., 2009). Studies by Kawakishi and Kaneko (1985, 1987) observed the ability of AITC to initiate disulfide bond oxidative cleavage in cysteine moieties and to react with free amino groups of lysine and arginine. Luciano and Holley (2009) found that AITC was able to inhibit thioredoxin reductase and acetate kinase, two unrelated enzymes integral in the metabolism of *E. coli*. These results indicate that AITC may possess a broad range of antimicrobial activity that affects many different processes in the target bacteria.

ITCs have also been found to affect respiratory enzymes. In yeast, several ITCs (AITC, methyl ITC, phenyl ITC, and phenethyl ITC) were found to reduce oxygen uptake (Kojima and Ogawa, 1971). However, levels of ITCs used in this study exceeded the MIC by over 200 times. Jakobsen and colleagues (2012) investigated the effect of iberin, a medium chain aliphatic ITC, on *Pseudomonas aeruginosa* and found that the ITC induced the expression of two respiratory genes encoding for cytochrome O ubiquinol oxidase, while simultaneously downregulating a putative cytochrome c oxidase.

The objectives of the present study were to assess the microbiocidal effects of AITC and several antibiotics encompassing a wide range of antibiotic classes against a variety of bacteria; to compare the resulting MIC profiles in order to determine the antibiotics that AITC action is most similar to; and to draw parallels between AITC and analogous antibiotics to gain insights on AITC antibacterial activity.

3.3 Materials and Methods

3.3.1 Chemicals and antibiotics

Sinigrin hydrate (≥99%), potassium hydrogen phthalate, and tetrabutylammonium hydrogen sulfate (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA); AITC at 94% purity was purchased from Acros Organics (Ottawa, ON).

The 12 antibiotics used in this experiment were selected from an array of classes to represent a span of mechanisms of action. Antibiotics selected for testing included ampicillin (AMP), cephalothin (CET), lincomycin (LCM), vancomycin (VAN), erythromycin (ERY), azithromycin (AZI), neomycin (NEO), ciprofloxacin (CIP), polymyxin B (PLB), sulfamethazine (SMT), oxytetracycline (OTC) and chloramphenicol (CAM). The antibiotics were purchased in powdered form from commercial sources. Oxytetracycline dihydrate, erythromycin, sulfamethazine, chloramphenicol were obtained from Acros Organics; ampicillin sodium salt, ciprofloxacin, polymyxin B sulfate, cephalothin sodium salt, azithromycin, and vancomycin

dihydrate were from Sigma-Aldrich Canada (Oakville, ON); neomycin was from Fisher Scientific (Fair Lawn, NJ, USA); and lincomycin was from Alfa Aesar (Ward Hall, MA, USA).

3.3.2 Bacterial strains and culture conditions

Twelve bacterial isolates were tested. Bacterial strains used were selected from groups having resistance or sensitivity to AITC, the ability to degrade sinigrin, or were thought to have useful genetic determinants (genes) that might be helpful in comparisons. Cultures of *Lactobacillus plantarum* UM 134L and UM 131L, *Pediococcus acidilactici* UM 119P and UM 129P, *Pediococcus pentosaceus* UM 121P, and *Staphylococcus carnosus* UM 136 were obtained from the Department of Food Science culture collection, University of Manitoba. *Escherichia coli* K-12 DH5α, *Pseudomonas aeruginosa* PAO1, and its mutant, *Ps. aeruginosa* PAO750, were obtained from Dr. A. Kumar (Department of Microbiology, University of Manitoba, Winnipeg, MB). The *Ps. aeruginosa* strains were selected due to the wild type (PAO1) possessing efflux pumps, conferring antibiotic resistance, and the lack of 5 operons encoding for resistance nodulation cell division (RND) efflux pumps in the mutant (PAO750). Methicillin-resistant *Staphylococcus aureus* ST398 was obtained from Dr. G. Golding (Public Health Agency of Canada, Winnipeg, MB). *Escherichia coli* O157:H7 1931 was obtained from the culture collection in the Department of Food Science, University of Manitoba. Isolates of *P. pentosaceus*, *P. acidilactici*, and *L. plantarum* strains were regularly grown aerobically on All Purpose Tween (APT) agar (Difco), whereas *E. coli* O157:H7, *E. coli* K-12, *Ps. aeruginosa*, *Ps. fluorescens*, *S. aureus* and *S. carnosus* were grown aerobically on Tryptic Soy Agar (TSA; Oxoid, Fisher Scientific, Edmonton, AB) at 35°C.

From these plates, 1-3 isolated colonies were inoculated into 0.05M potassium phthalate-buffered (Sigma-Aldrich) (pH 5.5) Mueller-Hinton broth (MHB) (Oxoid) for *Escherichia* spp., All-purpose Tween media (APT) (Difco) for *L. plantarum* spp., *P. pentosaceus* spp., and *P. acidilactici* spp. or Tryptone Soy Broth media (TSB) (Oxoid) for pseudomonads and staphylococci. These tubes were then incubated overnight (18 h). Aliquots of 0.1 ml were then transferred into 9.9 ml of the corresponding broth and incubated at 21°C with shaking at approximately 150 rpm (2314Q Benchtop Lab Rotator, Thermo Scientific, Waltham, MA, USA) until the mid-exponential phase was reached, where optical density reached $OD_{600} = 0.6$ (approximately 7.7 log CFU ml^{-1}) using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England). Further dilutions of 0.1 ml aliquots of these cultures were made in the corresponding broth for use in AITC susceptibility determinations or in sterile 0.1% peptone water for antibiotic susceptibility testing. The final concentration of bacteria used in both these tests was approximately 5.7 log CFU ml^{-1} (CLSI, 2012).

3.3.3 Antimicrobial agents

Ethanol (2% v/v) was used to solubilize AITC in each of MHB, TSB and APT broths. The concentration of ethanol in the first tube of each trial was no more than 0.5%, and the effect of this solvent on the growth of bacteria was tested during the susceptibility tests. Concentrations of AITC ranged from 0.81 mg/L to 832 mg/L.

The range of antibiotic concentrations used was 0.20 μg/mL to 400 μg/mL. AMP, CET, LCM, VAN, AZT, ERY, and NEO were dissolved in each of double strength MHB, TSB and APT broth. For OTC, CAM, and SMT, dimethyl sulfoxide (DMSO) (Sigma-Aldrich) (4% v/v) was used as a solubility mediator in order to dissolve the agents in the appropriate broths. The starting well of these antibiotics contained no more than 1% DMSO, and the effect of DMSO against the growth of bacteria was tested during the susceptibility tests. For CIP, 0.1 M HCl (8%) was used as a solubility mediator. Again, the starting well of each CIP test contained no more than 2% 0.1 M HCl and its effect on the growth of the test strains was monitored. Following dissolution in double strength broth, the antibiotics were filter sterilized using 0.20 μm syringe filters (Whatman, GE Science, Little Chalford, Buckinghamshire, UK).

3.3.4 AITC minimum inhibitory concentration (MIC) testing

The resistances of bacteria to AITC were determined via standard broth macrodilution (Clinical Laboratory Standards Institute, CLSI, 2012) in autoclaved, 10 ml screw-capped tubes. Each tube was filled with 5 ml of the corresponding broth. MHB was used for *E. coli* O157:H7 1931 and *E. coli* K-12 DH5α. APT was used for *L. plantarum*, *P. pentosaceus* and *P.acidilactici.* TSB was used for *Ps. aeruginosa* PAO1, *Ps. aeruginosa* PAO750, *Ps. fluorescens*, *S. carnosus* and *S. aureus*. The first tubes of each trial were filled with 5 ml 3200 μL mL^{-1} AITC solution and serial two-fold dilutions were performed so that the series of 10 tubes ranged in concentrations from 832 mg/L to 0.81 mg/L. Each tube was then inoculated with 5 ml of bacterial suspension; totalling a volume of 10 ml. Tubes were capped and incubated with shaking at approximately 150 rpm for 24 h at 21°C. Following incubation, tubes were examined visually to determine the

MIC, which was considered to be the lowest concentration of AITC that resulted in a non-turbid tube (signifying inhibited growth). Each trial was conducted in triplicate. Control tests were also performed alongside the MIC following the guidelines of the CLSI (2012). Each experiment was performed twice in triplicate.

3.3.5 Antibiotic MIC determination

The resistances of bacteria to antibiotics were determined via standard broth microdilution in sterile, 96-well microtitre plates (Falcon, Corning Inc., Corning, NY, USA) (Holley and Blaszyk, 1998). Each well was filled with 50 µL potassium phthalate-buffered, double-strength MHB/TSB/APT broth. The first wells were filled with 50 µL of the prepared 1600 µg/mL antibiotic solutions and serial two-fold dilutions were performed. Following, 50 µL of bacterial suspension was added to each well, totalling a volume of 100 µL. Plates were covered and taped and were placed on a shaker for 24 h at 21°C. Afterward, 40 µL of p-iodonitrotetrazolium violet (p-INT) indicator was added to each well and plates were incubated at 35°C for an additional 2 h (Eloff, 1988) with the exception of *P. acidilactici* and *P. pentosaceus*, which did not react with the indicator. The MIC was determined to be the lowest concentration of antibiotic where the well remained colourless (no metabolic activity). For *P. acidilactici* and *P. pentosaceus*, the MIC was determined as the well with the lowest concentration of antibiotic that yielded no turbidity after 24 h. Each experiment was performed twice in triplicate.

3.3.6 Statistical analysis of MICs

All statistical analyses were performed using JMP 10 software (SAS Institute, Inc., Cary, NC, USA). The data reported are the mode values (the value that appeared most often in a set of data) of a minimum of 6 experiments. To determine the correlation between the antibiotic and AITC MICs against the bacteria tested, a correlation matrix was created. Relationships among test parameters were evaluated using Pearson's correlation analysis (Pearson, 1895; Microsoft, 2007). Significance was determined at $p < 0.05$.

3.4 Results

3.4.1 Bacterial sensitivity to AITC and correlation with antibiotic sensitivity

Bacterial sensitivity to AITC was determined through broth macrodilution using pH 5.5 phthalate-buffered Mueller-Hinton, All-Purpose Tween, or Tryptone Soya Broths. Table 3.1 lists the MIC values of AITC against the 12 bacterial isolates. The results demonstrated the efficacy of AITC against different genera of bacteria, and showed that even species within the same genera can possess diversity in susceptibility to the compound. The LAB, as a group, were the most resistant to AITC compared all other groups of bacteria, where all strains of *L. plantarum* and *P. acidilactici* had an MIC of 104 mg/L and *P. pentosaceus* was inhibited at 208 mg/L. Pseudomonads were the least resistant to the compound, with all three species having been inhibited at less than 1.63 mg/L, the lowest concentration of AITC tested. Within *Escherichia*, the 26 mg/L MIC required for pathogenic *E. coli* O157:H7 was almost 8-fold higher than the MIC required for *E. coli* K-12 (3.25 mg/L). The staphylococci also showed variation in resistance to AITC within the genus. Despite both organisms being very sensitive to the compound, pathogenic *S. aureus* was slightly more resistant to AITC, having been inhibited at 6.50 mg/L, compared to *S. carnosus*, which was inhibited at 3.25 mg/L.

Antibiotic breakpoints for the 12 bacterial isolates were established by microdilution using pH 5.5 phthalate-buffered broth (MHB, APT, or TSB). Table 3.1 lists the antibiotic MIC values of the isolates tested. Similarities in the bacterial MIC profiles of the antibiotics were compared with the MIC profile of AITC. Table 3.2 shows the correlation matrix describing the associations between AITC sensitivity and antibiotic sensitivity. Of the 12 antibiotics tested, the MIC values

of ciprofloxacin and polymyxin B showed the highest positive correlation with those of AITC (81.7% and 76.2% correlation, respectively). Conversely, azithromycin and lincomycin possessed the highest negative correlations to the resistance profile of AITC (-90.0% and -78.1%, respectively).

Table 3.1: Minimum inhibitory concentration values of AITC and antibiotics against 12 bacterial isolates.

Organism	MIC values of AITC and antibiotics (ppm)												
	AITC	AMP[a]	CET	NEO	OTC	ERY	AZI	CIP	LCM	PLB	VAN	SMT	CAM
Escherichia coli O157:H7 1931	25	1.56	6.25	200	1.56	**>400**	**>400**	**0.78**	**>400**	**0.78**	400	**>400**	3.13
Escherichia coli K-12 DH5α	3.13	0.78	0.78	50	0.78	**>400**	**>400**	**<0.20**	**>400**	**<0.20**	50	**>400**	1.56
Pseudomonas aeruginosa PAO1	**<1.56**	400	**>400**	**>400**	**>400**	**>400**	**>400**	3.13	**>400**	1.56	**>400**	**>400**	100
Pseudomonas aeruginosa PAO750	<1.56	1.56	100	200	0.20	400	400	**<0.20**	**>400**	**<0.20**	**>400**	1.56	0.39
Pseudomonas fluorescens UM-1	<1.56	**>400**	**>400**	400	1.56	**>400**	**>400**	3.13	**>400**	**<0.20**	**>400**	**>400**	12.5
Staphylococcus aureus ST398	6.25	0.78	**0.20**	25	6.25	6.25	400	3.13	100	400	0.78	**>400**	3.13
Staphylococcus carnosus	3.13	0.40	12.5	12.5	50	12.5	400	1.56	400	60	**<0.2**	**>400**	1.56
Pediococcus pentosaceus UM 121P	200	3.13	3.13	**>400**	3.13	**0.78**	50	**>400**	12.5	**>400**	**>400**	**>400**	1.56
Pediococcus acidilactici UM 119P	100	6.25	1.56	**>400**	1.56	**0.78**	25	100	1.56	**>400**	**>400**	**>400**	**0.78**
Pediococcus acidilactici UM 129 P	100	6.25	1.56	**>400**	**0.78**	**0.78**	25	**>400**	1.56	**>400**	**>400**	**>400**	**0.78**
Lactobacillus plantarum UM 131 L	100	**1.56**	6.25	**>400**	**1.56**	4.56	50	50	200	**>400**	**>400**	**>400**	**1.56**
Lactobacillus plantarum UM 134 L	100	**0.78**	400	**>400**	3.13	1.56	100	**>400**	200	**>400**	**>400**	**>400**	1.56

[a]AMP = ampicillin; CET = cephalothin; NEO = neomycin; OTC = oxytetracycline; ERY = erythromycin; AZI = azithromycin; CIP = ciprofloxacin; LCM = lincomycin; PLB = polymyxin B; VAN = vancomycin, SMT = sulfamethazine; CAM = chloramphenicol

Table 3.2: AITC minimum inhibitory concentration and antibiotic minimum inhibitory concentration correlation matrix. R values represent the degree of correlation between the mode MIC values of two antimicrobials, where 1.00 denotes 100% linear correlation and -1.00 denotes 100% inverse linear correlation. R^2 values represent the degree of fit of data to the regression line.

Correlation with AITC		
Antibiotic	r	r^2
Ampicillin	-0.371[a]	0.135
Cephalothin	-0.226[a]	0.0499
Neomycin	0.597[a]	0.359
Oxytetracycline	-0.254[a]	0.0653
Erythromycin	-0.650[a]	0.420
Azithromycin	**-0.900[b]**	0.809
Ciprofloxacin	**0.816[b]**	0.667
Lincomycin	-0.781[a]	0.609
Polymyxin B	0.764[a]	0.581
Vancomycin	0.454[a]	0.209
Sulfamethazine	0.254[a]	0.064
Chloramphenicol	-0.290[a]	0.0828

[1]Significance was measured using a Pearson Correlation critical value table. r values not identified by the same letter are significantly different ($P<0.05$).

3.5 Discussion

3.5.1 Bacterial sensitivity to AITC and correlation with antibiotic sensitivity

AITC showed the strongest positive correlation to the MIC profiles of ciprofloxacin and polymyxin B (Table 3.2), suggesting that it may have a mechanism of action similar to these antimicrobials. Previous research by Lin and colleagues (2000) has indicated that the mechanism of AITC antimicrobial action was similar to that of polymyxin B, where 1000 μg/mL gaseous AITC was comparable in inhibitory effect to 10 μg/mL of polymyxin B in killing *E. coli* O157:H7 and *Salmonella* Montevideo. In comparison to the present study, 26 mg/L AITC was as effective as 0.78 μg/mL polymyxin B in inhibiting *E. coli* O157:H7, which supports that idea these two antimicrobial compounds are correlated in antibiotic magnitude of strength and mechanism of action. Polymyxins are effective against Gram-negative bacteria as their mode of action involves attacking the bacterial cell outer membrane. These antibiotics have the ability to bind to LPS of the outer membrane, and then act to destabilize these phospholipid molecules and result in the distortion and eventual rupture of the membrane, allowing the polymyxin to further enter the cell. The drug destroys the cytoplasmic membrane, releasing cytoplasmic components (Hancock, 1997; Lambert, 2011). Benzyl isothiocyanate (BITC) at concentrations of 2.8 μmol, (0.418 mg/L) has been observed to cause protrusions or "blebbing" in the cell membrane of Gram-negative *Aggregatibacter actinomycetemcomitans* HK1519. These changes in cell physiology were thought to occur due to the penetration of the lipophilic and electrophilic BITC through the outer membrane, preventing the cell from maintaining its membrane potential (Sofrata et al., 2011). Additionally, AITC has been found to cause cellular leakage in *E. coli* O157:H7 and *Salmonella* Montevideo, but was largely ineffective at causing metabolite leakage

from *Listeria monocytogenes* (Lin et al., 2000a). However, further research by Ahn and collaborators (2001) determined that *L. monocytogenes* treated with AITC did not cause leakage of ATP nor was cell wall damage observed. The results from the present study, as well as the evidence of cell membrane interference and cellular leakage of metabolites from previous studies, indicate that it is likely that ITCs may possess an antibacterial mechanism similar to that of polymyxin antibiotics, where an attachment to the LPS of Gram-negative cellular membranes and subsequent destruction of the membrane is involved. However, this mechanism does not explain why several Gram-positive strains tested in the present study were susceptible to AITC but not to polymyxin B.

In addition to acting on the outer membrane, polymyxins also possess a secondary mechanism of action involving the inhibition of bacterial respiration. Polymyxin B has been shown to inhibit type-II NADH-quinone oxidoreductase (NDH-2) enzymes involved in the electron transport chain in the inner membrane of Gram-negative bacteria (Deris et al., 2014). Similarly, ITCs have been found to affect bacterial respiration processes. A study by Kojima and Ogawa (1971) indicated a decreased oxygen uptake was observed in yeast treated by various ITCs including AITC. However, this study was performed with high levels of ITCs at almost 200 times the MIC. A report by Jakobsen et al. (2012), where *Ps. aeruginosa* was exposed to iberin, showed that genes encoding for cytochrome oxidases were both downregulated and upregulated in the presence of the ITC. The authors also noted the upregulation of several genes encoding for oxidoreductases, suggesting that the presence of ITCs altered cellular respiration processes. In *C. jejuni*, it was found that exposure to BITC stimulated the expression of genes required in electron transport and energy metabolism, including those that encoded for alternate electron donors and

acceptors, as well as those that encoded for putative cytochromes (Dufour et al., 2013). Additionally, Dufour and colleagues (2013) found the upregulation of genes involved in metabolic pathways, including genes responsible for facilitating the relationship between carbon metabolism and respiration or redox homeostasis. From these studies, it seems that ITCs may induce an oxidative response in bacteria similar to that of polymyxin B, however further examination and comparison of these two mechanisms is required to help explain the antibacterial activity of ITCs.

The MIC profile of AITC also correlated highly with that of ciprofloxacin. Similar to AITC, ciprofloxacin was not as potent against Gram-positive organisms as it was against Gram-negative organisms. Amongst Gram-positive organisms, however, the staphylococci were the most susceptible to ciprofloxacin and were inhibited at concentrations ≤ 1 μg/mL (Barry and Jones, 1987; Sanders, 1988). Similar to other quinolones, ciprofloxacin interferes with DNA gyrase (topoisomerase II), an enzyme vital for the synthesis of DNA (Domagala et al., 1986). Although, it has also been reported that ciprofloxacin attacks two targets in bacteria, but with different precedence, depending on the Gram-reaction of the organism. In Gram-positive organisms, it is believed that ciprofloxacin primarily targets topoisomerase IV and attacks DNA gyrase as a secondary target. In Gram-negative organisms, these targets are reversed (Berlanga et al., 2004). There have been few studies regarding the effect of ITCs on any of the enzymes involved with DNA replication; however, a recent study employed *Acinetobacter baylyi* with a chromosomally-integrated *recA-luxCDABE* transcriptional fusion to examine the effects of AITC on DNA. The authors determined that AITC did not provoke the expression of the labelled gene, suggesting that the ITC was not affecting the genome itself (Chan et al., 2013). AITC, PEITC, and methyl

ITC (MITC) were all shown to cause DNA damage in *Salmonella*, *E. coli*, and human Hep G2 cells, which the authors attributed to the reactive ITC group attacking proteins (Kassie and Knasmüller, 2000; Kassie et al., 2001). The presence of other proteins reduced the genotoxic action of ITCs, suggesting that detoxification occurred by direct non-enzymatic binding to these proteins (Kassie and Knasmüller, 2000). Against *E. coli*, PEITC seemed to induce a strong inhibition of RNA synthesis in the organism (Nowicki et al., 2014). However, this inhibition did not seem to be due to direct attack on the RNA synthetic process itself, but instead the authors determined that PEITC depleted cellular amino acids, therefore indirectly halting the synthesis of RNA (2014).

Although evidence exists to suggest that AITC may not directly affect DNA replication, the ITC has been identified to react readily with hydroxyls, amines, and thiols (Zhang and Talalay, 1994). Furthermore, AITC is able to react with amino acids, proteins (Kawakishi and Kaneko, 1987) and cysteine-containing enzymes (Luciano and Holley, 2009). The AITC is also known to cleave disulfide bonds (Kawakishi and Kaneko, 1987), and may affect disulfide bonds such as those found in DNA polymerase I, an enzyme heavily involved in the repair and replication of DNA (Jovin et al., 1969). Additionally, disulfide bonds play a role in protein folding, and it is likely that ITCs disrupt this process, as Dufour and colleagues (2013) determined that, in *Campylobacter jejuni*, genes for protein refolding were upregulated in the presence of BITC. This effect may also be a response to the ITC's ability to inhibit thioredoxin reductase, an enzyme important in the regeneration of proteins containing disulfide bonds in the cytoplasm (Stewart et al., 1998). It seems that the activity of ITCs against bacteria is associated with their reactivity to sulfhydryl groups, as well as their ability to attack thiols and amines. Additionally,

ITCs seem to react preferentially with amino groups and sulfhydryl side chains of proteins (Rawel and Kroll, 1995). Thus, it seems that ITCs may affect DNA and RNA in bacterial cells by reacting with amino groups of proteins important in replication. As the present study indicated that AITC sensitivity correlated highly with ciprofloxacin MIC profile, a focus on investigating ITCs' role(s) in DNA and RNA synthesis would be valuable.

The MIC profile of AITC was also inversely correlated with azithromycin, indicating that the more resistant an organism was to AITC, the more susceptible it was to azithromycin. This inverse correlation was expected as azithromycin, a macrolide antibiotic, is limited in its activity to Gram-positive cocci and bacilli (Leclercq, 2002). As anticipated, both strains of *E. coli* and all the pseudomonads were resistant to azithromycin and all strains of LAB showed lower resistance; however, the staphylococci in the present study were unexpectedly resistant to the drug. As *S. aureus* is livestock-associated, perhaps the use of azithromycin in a farm environment promoted the development of resistance in this strain (Sharma et al., 2015). In *S. carnosus*, resistance to macrolide antibiotics can occur in approximately 14.6% of coagulase-negative strains (Lina et al., 1999).

Due to the fact that *E. coli* has been shown to exhibit only a transient, phenotypic resistance to AITC instead of acquiring sustained resistance (Cordeiro et al., 2014), and that this compound is able to disrupt bacterial outer membranes of both Gram-negative and Gram-positive bacteria, it is likely that AITC may act as a surfactant with similar properties to polymyxin B rather than an antibiotic. Surfactants are able to disrupt the interface that exists between hydrophobic and hydrophilic structures, such as those that exist in bacterial membranes (Burden, 2012), and have

been shown to be bactericidal towards both Gram-positive and Gram-negative bacteria (Hamouda and Baker, 2000). These observations are consistent with previous research involving the effect of AITC on bacterial outer membranes. Further investigation of this relationship would be useful in identifying the antimicrobial nature of AITC.

Allyl isothiocyanate showed a minimum inhibitory concentration of 26 mg/L against *E. coli* O157:H7 and an MIC of 3.25 mg/L against *E. coli* K-12 (Table 3.1), indicating that *E. coli* O157:H7 had greater resistance to the compound compared to its counterpart, *E. coli* K-12. *E. coli* O157:H7 is known to be susceptible to AITC (Luciano and Holley, 2009; Cordeiro et al., 2014), but in comparison with *E. coli* K-12, the latter organism was much more sensitive. According to Lim et al. (2010), the genome of *E. coli* O157:H7 contains 1.4 Mb of genetic data that is unique and different from other *E. coli* strains, suggesting that this organism's relative resistance to AITC in comparison to that of *E. coli* K-12 may be attributed to the foreign DNA that it acquired horizontally. Of this acquired DNA, *E. coli* O157:H7 possesses a plasmid (pO157) that is responsible for virulence factors such as hemolytic activity and adherence to intestinal cells; however, the full role of pO157 has not been identified (Johnson and Nolan, 2009). It may be that this plasmid contains genes that facilitate AITC tolerance, as the plasmid's sequence was identified as similar to an F transmissible factor and the drug resistance plasmid R100 (Makino et al., 1998; Fratamico et al., 2011). Resistances found on the R100 plasmid include those to the mercuric ion, sulfonamides, streptomycin, fusidic acid, chloramphenicol, and tetracycline (Birge, 2000).

Allyl isothiocyanate did not show a large difference in MICs between *S. carnosus* and *S. aureus*, although both were susceptible to the compound. Of the two staphylococci, methicillin-resistant *S. aureus* (MRSA) is recognized as the prominent pathogen of the genus while *S. carnosus* is regarded as a "generally recognized as safe" (GRAS) starter culture used in the food industry (Rosenstein et al., 2009). The pathogenicity of *S. aureus* is based on, in part, its multitude of virulence factors but is also enhanced due to the possession a variety of genes that facilitate the organism's resistance to antibiotics and other antibacterial compounds (Rosenstein and Götz, 2010). These antibiotic resistances include resistance to methicillin, oxacillin, ciprofloxacin, and clindamycin (Velebit et al., 2010). However, this study did not find ciprofloxacin resistance in this strain of *S. aureus* ST398, which is common in approximately 67% of MRSA ST398 strains (Camoez et al., 2013). In fact, the MIC profile of this strain did not seem to match that of a typical MRSA strain, and in comparison to *S. carnosus*, seemed to be just as susceptible to the range of antibiotics used. Work investigating the antibiotic susceptibilities of *S. aureus* ST398 has indicated that antibiotic susceptibility in this strain is common towards erythromycin, aminoglycosides, and vancomycin (Morcillo et al., 2015). Any antibiotic resistances inherent in ST398 did not contribute to its resistance to AITC. *S. carnosus*, conversely, has few antibiotic resistances and is susceptible to most clinically important antibiotics (Resch et al., 2008); however, in the present study it seemed to be quite resistant to the range of antibiotics used. This was unexpected as less than 3% of coagulase-negative staphylococci show resistance against ampicillin and tetracycline, and only a small percentage possess resistance against lincomycin, all of which were demonstrated in this study (Resch et al., 2008). However, this is not to say that it is unheard of as Resch and colleagues (2008) determined that approximately 12% of *S. carnosus* strains possessed antibiotic resistances. With regards to AITC, *S. carnosus* proved to be

as sensitive as *S. aureus* to the compound in the present study. Dias and colleagues (2014) also remarked on the effectiveness of ITCs against 15 isolates of MRSA. The results from the present study are supported by their findings: BITC and AITC were effective against the strains with MICs ranging from 2.9 to 110 μg/mL and 27.9 to 220 μg/mL, respectively. Comparing the two species, it seems that neither *S. carnosus* nor its pathogenic relative, *S. aureus*, are particularly resistant to ITCs, which may indicate that the mechanism of AITC antimicrobial action is not related to the antimicrobial resistance mechanisms employed by either species.

Similar to the pseudomonads in the present study, other *Ps. aeruginosa* strains have been found to be susceptible to both ciprofloxacin and colistin, a polymyxin (Henwood et al., 2001; Hill et al., 2005). It has been indicated that *Ps. aeruginosa*, although not inhibited in growth by the ITCs iberin and sulphorophane, was found to have been inhibited in its quorum sensing activation ability and subsequent virulence (Ganin et al., 2013; Jakobsen et al., 2012; Tan et al., 2014). Additionally, it is thought that *Ps. aeruginosa* employs the use of several resistance mechanisms against ITCs, including the upregulation of efflux pump genes to expel the compound from the cell as well as the upregulation of a glutathione S-transferase gene in order to convert the toxic ITCs to glutathione (Jakobsen et al., 2012). The *Ps. aeruginosa* strains (PAO1 and PAO750) used in this study were chosen based on the multidrug resistance of the wild type and the opposing antibiotic susceptibility of its mutant (Kumar et al., 2006). Five operons encoding for resistance nodulation cell division (RND) efflux pumps, as well as a gene shown to function with other various RND pumps of PAO1 were eliminated in PAO750. All three *Pseudomonas* strains tested (PAO1, PAO750, and *Ps. fluorescens* UM-1) were inhibited at the lowest concentration of AITC, indicating that, despite its multidrug resistance, PAO1 was as unable to withstand the

antibiotic challenge presented by AITC as its mutant, PAO750, and thus the antibiotic efflux mechanisms that these organisms employ were ineffective against AITC. The hyper-susceptibility in these pseudomonads may indicate that AITC may possess stronger or more effective antimicrobial power than the other ITCs, iberin and sulphorophane, which were unable to inhibit the growth of *Ps. aeruginosa* to a similar degree. However, work by Pang et al. (2013) also found that *Ps. aeruginosa* was highly susceptible to gaseous AITC but the authors did observe that, although AITC did not affect the maximum growth rate, it seemed to extend the lag phase of the organism, especially when paired with lower growth temperatures. Therefore, the high susceptibility of the pseudomonads witnessed in the present study may have been a result of a combination of the low temperature and low pH of the growth conditions.

As expected, the LAB showed strong resistance to AITC, which has previously been found (Kamii and Isshiki, 2009; Pérez-Díaz and McFeeters, 2010). *Lactobacillus* strains have been reported to be naturally resistant to aminoglycosides, fluoroquinolones, and glycopeptides (Liu et al., 2009). As well, LAB resistance to ciprofloxacin has been reported as intrinsic (Jacoby, 2005; D'Aimmo et al., 2007; Hummel, 2007). Typically, ciprofloxacin resistance is attributed to mutations in the *gyrA* and *parC* genes, which result in amino acid substitutions in the DNA gyrase or topoisomerase IV subunits prone to quinolone attack (Jacoby, 2005). However, these substitutions were not observed in the DNA sequences of ciprofloxacin-resistant strains of *L. plantarum* or *P. pentosaceus* (Hummel et al., 2006). Recent reports indicated that some LAB strains possessed mutations on the *gyrA* gene but not the *parC* gene (Li et al., 2015). However, only one strain of *L. plantarum* was screened in the aforementioned study, and no mutations were found, indicating that perhaps lactobacilli and pediococci do not possess these mutations.

As such, the basis for LAB fluoroquinolone resistance has not been established and cannot be linked to AITC resistance in these species. Identifying the AITC resistance profile of strains that possess mutated *parC* and *gyrA* genes may help identify a mechanism for ITC activity against these bacteria. All Gram-positive bacteria exhibit intrinsic resistance to polymyxin B (Tille, 2013). Few studies have investigated the mechanism of polymyxin resistance in LAB, although many have determined that *Lactobacillus* strains are resistant to the drug (Sozzi and Smiley, 1980; Vidal, 1987; Mathur and Singh, 2005; D'Aimmo, et al., 2007). Inherent resistance in Gram-positive bacteria is attributed to cell membrane structure: the method of attack of polymyxin B requires its attachment to positively-charged LPS in the outer membrane of Gram-negative bacteria (Newton, 1956). As LPS is not found in the peptidoglycan-rich cell wall of Gram-positive bacteria, polymyxin B is generally ineffective against these organisms. However, the possibility that AITC is unable to penetrate the Gram-positive cell wall is unlikely due to the susceptibility of *S. carnosus*, another Gram-positive organism. In *L. monocytogenes*, Ahn and colleagues (2001) showed that exposure to AITC did not do any cell wall damage. However, the treatment seemed to alter internal structures in this organism. Previous work investigating the effect of BITC on bacterial cell membranes indicated that the compound caused a loss of membrane integrity in *Aggregatibacter actinomycetemcomitans* HK1519 after 2 min exposure (Sofrata et al., 2011). As BITC is both lipophilic and electrophilic, the authors postulated that the compound may penetrate the outer membrane and prevent the bacterium from maintaining its membrane potential. AITC, possessing the same lipophilic and electrophilic properties, is thought to behave in a similar manner, which may explain its ability to cause membrane damage to Gram-negative bacteria. Against Gram-positive organisms, however, AITC may enter the cell

via a different route or mechanism. Further studies examining the method in which ITCs enter Gram-positive bacteria are recommended.

AITC and other ITCs, as stated by Luciano and Holley (2009), likely possesses multi-targeted antimicrobial activity as they, as indicated in the present study, are similar in MIC profiles to polymyxin B and ciprofloxacin, two antibiotics with different mechanisms of action. However, concentrations above and below the range of AITC and antibiotic levels tested in this work should be investigated further as changing these values will alter the level of correlation. Additionally, a wider range of bacteria should be examined in order to identify a better relationship between the ITC and antibiotics. Once more focused values have been determined with a larger sample set of bacteria, a more confident relationship can be identified between AITC and antibiotic breakpoints, which may help in confirming the mechanism of action of AITC. Genotyping two different strains of the same bacterial species that each possess different levels of resistance to AITC would also help to illustrate which genes may be responsible for resistance/sensitivity.

CHAPTER 4

4. Investigating myrosinase-like activity and carbon catabolite repression in *E. coli* O157:H7

4.1 Abstract

Glucosinolates are sulfur- and nitrogen-containing compounds found in plants of the family *Brassicaceae*. In these plants, glucosinolates act as part of a defense mechanism by reacting with the enzyme myrosinase in the event of cellular damage to form antimicrobial isothiocyanates (ITCs). Sinigrin, the glucosinolate found in Oriental and brown mustards (*Brassica juncea*), is hydrolysed by myrosinase to form allyl isothiocyanate (AITC), a compound used in the food industry in Japan as a safe and effective antimicrobial. Studies have identified bacteria possessing "myrosinase-like" activity, where the glucosinolates present in deodorised (myrosinase-inactivated) mustard flour are hydrolysed by the organisms to form the lethal ITC. The mechanism behind this phenomenon has, at present, not been identified, although it is speculated that the organism utilises the glucose component as a carbon source following its generation by hydrolysis of the glucosinolate. Previous studies have found glucose, at small concentrations, increased the amount of sinigrin hydrolysed by *E. coli* O157:H7, while larger concentrations reduced the level of glucosinolate utilised. The present work investigated the sinigrin-hydrolysing abilities of a range of 12 bacteria, determining that *E. coli* O157:H7, *E. coli* K-12, *Staphylococcus carnosus, S. aureus*, *Pseudomonas aeruginosa*, a *Ps. aeruginosa* mutant, *Ps. fluorescens*, *Pediococcus pentosaceus*, *P. acidilactici*, and *Lactobacillus plantarum* were

able to utilise the glucosinolate while other strains of *P. acidilactici* and *L. plantarum* were unable. Additionally, the ability of *E. coli* O157:H7 to hydrolyse sinigrin in the presence of 0.025 M and 0.1 M glucose as well as with the addition of 0.025 M and 0.01 M sorbitol, galactose, mannose, or ribose was investigated. Supplementation of the growth medium with 0.1 M glucose and 0.025M of a monosaccharide or sorbitol reduced the amount of sinigrin hydrolysed by 35-41%, although no significant difference from the control was noted. The addition of 0.025 M glucose and 0.025 M monosaccharide or sorbitol did not affect the sinigrin degradation activity of the organism, nor did the addition of 25 mM glucose and 0.1 M monosaccharide. pH measurement during this experiment suggested that the organism hydrolysed sinigrin and fermented the resulting glucose before utilising the amino acids present as a growth source. Growth of *E. coli* O157:H7 during the first three days of this experiment indicated that treatments omitting sinigrin consistently exhibited the lowest OD_{max}, whereas treatments containing sinigrin and monosaccharide/sorbitol exhibited the highest maximum optical densities.

4.2 Introduction

Glucosinolates, found in plants of the family *Brassicaceae*, are sulfur- and nitrogen-containing compounds that act as part of a defense system in the plant. This defense system, called the "glucosinolate-myrosinase complex" or "mustard oil bomb" is activated when cellular damage occurs in the plant, releasing the glucosinolate and allowing it to interact with the enzyme myrosinase. This interaction cleaves the glucose unit from the glucosinolate, forming pungent isothiocyanates (ITCs), among other secondary breakdown products (Delaquis and Mazza,

1995). Sinigrin, the primary glucosinolate in commercial mustard *Brassica juncea* (brown and Oriental mustards), forms allyl isothiocyanate (AITC) upon reaction with myrosinase.

Myrosinase production is not unique to glucosinolate-producing plants; enzymes able to hydrolyze glucosinolates have been identified in fungi and bacteria (Ohtsuru et al., 1969; Palop et al., 1995; Rakariyatham et al., 2002). In bacteria, "myrosinase-like" activity has also been observed in a range of organisms including lactic acid bacteria (LAB) and foodborne pathogens such as *E. coli*, *Salmonella*, *Campylobacter* and *Listeria monocytogenes* (Ekanayake et al., 2006; Herzallah et al., 2011; Lara-Lledó et al., 2012; Luciano and Holley, 2010; Olaimat et al., 2014). It was found that, in the presence of myrosinase-deactivated (deodorized) mustard powder in dry fermented cured sausages, *E. coli* O157:H7 was able to convert the available glucosinolate into ITC, effectively inhibiting their own growth in the environment (Graumann and Holley, 2008). Further investigation has identified a wide range of bacteria possessing this myrosinase-like activity, including *Pediococcus pentosaceus, Staphylococcus carnosus, S. aureus, Salmonella* Typhimurium, *Enterococcus faecalis*, many LAB and staphylococci strains (Luciano and Holley, 2010; Herzallah et al., 2011; Luciano and Holley, 2011).

Although not fully characterized, it is believed that bacterial myrosinase acts intracellularly to transform glucosinolate substrates into ITCs, which reduce bacterial viability (Luciano et al., 2010). Computer analysis has indicated that, due to its similarity to plant myrosinase, 6-phospho-β-glucosidase-encoding genes *bglA* and *ascB* in *E. coli* O157:H7 may be responsible for producing enzymes linked to myrosinase-like activity (Cordeiro et al., 2015). In the latter study it was concluded that the deletion of these genes reduced the organism's ability to hydrolyse

sinigrin and indicated that 6-phospho-β-glucosidase seemed to be the organism's analogue for plant myrosinase (Cordeiro et al., 2015). In the absence of an energy source, it was hypothesized that *E. coli* O157:H7 will hydrolyse the glucosinolate and utilize the glucose produced, inadvertently forming the lethal ITC (Luciano and Holley, 2010). When in an environment containing an excess concentration of a preferred energy source (i.e. glucose), *E. coli* reduces synthesis of myrosinase-like enzymes and utilization of glucosinolates, supporting the idea that the organism will utilise glucosinolates as an alternative energy source (Cordeiro, unpublished). This phenomenon, known as carbon catabolite repression, is observed when, in the presence of a rapidly metabolizable carbon source in the growth medium, the organism down-regulates gene expression and/or protein activity (Hogema et al., 1998). A well-studied example is the repression of the *lac* operon in the presence of glucose and lactose, where *E. coli* consumed glucose first before switching to lactose (Loomis and Magasanik, 1965). It is thought that if glucosinolates are indeed being metabolized by *E. coli* as an energy source, then the organism may utilise glucosinolates preferentially, placing it in a hierarchy of metabolisable carbon sources. The objective of this work was to identify myrosinase-like activity in a range of organisms; to investigate the effect of glucose and other monosaccharides on the sinigrin degradation ability of *E. coli* O157:H7; and to monitor the growth rate of the organism during the first three days of incubation. Identifying myrosinase-like activity in other organisms may help characterize this ability and aid in its understanding. Observing the effect of glucose and other monosaccharides on myrosinase-like activity of *E. coli* will aid in the optimization of conditions in dry-fermented sausages for pathogen control, and will help to identify where sinigrin is located on the hierarchy of utilisable carbon sources. Lastly, observing the growth rate of *E. coli* O157:H7 in the presence of sinigrin as well as glucose and monosaccharides may help

in understanding the effect of these components on the growth rate and will identify if the organism is utilising sinigrin as a carbon source.

4.3 Materials and Methods

4.3.1 Identification of myrosinase-like activity

Twelve bacterial isolates including *E. coli* O157:H7 1931, *E. coli* K-12 DH5α, *S. carnosus* UM 136, *S. aureus* ST398, *Pseudomonas aeruginosa* PAO1, *Ps. aeruginosa* PAO750, *Ps. fluorescens* UM-1, *P. pentosaceus* UM 121P, *P. acidilactici* UM 119P, *P. acidilactici* UM 129P, *L. plantarum* UM 131L, and *L. plantarum* UM 134L were examined for their ability to degrade sinigrin. Cultures of *Lactobacillus plantarum* UM 134L and UM 131L, *Pediococcus acidilactici* UM 119P and UM 129P, *Pediococcus pentosaceus* UM 121P, *Staphylococcus carnosus* UM 136, and *E. coli* O157:H7 were obtained from the Department of Food Science culture collection, University of Manitoba. *Escherichia coli* K-12 DH5α, *Pseudomonas aeruginosa* PAO1, and *Ps. aeruginosa* PAO750 were obtained from Dr. A. Kumar (Department of Microbiology, University of Manitoba, Winnipeg, MB). The *Ps. aeruginosa* strains were selected due to the lack of efflux pumps in the wild type (PAO1), which confers antibiotic resistance. Its mutant lacks 5 operons encoding for resistance nodulation cell division (RND) efflux pumps, which was thought to be useful in comparison of MICs. Methicillin-resistant *Staphylococcus aureus* ST398 was obtained from Dr. G. Golding (Public Health Agency of Canada, Winnipeg, MB). Levels of sinigrin were observed over 13 d, with samples being collected on days 0, 1, 5, 9, and 13 for *E. coli* O157:H7, *E. coli* K-12, *S. carnosus*, *S. aureus*, *Ps. aeruginosa* PAO1, *Ps. aeruginosa* PAO750, and *Ps. fluorescens*, and days 0, 1, 4, 7, 10, and 13

for *P. pentosaceus*, *P. acidilactici* UM 119P, *P. acidilactici* UM 129P, *L. plantarum* UM 131L, and *L. plantarum* UM 134L. The experiment was conducted using autoclaved, 10 ml screw-capped tubes. Tubes were filled with 8.9 ml of pH 5.5 buffered broth. Sinigrin (Sigma-Aldrich) was dissolved in 0.05 M potassium phthalate-buffered MHB, TSB and APT broths (Oxoid, Fisher Scientific, Edmonton, AB) and 1 ml of this solution was added to each tube of the experiment, resulting in a 1000 ppm concentration of sinigrin in each tube (2.5 mM) (Brabban and Edwards, 1994). Each trial was performed in duplicate. On days of examination, samples were collected, centrifuged at 22 673 x *g* for 10 min (Micro 12 Microcentrifuge, Fisher Scientific, Fair Lawn, NJ, USA) and filtered through sterilized 0.2 μm filters.

4.3.2 Effect of glucose and monosaccharides on degradation of sinigrin in E. coli *O157:H7*

E. coli O157:H7 1931 was analyzed for its ability to degrade sinigrin in the presence of low and high concentrations of glucose, sorbitol, and three monosaccharides. Sorbitol and mannose were obtained from Difco (Becton-Dickinson and Co., Sparks, MD, USA), whereas galactose and ribose were purchased from Sigma-Aldrich.

E. coli O157:H7 1931 was grown in 2.5 mM sinigrin-supplemented pH 5.5-buffered MHB (adapted from Brabban and Edwards, 1994), and levels of sinigrin were observed over 13 d, with samples being collected on days 0, 3, 8 and 13. The experiment was conducted using autoclaved, 10 ml screw-capped tubes. Tubes were filled with 6.9 ml of pH 5.5 buffered MHB, plus 1 mL of glucose, sorbitol or monosaccharide, and sinigrin, and 0.1 mL of bacterial suspension to total a volume of 10 mL. Glucose was dissolved in potassium phthalate-buffered MHB and 1 ml of this

solution was added to each tube, resulting in a 0.025M or 0.1M concentration of glucose (Table 4.1) (Cordeiro, unpublished). Sorbitol, galactose, mannose and ribose were also prepared in the same manner, resulting in a 0.025M or 0.1M concentration of each monosaccharide or sorbitol in each tube.

E. coli O157:H7 was grown overnight in pH 5.5- and 0.05 M potassium phthalate-buffered MHB at 21°C. Following, 0.1 ml was transferred and incubated at 21°C until an OD_{600} of about 0.60 (10^7-10^8 CFU/ml) was reached. The cells were then centrifuged at 3900 x *g* for 20 min at 4°C to obtain a pellet (Avanti® J-26 XP, Beckman-Coulter, Pasadena, CA, USA), washed and re-suspended with 0.1% peptone water and centrifuged again under the same conditions. The resulting pellet was again washed and re-suspended; 0.1 ml of this suspension was then added to the prepared tubes so that the concentration of cells was approximately 5.5 x 10^3 CFU ml^{-1}(3.74 log) per tube. On days of examination samples were collected, centrifuged at 22 673 x *g* for 10 min and filtered through sterile 0.2 μm filters and the filtrate examined for residual sinigrin.

Table 4.1: Concentrations of glucose, sorbitol, or monosaccharide[a] in each of the treatments used to determine sinigrin degradation in Mueller-Hinton broth.

Treatment No.	Sinigrin (mM)	Glucose (M)	Monosaccharide (M)
1	2.5	0.025	0.025
2	2.5	0.1	0.025
3	2.5	0.025	0.1
Control 1	2.5	0	0

Control 2	2.5	0.025	0
Control 3	2.5	0.1	0

[a]Galactose, mannose, or ribose

4.3.3 Growth rate of E. coli *O157:H7 via change in optical density*

The growth rate of *E. coli* O157:H7 was measured in 2 h intervals over the first 72 h of incubation in the presence of 0.025M or 0.1M glucose and 0.025 M or 0.1 M concentrations of monosaccharide or sorbitol. Microplates (96 well) were prepared in accordance with the three treatments and accounting for all permutations of sinigrin, glucose, and monosaccharide or sorbitol additions to a total volume of 90 μL (Table 4.2). *E. coli* O157:H7 was prepared in pH 5.5 and 0.05 M potassium phthalate-buffered Mueller-Hinton Broth (Criterion, Hardy Diagnostics, Santa Maria, CA, USA) and 10 μL was added to each well of a 96 well plate, totalling an initial cell concentration of approximately 5.5×10^3 CFU ml^{-1} (3.72 log CFU mL^{-1}) per well. The controls were prepared in pH 5.5 and 0.05 M potassium phthalate-buffered MHB (Oxoid). The plates were incubated on a shaker at 21°C and optical density at 630 nm was determined at 2 h intervals using a microplate reader (Elx800, BioTek, Winooski, VT, USA) (Horáková et al., 2004).

Table 4.2: Concentrations of glucose, sorbitol, or monosaccharide[a] in each of the treatments used to determine growth rate via change in optical density.

Treatment No.	Tube No.	[Sinigrin] (mM)	[Glucose] (M)	[Monosaccharide/Sorbitol] (M)
Control[b]	A	0	0	0
1	A	2.5	0.025	0
	B	2.5	0.025	0.025
	C	2.5	0	0.025
	D	0	0.025	0.025
	E	0	0	0.025
	F	0	0.025	0
2	A	2.5	0.1	0
	B	2.5	0.1	0.025
	C	0	0.1	0.025
	D	0	0.1	0
3	A	2.5	0.025	0.1
	B	2.5	0	0.1
	C	0	0.025	0.1
	D	0	0	0.1

[a]Galactose, mannose, or ribose

[b]Additions were made in pH 5.5 and 0.05 M potassium hydrogen phthalate-buffered MHB

4.3.4 Determination of pH change and cell population during sinigrin degradation

Change in pH was monitored in tubes containing *E. coli* O157:H7 grown in pH 5.5 and 0.05 M potassium phthalate-buffered MHB in the presence of 2.5 mM or 0.1 M concentrations of glucose, monosaccharide, or sorbitol (Table 4.3) by analyzing tubes via pH meter (SevenExcellence, Mettler Toledo, Columbus, OH, USA) on days 0, 1, 3, 5, 7, and 13 in duplicate. Cell population was measured on the same days as pH analysis. A sample consisting of 0.1 mL of each duplicate tube was serially diluted and spread-plated on Violet Red Bile (VRB) agar. Cell numbers were determined by counting colonies after 24 h incubation at 35°C.

Table 4.3: Concentrations of glucose, sorbitol, or monosaccharide[a] in each of the treatments used to determine change in pH.

Tube No.	Sinigrin (mM)	Glucose (mM)	Monosaccharide/Sorbitol (25 mM)
1	0	0	N/A
2	2.5	0	N/A
3	0	25	N/A
4	0	100	N/A
5	2.5	25	Sorbitol
6	2.5	25	Galactose
7	2.5	25	Mannose
8	2.5	25	Ribose

[a]Galactose, mannose, or ribose

4.3.5 HPLC apparatus

Separation and quantification of sinigrin were conducted on an HPLC system (Waters 2695, Waters Corp., Milford, MS, USA) equipped with Empower software and an autosampler (Waters 717 plus). Elution was performed isocratically at room temperature for 15 min at a flow rate of 1ml min^{-1} using a Symmetry C18 column (Gemini, 5 µm, Phenomenex, Torrance, CA, USA). The solvent system used contained 20% (v/v) acetonitrile and 80% water + 0.02M tetrabutylammonium hydrogen sulfate (w/v) adjusted to pH 5.5. Injection volume was 10 µl. A photodiode array detector (Waters 996) was used to measure the absorbance at 227 nm in order to verify and quantify the presence of sinigrin. The calibration curve used for sinigrin quantification was established with a sinigrin standard under the same analytical conditions used for sample analyses.

4.36 Statistical analyses

Statistical analyses were performed using JMP 10 software (SAS Institute, Inc., Cary, NC, USA) and statistical differences among treatments were compared using Tukey's test. The sinigrin degradation data reported represent the mean values of a minimum of 4 experiments and are presented as means ± SEM. Growth rate OD data reported represent the mean values of a minimum of 6 replicates and are presented as means. pH data reported represent the mean values of 2 replicates and are presented as means ± SEM. Cell enumeration data are reported as the mean values of 2 replicates.

4.4 Results

4.4.1 Identification of myrosinase-like activity

Twelve bacterial isolates were monitored for sinigrin consumption over 13 d using pH 5.5 buffered MH, APT or TS broth as growth media (Figs. 4.01-4.04). Each strain showed an ability to hydrolyse the glucosinolate. *E. coli* O157:H7 produced a similar reduction of sinigrin levels, hydrolysing 347.2 ± 33.8 μM in 13 d (Table 4.4) as *E. coli* K-12, which produced a reduction of 299.4 ± 67.4 μM in the same time period,. Of the staphylococci, *S. carnosus* was able to consume a total of 306.9 ± 9.0 while *S. aureus* hydrolysed 276.3 ± 50.9 μM. Among the pseudomonads, wild-type *Ps. aeruginosa* PAO1 was able to consume the most sinigrin (246.6 ± 45.6 μM), while its mutant, PAO750, degraded 208.8 ± 26.9 μM and *Ps. fluorescens* was able to produce a reduction of 213.9 ± 23.0 μM. The LAB showed a wide range of degradation activity: *P. pentosaceus* was able to degrade 286.8 ± 13.7 μM of sinigrin while both *P. acidilactici* UM 119P and *L. plantarum* UM 134L produced a 246.6 ± 20.9 and a 239.0 ± 40.2 μM reduction, respectively. Conversely, *P. acidilactici* UM 129P and *L. plantarum* UM 131L were not able to degrade sinigrin to a great degree. Both strains consumed 85.5 ± 17.5 and 88.1 ± 12.3 μM of the glucosinolate in the 13 d period, respectively, which was significantly different from the other LAB degradation values ($P<0.05$). Excluding the LAB, there were no significant differences in the levels of sinigrin hydrolysed in any of the bacteria tested ($P>0.05$).

The activity and level of sinigrin degradation of these organisms were compared with their antibiotic MIC values. Table 4.5 shows the correlation between these two attributes. Of the 13 antibiotics tested, azithromycin possessed the highest correlation (51%) with sinigrin degradation

levels. No substantial correlation (-24.1%) was observed between the ability of an organism to degrade sinigrin and its MIC in response to AITC challenge.

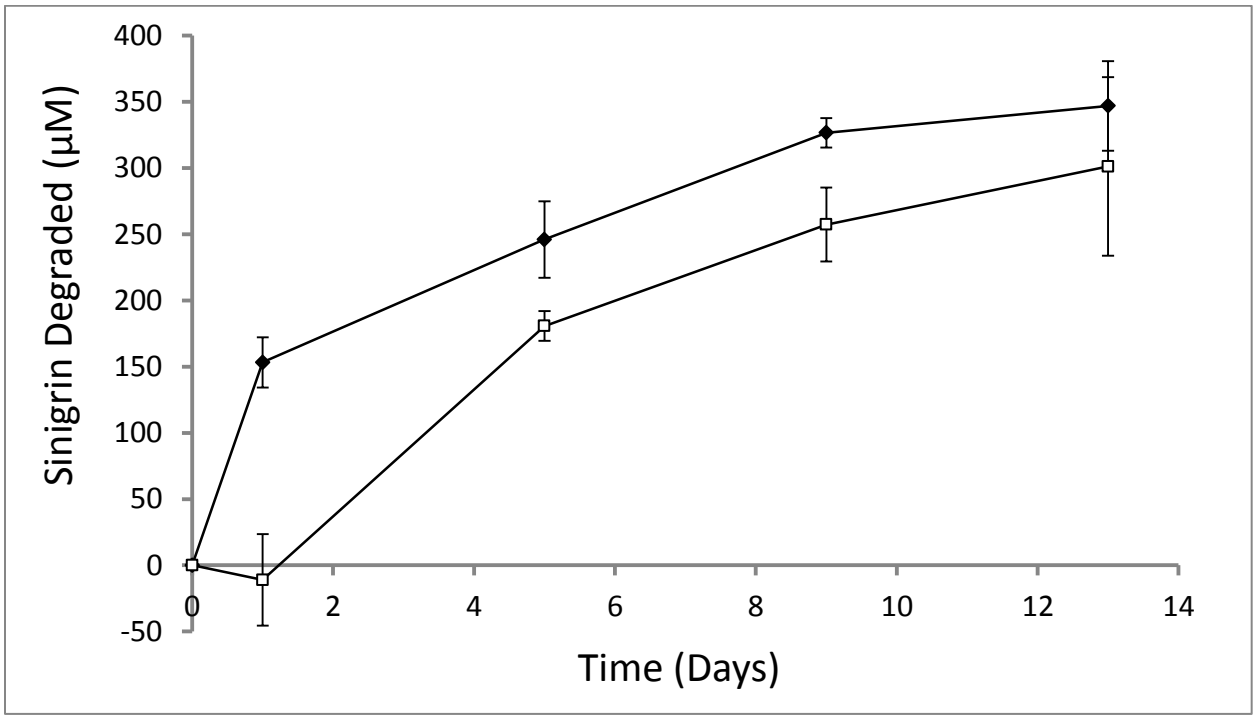

Figure 4.01: Degradation of sinigrin by *Escherichia coli* O157:H7 (◆) and *Escherichia coli* K12 (□) grown in pH 5.5 and 0.05 M potassium phthalate-buffered MHB. Data points represent the mean of 4 replicates ± SEM.

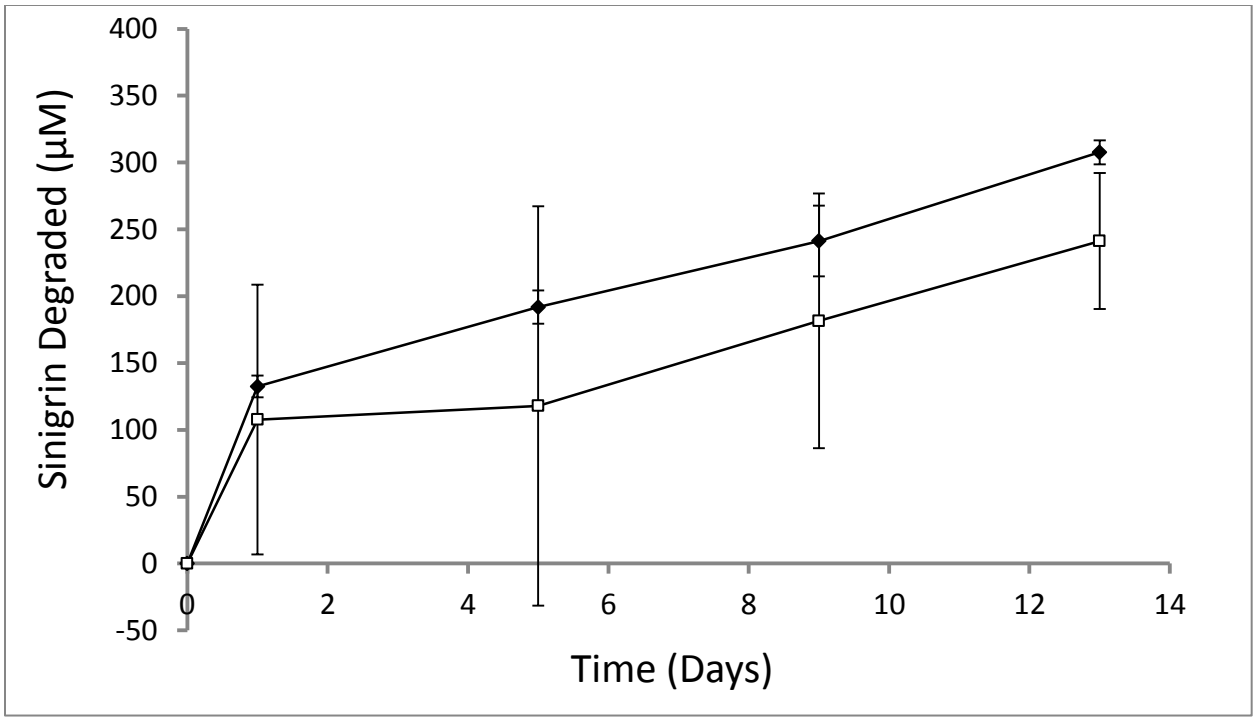

Figure 4.02: Degradation of sinigrin by *Staphylococcus carnosus* (◆) and *Staphylococcus aureus* (□) grown in pH 5.5- and 0.05 M potassium phthalate-buffered TSB. Data points represent the mean of 4 replicates ± SEM.

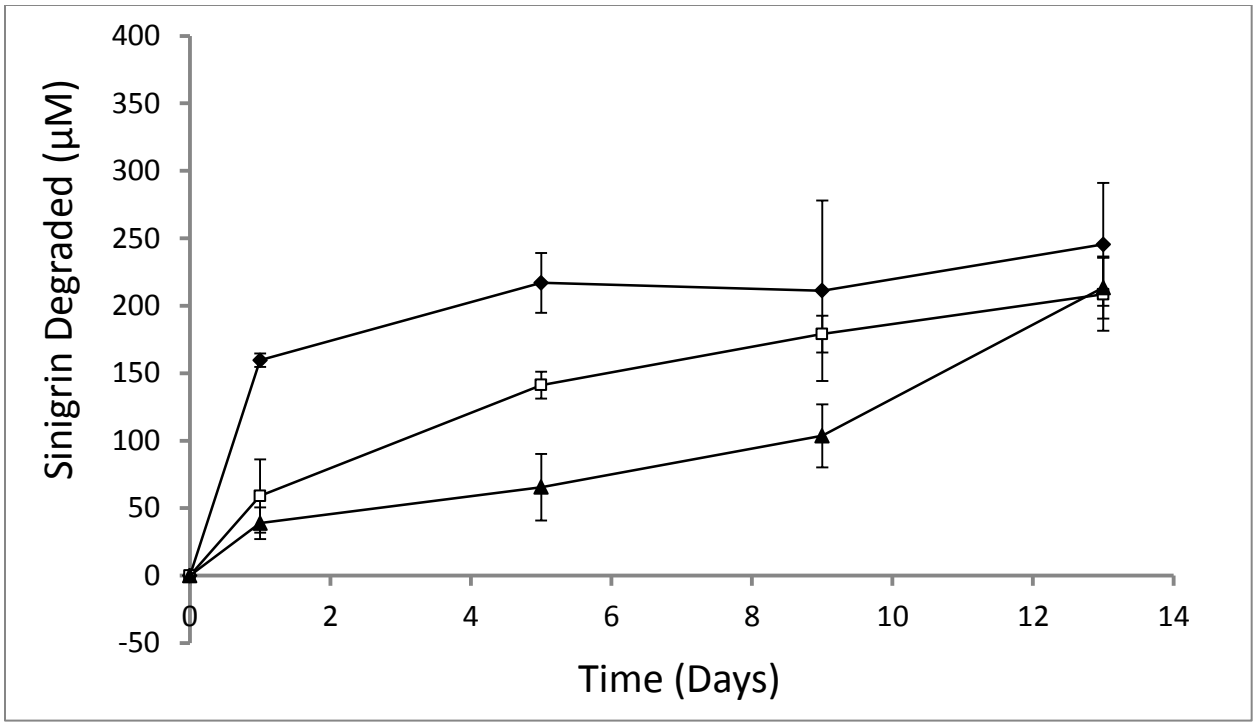

Figure 4.03 Degradation of sinigrin by *Pseudomonas aeruginosa* wild type (PAO1) (◆), *Pseudomonas aeruginosa* mutant (PAO750) (□), and *Pseudomonas fluorescens* (▲) grown in pH 5.5 and 0.05 M potassium phthalate-buffered TSB. Data points represent the mean of 4 replicates ± SEM.

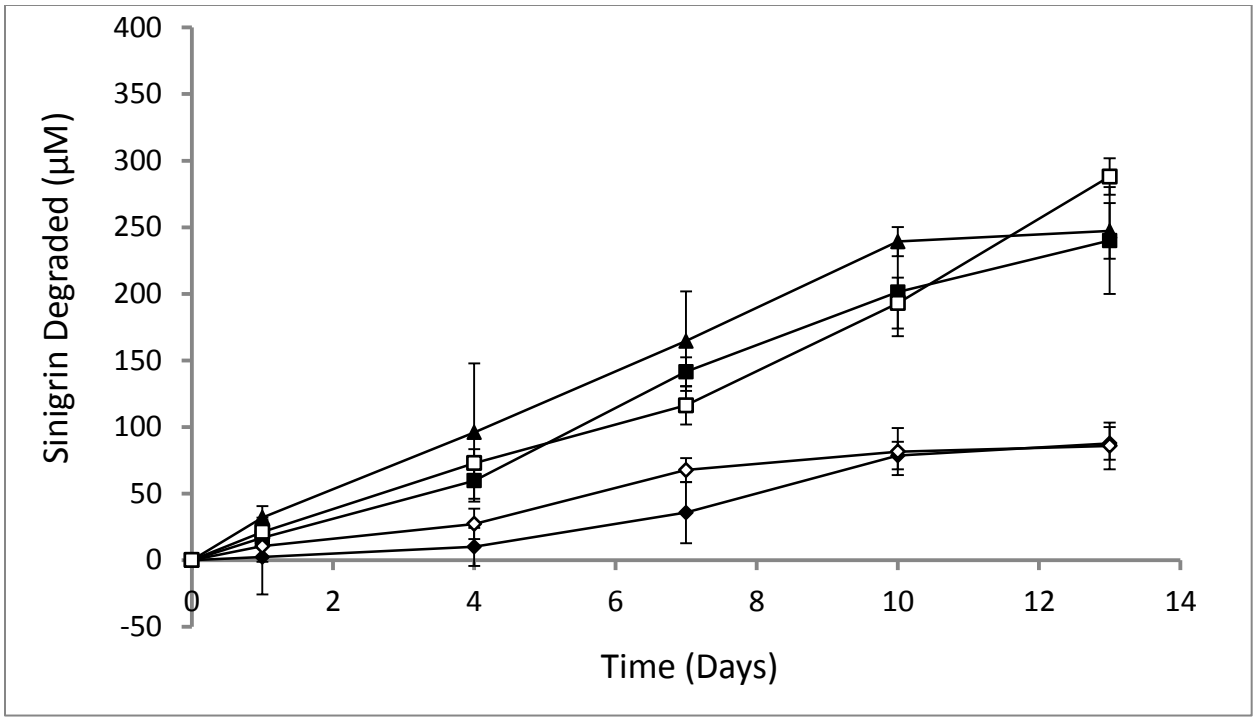

Figure 4.04: Degradation of sinigrin by *Lactobacillus plantarum* UM 131L (◆), *Lactobacillus plantarum* UM 134L (■), *Pediococcus acidilactici* UM 119P (▲), *Pediococcus acidilactici* UM 129P (◇), and *Pediococcus pentosaceus* UM 121P (□) grown in pH 5.5 and 0.05 M potassium phthalate-buffered APT. Data points represent the mean of 4 replicates ± SEM.

Table 4.4: The total amount of sinigrin utilised on day 13 of the sinigrin analysis experiment[1].

Organism	Sinigrin Degraded (μM)
E. coli O157:H7	347.2 ± 33.8[a]
E. coli K-12	299.4 ± 67.4[a]
S. carnosus ST398	306.9 ± 9.0[a]
S. aureus UM 136	276.3 ± 50.9[ab]
Ps. aeruginosa PAO1	246.6 ± 45.6[ab]
Ps. aeruginosa PAO750	208.8 ± 26.9[ab]
Ps. fluorescens UM-1	213.9 ± 23.0[ab]
P. pentosaceus UM 121P	286.8 ± 13.7[a]
P. acidilactici UM 119P	246.6 ± 20.9[ab]
P acidilactici UM 129P	85.5 ± 17.5[b]
L. plantarum UM 131L	88.1 ± 12.3[b]
L. plantarum UM 134L	239.0 ± 40.2[ab]

[1]Levels not identified by the same letter are significantly different ($P<0.05$).

Table 4.5: Sinigrin degradation and antibiotic minimum inhibitory concentration correlation matrix. R values represent the degree of correlation between the mode MIC values of two antimicrobials, where 1.00 denotes 100% linear correlation and -1.00 denotes 100% inverse linear correlation.

Correlation with Sinigrin	
Antibiotic	r
AITC	-0.2406
Ampicillin	-0.03425
Cephalothin	-0.02347
Neomycin	-0.49043
Oxytetracycline	0.312912
Erythromycin	0.322893
Azithromycin	0.513395
Ciprofloxacin	-0.27002
Lincomycin	0.383935
Polymyxin B	-0.45992
Vancomycin	-0.3621
Sulfamethazine	0.10201
Chloramphenicol	0.047331

4.4.2 Effect of sinigrin, glucose, and monosaccharide concentration on cell population and on growth rate in E. coli *O157:H7 as measured by change in optical density*

Growth of *E. coli* O157:H7 in pH 5.5-buffered MHB containing 2.5 mM sinigrin supplemented with 0.025 M or 0.01 M glucose and one of 0.025 M or 0.1 M sorbitol, galactose, mannose, or ribose was monitored during the first 3 d of the experiment, with measurements every 2 h. Figures 4.05-4.08 depict the growth curves of the organism in response to different concentrations of added monosaccharide or sorbitol. In all replicates, a 20-24 h lag was observed as the organism adjusted to the new media. When comparing the change in optical density of *E. coli* O157:H7 between the controls (MHB, MHB with 25 mM glucose, or MHB with 0.1 M glucose ± sinigrin), the final ODs revealed that a significant difference was observed with the addition of 25 mM glucose, and that the addition of sinigrin to this concentration of glucose significantly reduced the OD. However, no significant difference was observed between the MHB control and the 0.1 M glucose control, nor was there a significant difference observed upon the addition of sinigrin to these two concentrations (Table 4.6, Fig 4.05). The effect of the addition of 2.5 mM sinigrin, 25 mM glucose, and 25 mM monosaccharide/sorbitol on the cell density of *E. coli* was investigated (Fig. 4.06). No consistent difference in final ODs between the three monosaccharides and sorbitol existed across treatments (Table 4.6). However, the omission of sinigrin seemed to depress the maximum optical density of *E. coli* O157:H7 in the treatments involving only 25 mM glucose and 25 mM sorbitol or monosaccharide, although the final ODs did not indicate this reduction to be significant. Additionally, the treatment omitting glucose from the growth media seemed to result in the highest maximum optical density in all 4 treatments containing the three monosaccharides and sorbitol. In the second treatment where the

growth medium was supplemented with 2.5 mM sinigrin, 0.1 M glucose, and 25 mM monosaccharide or sorbitol (Fig. 4.07), *E. coli* O157:H7 experienced consistent growth across treatments containing sinigrin. Again, the treatment resulting in the lowest OD_{max} involved the omission of sinigrin (0.1 M glucose and 25 mM sorbitol or monosaccharide), which corroborates with the growth curves of the other two treatments but contradicts the control curves (Fig. 4.05). The treatment containing only 2.5 mM sinigrin and 25 mM sorbitol or monosaccharide seemed to yield the highest OD regardless of the addition of sorbitol or monosaccharide. When change in OD was measured in the presence of 2.5 mM sinigrin, 25 mM glucose, and 0.1 M sorbitol or monosaccharide, similar trends in the lowest and highest OD_{max} were observed. Omission of sinigrin, supplementation with only 25 mM glucose and 0.1 M sorbitol or monosaccharide, yielded the lowest maximum OD in *E. coli* O157:H7. This similarity was seen across all 4 OD curves of sorbitol, galactose, ribose, or mannose. However, the treatment containing 2.5 mM sinigrin, 25 mM glucose, and 0.1 M sorbitol (Fig 4.08A) seemed to yield a diminished maximum optical density curve. Upon the removal of 25 mM of glucose from the environment, the organism experienced its highest OD_{max} values of any of the other treatments across all three monosaccharides or sorbitiol, which was surprising considering the high concentration of these sugars present in the growth medium.

The change in numbers of *E. coli* O157:H7 was monitored in tubes containing pH 5.5 and 0.05 M potassium phthalate-buffered MHB on days 0, 1, 3, 5, 7, and 13 (Fig. 4.09). All tubes had initial cell numbers of approximately 3.62 log CFU mL^{-1} and grew to a maximum number of approximately 8.50 log CFU/mL^{-1} on day 3 of the experiment. Enumeration on day 13 of the

experiment showed that all tubes decreased to numbers of approximately 7.05-7.89 log CFU mL^{-1}. No statistical differences were found among day 13 cell populations ($P>0.05$).

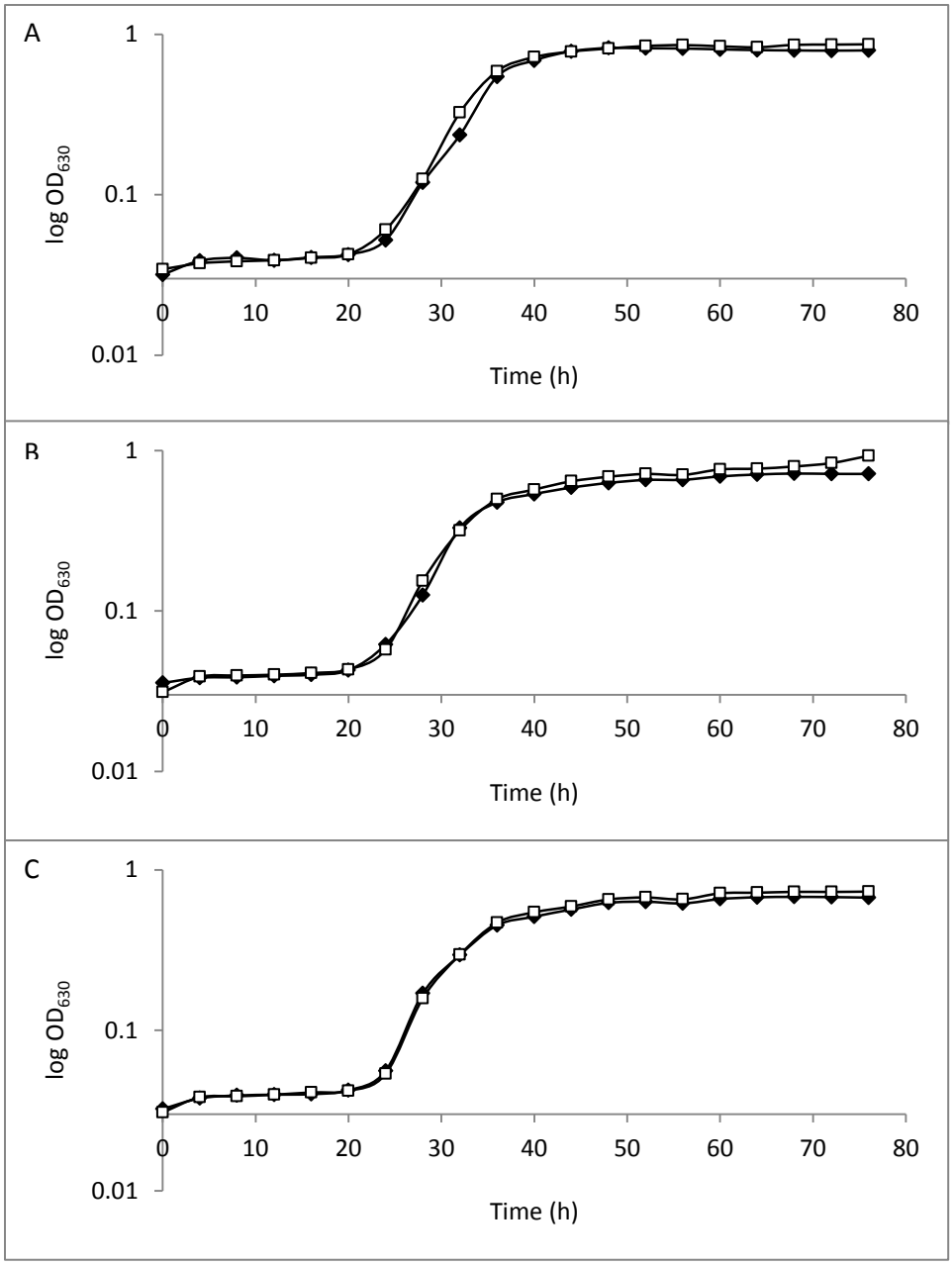

Figure 4.05: Optical density of *E. coli* O157:H7 in A) 2.5 mM sinigrin (□), and MHB control (◆); B) 25 mM glucose and 2.5 mM sinigrin (□), and 25 mM glucose in MHB control (◆); C) 0.1 M glucose and 2.5 mM sinigrin (□) with 0.1 M glucose in MHB control (◆) plotted on a $\log_{10}$ scale. Culture was incubated, shaking, at 21°C and microplate readings were taken every 4 h. Data points were the result of 9 replicates.

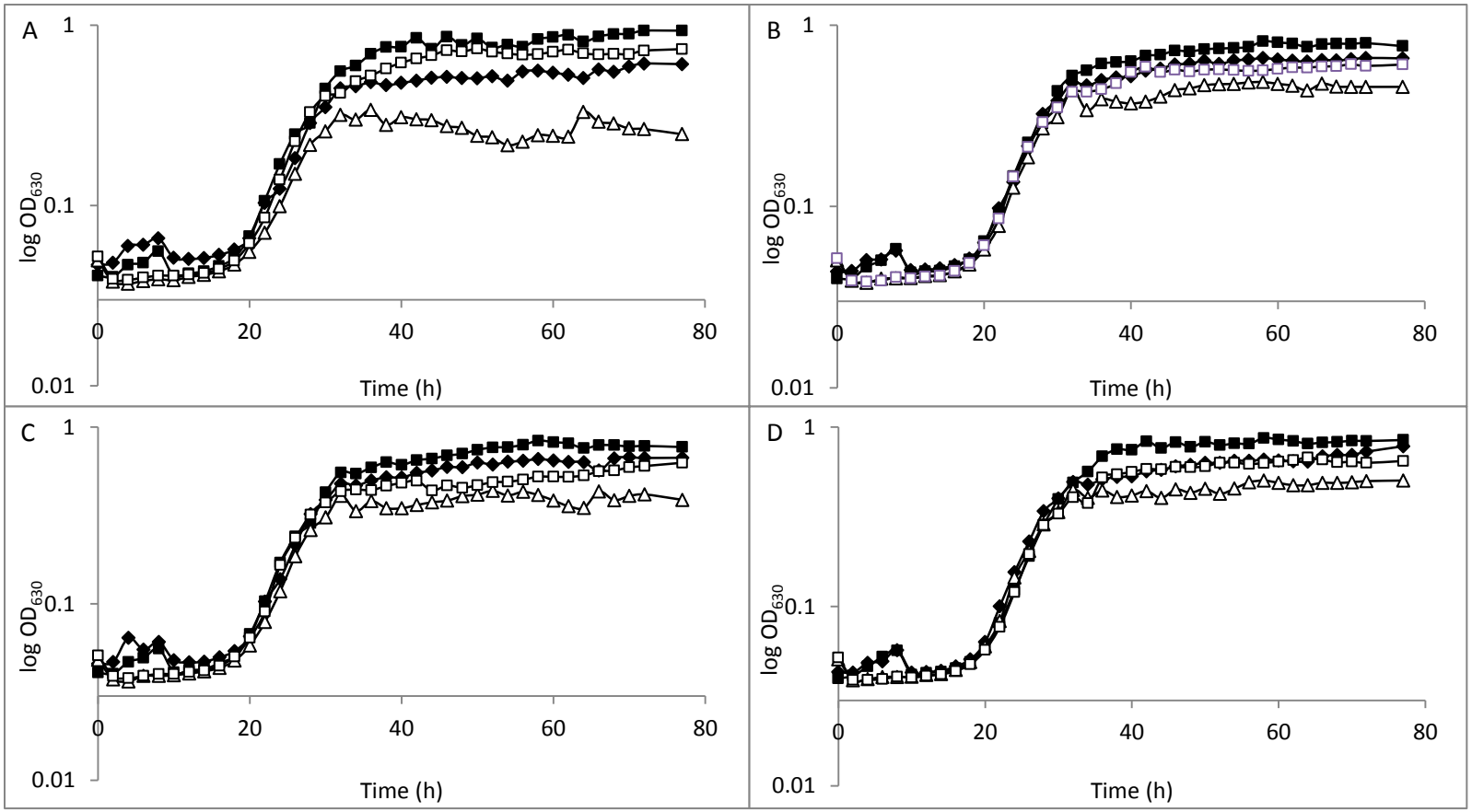

Figure 4.06: Effect of different concentrations of glucose, sinigrin, and sorbitol or monosaccharide on the optical density of *E. coli* O157:H7 in pH 5.5 and 0.05 M potassium phthalate-buffered MHB supplemented with 2.5 mM sinigrin, 25 mM glucose, and 25 mM sorbitol or monosaccharide (◆), 2.5 mM sinigrin and 25 mM sorbitol or monosaccharide (■), 25 mM glucose and 25 mM sorbitol or

monosaccharide (Δ), or 25 mM sorbitol or monosaccharide (□). A) Sorbitol. B) Galactose. C) Mannose. D) Ribose. Culture was incubated, shaking, at 21°C and microplate readings were taken every 2 h. Data points were the result of 6 replicates.

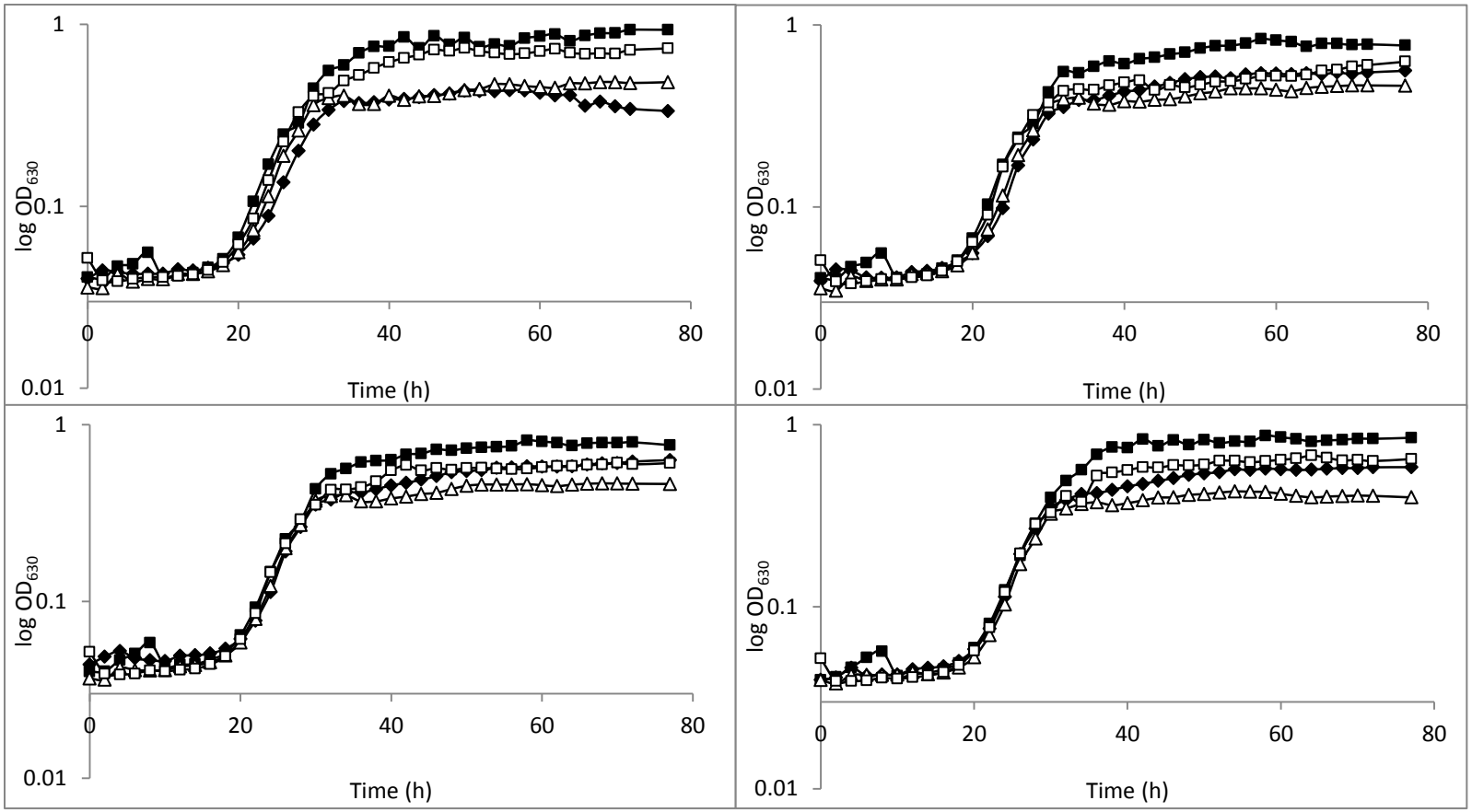

Figure 4.07: Effect of different concentrations of glucose, sinigrin, and sorbitol or monosaccharide on the optical density of *E. coli* O157:H7 in pH 5.5 and 0.05 M potassium phthalate-buffered MHB supplemented with 2.5 mM sinigrin, 0.1 M glucose, and 25 mM sorbitol or monosaccharide (◆); 2.5 mM sinigrin and 25 mM sorbitol or monosaccharide (■); 0.1 M glucose and 25 mM sorbitol or

monosaccharide (Δ); or 25 mM sorbitol or monosaccharide (□). A) Sorbitol. B) Galactose. C) Mannose. D) Ribose. Culture was incubated, shaking, at 21°C and microplate readings were taken every 2 h. Data points were the result of 6 replicates.

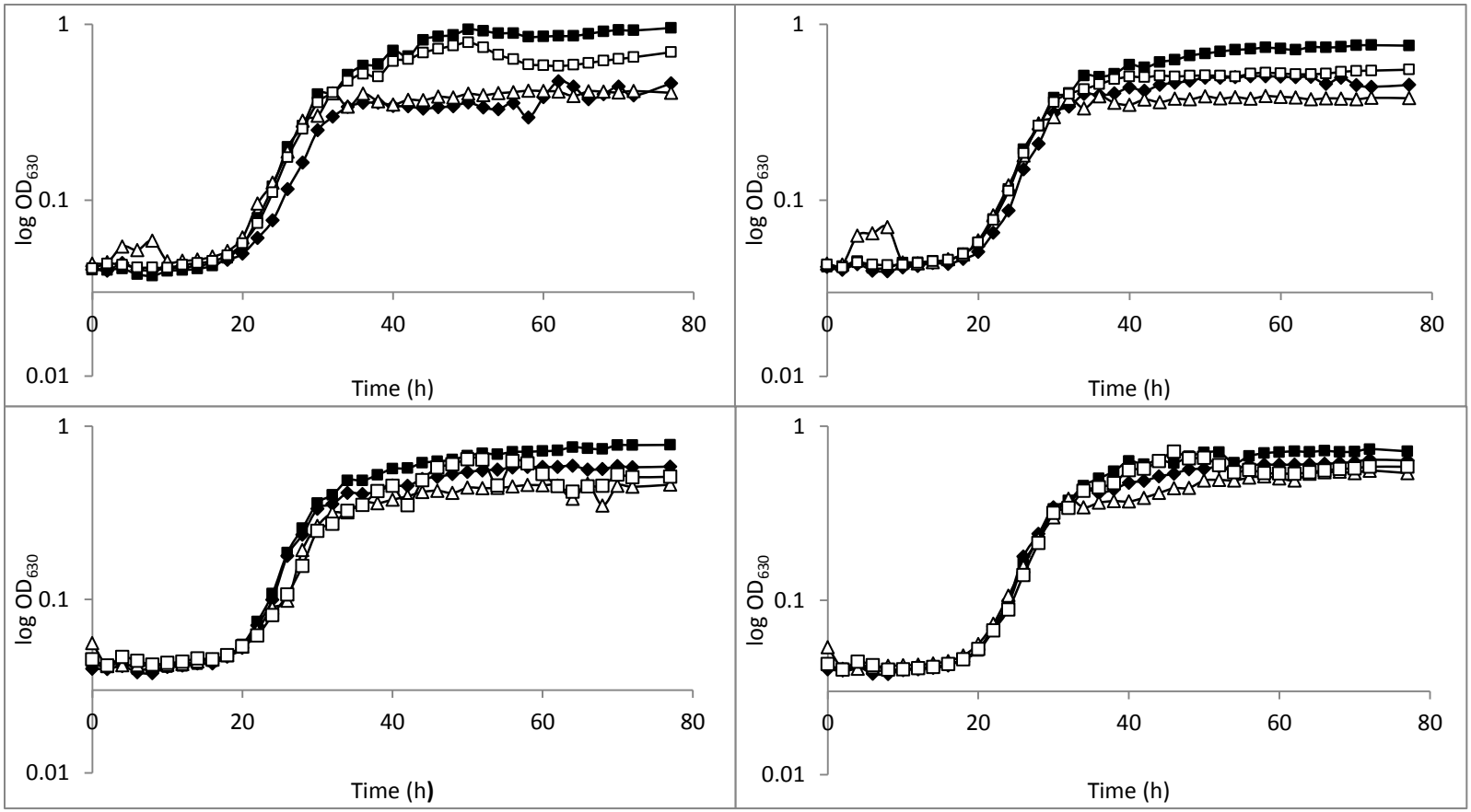

Figure 4.08: Effect of different concentrations of glucose, sinigrin, and sorbitol or monosaccharide on the optical density of *E. coli* O157:H7 in pH 5.5 and 0.05 M potassium phthalate-buffered MHB supplemented with 2.5 mM sinigrin, 25 mM glucose, and 0.1 M sorbitol or monosaccharide (◆); 2.5 mM sinigrin and 0.1 mM sorbitol or monosaccharide (■); 25 mM glucose and 0.1 M sorbitol or

monosaccharide (Δ); or 0.1 M sorbitol or monosaccharide (□). A) Sorbitol. B) Galactose. C) Mannose. D) Ribose. Culture was incubated, shaking, at 21°C and microplate readings were taken every 2 h. Data points were the result of 6 replicates.

Table 4.6: Final optical densities of *E. coli* O157:H7 as a result of the addition of different concentrations of glucose, sinigrin, and sorbitol or monosaccharide

Treatment[1]	Monosaccharide[2]	Final OD
MHB control	N/A	0.79 ± 0.011^{bcd}
25 mM glucose control	N/A	0.93 ± 0.025^{a}
0.1 M glucose control	N/A	$0.73 \pm 0.0095^{cdefghi}$
MHB + sinigrin control	N/A	0.87 ± 0.015^{a}
25 mM glucose + sinigrin control	N/A	$0.71 \pm 0.0084^{cdefghij}$
0.1 M glucose + sinigrin control	N/A	$0.67 \pm 0.011^{efghijkl}$
1A	Sorbitol	$0.61 \pm 0.026^{hijklmno}$
1A	Galactose	$0.67 \pm 0.0068^{defghijkl}$
1A	Mannose	$0.66 \pm 0.014^{efghijkl}$
1A	Ribose	0.78 ± 0.014^{bcde}
1B	Sorbitol	0.93 ± 0.0098^{a}
1B	Galactose	0.77 ± 0.023^{bcdefg}
1B	Mannose	0.77 ± 0.020^{bcdefg}
1B	Ribose	0.85 ± 0.018^{abc}
1C	Sorbitol	0.25 ± 0.054^{v}
1C	Galactose	0.39 ± 0.024^{tuv}
1C	Mannose	0.46 ± 0.024^{qrstu}
1C	Ribose	$0.50 \pm 0.039^{mnopqrst}$
1D	Sorbitol	$0.74 \pm 0.011^{bcdefghi}$

1D	Galactose	$0.63 \pm 0.015^{ghijklmn}$
1D	Mannose	$0.61 \pm 0.054^{ijklmnop}$
1D	Ribose	$0.65 \pm 0.050^{efghijklm}$
2A	Sorbitol	0.33 ± 0.042^{uv}
2A	Galactose	$0.56 \pm 0.012^{klmnopqr}$
2A	Mannose	$0.63 \pm 0.011^{fghijklm}$
2A	Ribose	$0.58 \pm 0.0043^{jklmnopq}$
2B	Sorbitol	$0.48 \pm 0.016^{nopqrstu}$
2B	Galactose	0.46 ± 0.023^{opqrstu}
2B	Mannose	0.46 ± 0.020^{opqrstu}
2B	Ribose	0.40 ± 0.011^{stu}
3A	Sorbitol	0.24 ± 0.071^{v}
3A	Galactose	0.45 ± 0.033^{qrstu}
3A	Mannose	$0.58 \pm 0.017^{jklmnopq}$
3A	Ribose	$0.63 \pm 0.0042^{efghijklm}$
3B	Sorbitol	0.95 ± 0.013^{a}
3B	Galactose	0.76 ± 0.013^{bcdefgh}
3B	Mannose	0.78 ± 0.019^{bcdef}
3B	Ribose	$0.72 \pm 0.026^{cdefghij}$
3C	Sorbitol	0.43 ± 0.022^{rstu}
3C	Galactose	0.38 ± 0.011^{tuv}
3C	Mannose	0.36 ± 0.046^{tuv}
3C	Ribose	$0.54 \pm 0.0052^{lmnopqrs}$

3D	Sorbitol	$0.69 \pm 0.016^{defghijk}$
3D	Galactose	$0.55 \pm 0.0097^{klmnopqr}$
3D	Mannose	0.46 ± 0.053^{pqrstu}
3D	Ribose	$0.58 \pm 0.016^{jklmnopq}$

[1]Treatments: 1A: 2.5 mM sinigrin, 25 mM glucose, 25 mM sorbitol or monosaccharide[2]; 1B: 2.5 mM sinigrin, 25 mM sorbitol or monosaccharide; 1C: 25 mM glucose, 25 mM sorbitol or monosaccharide; 1D: 25 mM sorbitol or monosaccharide; 2A: 2.5 mM sinigrin, 0.1 M glucose, 25 mM sorbitol or monosaccharide; 2B: 0.1 M glucose, 25 mM sorbitol or monosaccharide; 3A: 2.5 mM sinigrin, 25 mM glucose, 0.1 M sorbitol or monosaccharide; 3B: 2.5 mM sinigrin, 0.1 M sorbitol or monosaccharide; 3C: 25 mM glucose, 0.1 M sorbitol or monosaccharide; 3D: 0.1 M sorbitol or monosaccharide.

[2]Galactose, mannose, or ribose.

[3]Levels not identified by the same letter are statistically different ($P<0.05$).

[4]Control final ODs were read at 76 h; all other final ODs were read at 77 h.

Table 4.7: Cell enumeration of *E. coli* O157:H7 grown in pH 5.5 and 0.05 M potassium-phthalate-buffered MHB for 13 d (log_{10} CFU/mL). Values are represented as the means of two replicates.

Day	Treatment[1]							
	1	2	3	4	5	6	7	8
0	3.62	3.62	3.62	3.62	3.62	3.62	3.62	3.62
1	3.95	3.85	4.23	4.76	4.93	4.63	4.66	4.64
3	8.53	7.90	8.15	8.28	8.20	8.46	8.51	8.60
5	8.58	8.69	7.95	8.23	7.78	8.51	8.30	8.36
7	8.11	8.04	8.00	7.85	7.95	7.11	7.08	8.86
13	7.90	7.83	6.82	6.93	6.54	7.23	7.04	6.36

[1]Treatments: Control: MHB, no *E. coli* O157:H7; 1: MHB; 2: MHB and 25 mM sinigrin; 3: 25 mM glucose; 4: 0.1 M glucose; 5: 2.5 mM sinigrin, 25 mM glucose, and 25 mM sorbitol; 6: 2.5 mM sinigrin, 25 mM glucose, and 25 mM galactose; 7: 2.5 mM sinigrin, 25 mM glucose, and 25 mM mannose; 8: 2.5 mM sinigrin, 25 mM glucose, and 25 mM ribose.

4.4.3 Effects of sinigrin, glucose, and monosaccharide concentration on change in MBH pH

The change in pH was monitored in tubes containing *E. coli* O157:H7 grown in pH 5.5 and 0.05 M potassium phthalate-buffered MHB on days 0, 1, 3, 5, 7, and 13 (Fig. 4.09). Day 13 pH values are presented in Table 4.7. In inoculated tubes containing only MHB, the pH was seen to rise over the course of the experiment, reaching a final pH of 8.5. This pH was significantly different from the control tube, which did not contain *E. coli* O157:H7, and the other pH values; no other treatment reached as high a pH as this treatment. Conversely, tubes containing only 2.5 mM sinigrin and MHB were observed to increase in pH only slightly, resulting in a pH of 5.7 at the end of 13 d. This pH was not significantly different from the control pH but was different from all other day 13 pH values. As expected, both tubes containing 25 mM and 0.1 M glucose decreased in pH throughout the 13 d and resulted in final pH values of 5.1. Addition of sorbitol, galactose, mannose, or ribose did not seem to affect the change in pH to a great extent. Tubes containing these sugars had final pH values of 5.0, 5.1, 5.1, and 5.2, none of which were significantly different from each other, but were different from the control as well as from the tube containing only MHB ($P<0.05$).

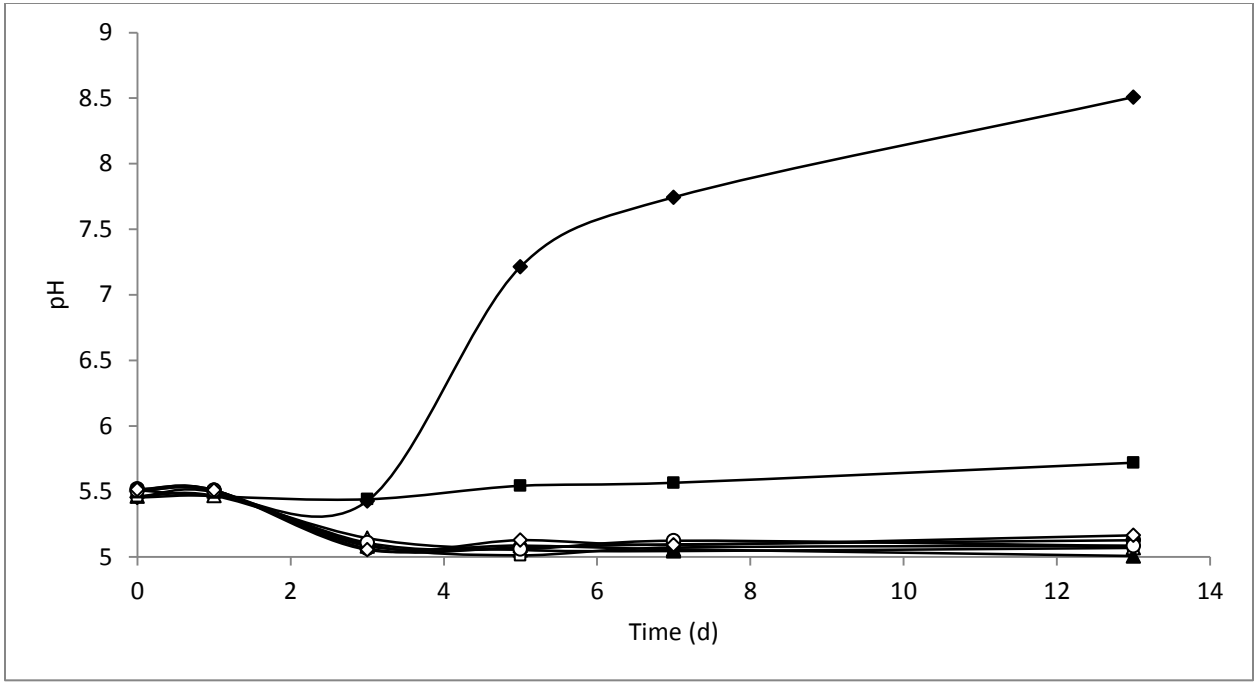

Figure 4.09: Change in pH of pH 5.5 and 0.05 M potassium phthalate-buffered MHB (◆) supplemented with 2.5 mM sinigrin (■), 25 mM glucose (△); 0.1 M glucose (□); 2.5 mM sinigrin, 25 mM glucose, and 25 mM sorbitol (▲); 2.5 mM sinigrin, 25 mM glucose, and 25 mM galactose (●); 2.5 mM sinigrin, 25 mM glucose, and 25 mM mannose (○); or 2.5 mM sinigrin, 25 mM glucose, and 25 mM ribose (◇). Samples were analysed on days 0, 1, 3, 5, 7, and 13 in duplicate.

Table 4.8: Effect of addition of sinigrin, glucose, and/or sorbitol, galactose, mannose, or ribose on pH of growth medium following 13 d growth of *E. coli* O157:H7[1].

Treatment[2]	pH
Control	5.511[b] ±0.005
1	8.508[a] ± 0.103
2	5.719[b] ± 0.012
3	5.069[c] ± 0.004
4	5.086[c] ± 0.047
5	5.008[c] ±0.008
6	5.128[c] ±0.025
7	5.084[c] ±0.048
8	5.165[c] ±0.006

[1] Values are the means of two replicates (± standard deviations, $n = 2$). Values not identified by the same letter are significantly different ($P<0.05$).

[2] Treatments: Control: MHB, no *E. coli* O157:H7; 1: MHB; 2: MHB and 25 mM sinigrin; 3: 25 mM glucose; 4: 0.1 M glucose; 5: 2.5 mM sinigrin, 25 mM glucose, and 25 mM sorbitol; 6: 2.5 mM sinigrin, 25 mM glucose, and 25 mM galactose; 7: 2.5 mM sinigrin, 25 mM glucose, and 25 mM mannose; 8: 2.5 mM sinigrin, 25 mM glucose, and 25 mM ribose.

4.4.4 Effect of glucose and monosaccharide concentration on sinigrin degradation activity in E. coli *O157:H7*

The ability of *E. coli* O157:H7 to degrade sinigrin in the presence of glucose and one of the monosaccharides (galactose, mannose, or ribose) or sorbitol was examined over a 13 d period in pH 5.5 and 0.1M potassium phthalate-buffered Mueller-Hinton broth containing 2.5 mM sinigrin. Figures 4.05-4.07 describe sinigrin degradation by *E. coli* O157:H7 at decreasing glucose concentrations in the presence of sorbitol, galactose, mannose, or ribose. Table 4.5 shows the total sinigrin concentration hydrolysed on day 13 of the experiment. No improvement after 13 d in sinigrin degradation was observed where the organism was exposed to 0.025 M glucose in addition to 0.025 M of one of the monosaccharides or sorbitol (Fig. 4.06). No significant difference in levels of sinigrin degradation between the different sugars existed. Conversely, treatment with 0.1 M glucose and 0.025 M monosaccharide seemed to inhibit sinigrin degradation by *E. coli* O157:H7. In comparison to the control, each of the sorbitol, galactose, mannose and ribose treatments reduced the amount of sinigrin degradation in comparison to a treatment without glucose. However, there were no significant differences in the levels of sinigrin hydrolysed between any of the sugars neither in this treatment nor in comparison to the control. The addition of 0.025 M glucose and 0.1 M monosaccharide also did not seem to affect the amounts of sinigrin hydrolysed in these treatments. No significant difference was identified between any of the sugars in this treatment or the control.

Table 4.9: The amount of sinigrin utilised by day 13 of analyses investigating the effect of addition of glucose and monosaccharide/sorbitol on sinigrin degradation by *E. coli* O157:H7 [1].

Treatment[2]	Monosaccharide/Sorbitol	Sinigrin Degraded (μM)
1	Sorbitol	114.4 ± 8.2^{ab}
	Galactose	121.5 ± 13.5^{ab}
	Mannose	130.2 ± 16.3^{ab}
	Ribose	103.0 ± 10.4^{ab}
2	Sorbitol	79.1 ± 21.2^{ab}
	Galactose	84.2 ± 2.6^{ab}
	Mannose	72.9 ± 14.8^{ab}
	Ribose	75.9 ± 18.1^{ab}
3	Sorbitol	97.8 ± 15.4^{ab}
	Galactose	129.6 ± 7.9^{ab}
	Mannose	104.2 ± 13.9^{ab}
	Ribose	105.0 ± 10.8^{ab}
MHB Control	N/A	124.1 ± 5.4^{ab}
0.025 M glucose Control	N/A	145.1 ± 15.1^{a}
0.1 M glucose Control	N/A	61.8 ± 4.8^{b}

[1]Levels not identified by the same letter are significantly different ($P<0.05$).

[2]Treatments: 1) 2.5 mM sinigrin, 25 mM glucose, 25 mM monosaccharide or sorbitol; 2) 2.5 mM sinigrin, 0.1 M glucose, 25 mM monosaccharide or sorbitol; 3) 2.5 mM sinigrin, 25 mM glucose, 0.1 M monosaccharide or sorbitol.

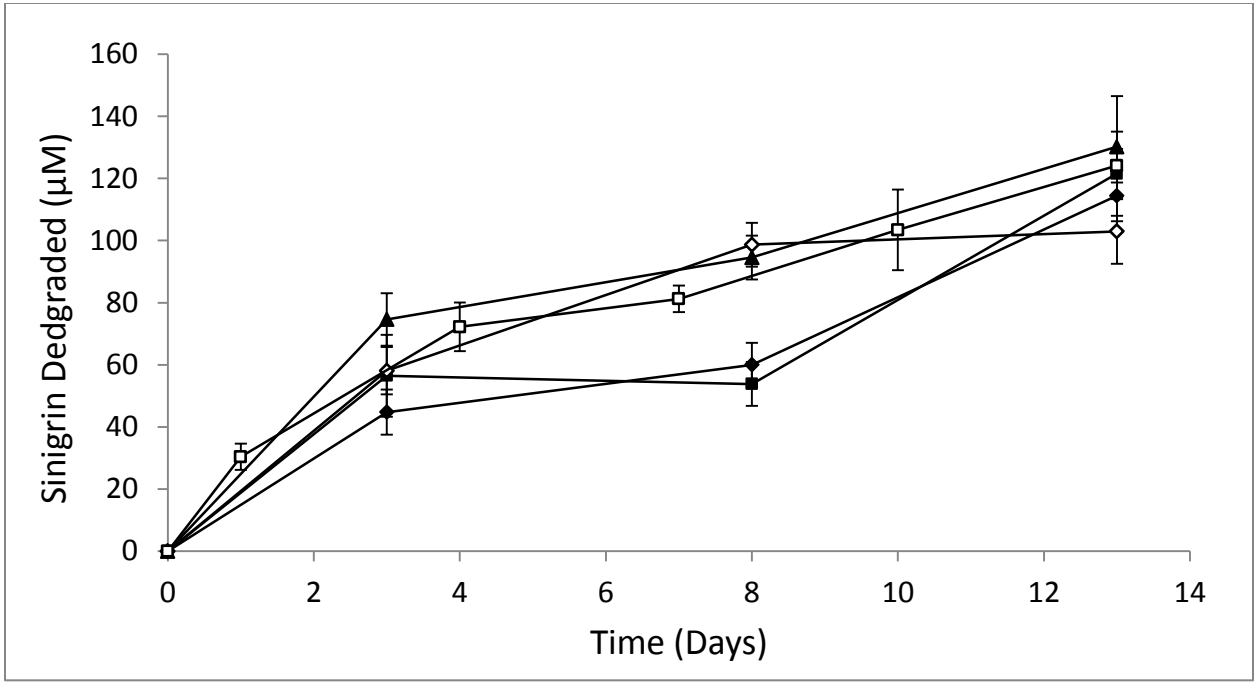

Figure 4.10: Degradation of sinigrin by *E. coli* O157:H7 grown in pH 5.5-buffered MHB (□) supplemented with 2.5 mM sinigrin, 0.025 M glucose, and 0.025 M of sorbitol (◆), galactose (■), mannose (▲), or ribose (◇).

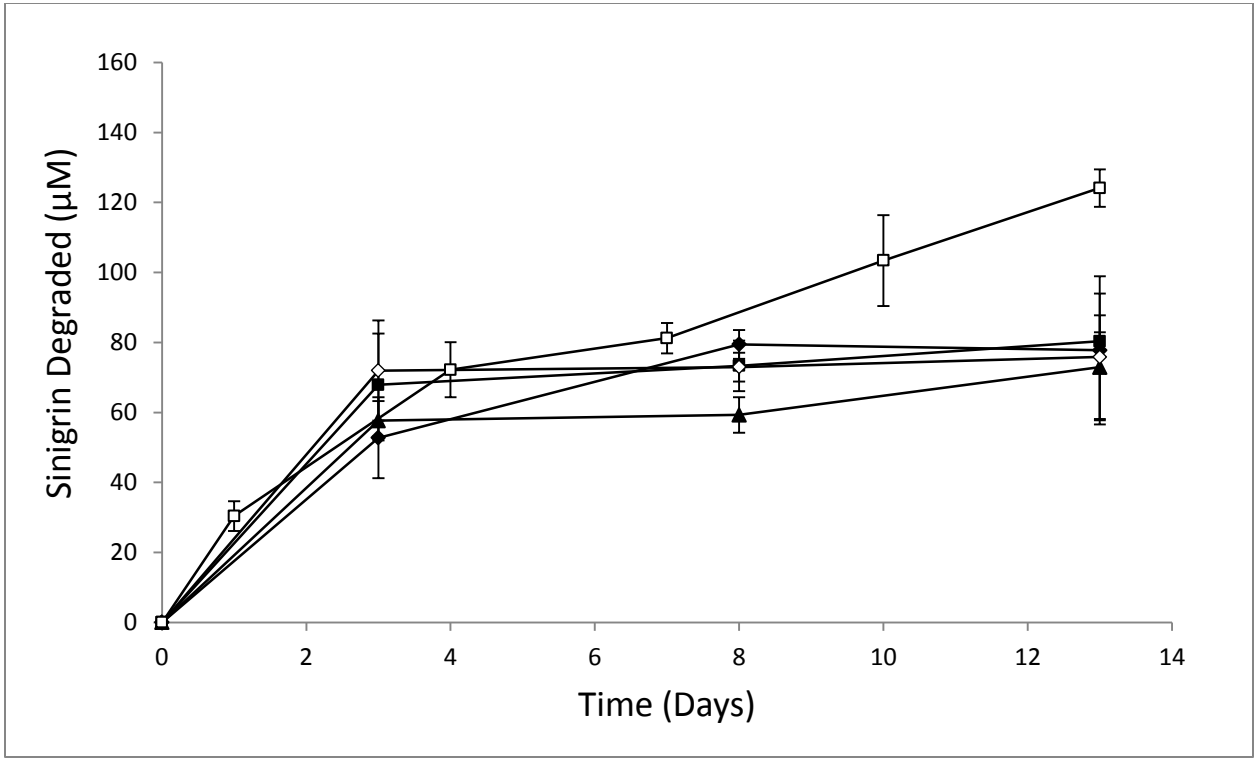

Figure 4.11: Degradation of sinigrin by *E. coli* O157:H7 grown in pH 5.5-buffered MHB (□) supplemented with 2.5 mM sinigrin, 0.1 M glucose, and 0.025 M of sorbitol (◆), galactose (■), mannose (▲), or ribose (◇).

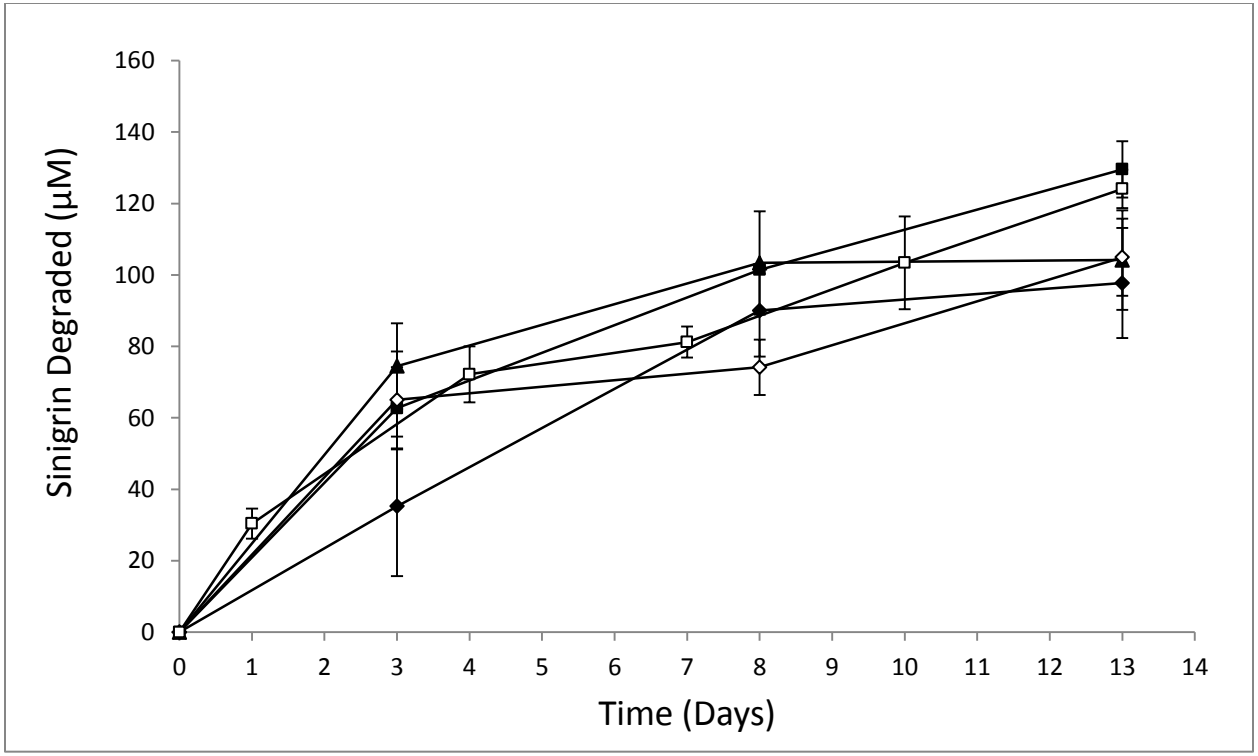

Figure 4.12: Degradation of sinigrin by *E. coli* O157:H7 grown in pH 5.5-buffered MHB (□) supplemented with 2.5 mM sinigrin, 0.025 M glucose, and 0.1 M of sorbitol (◆), galactose (■), mannose (▲), or ribose (◇).

4.5 Discussion

4.5.1 Identification of myrosinase-like activity and correlation with antibiotic sensitivity

Bacterial myrosinase-like activity has been reported in the past, and many bacterial species have been found to possess the ability to hydrolyse glucosinolates intracellularly (Brabban and Edwards, 1994; Palop et al., 1995; Krul et al., 2002; Cheng et al., 2004; Luciano and Holley, 2009; Herzallah et al., 2011; Cordeiro et al., 2014; Olaimat et al., 2014). All strains in the present study showed the ability to degrade sinigrin to some degree. In accordance with the results of Luciano and Holley (2009), the present work showed that *E. coli* O157:H7 was capable of utilising the most sinigrin, whereas *S. carnosus* was found to degrade slightly less and *P. pentosaceus* was found to hydrolyse the least of the three organisms. Additionally, these results are in agreement with those of Luciano and Holley (2011), where, of the LAB tested (which included the 5 strains examined in the present study), *L. plantarum* 131L and *P. acidilactici* 129P were both found to be unable to degrade the compound. Of the pseudomonads, all three strains were able to hydrolyse approximately the same amount of sinigrin, which agrees with research by Herzallah and colleagues (2011), where the authors found that *Ps. fluorescens* was only able to hydrolyse about 7% of the sinigrin present. It is hypothesized that bacteria hydrolyse glucosinolates in order to metabolise the glucose that results. The low level of sinigrin utilisation by the pseudomonads may be attributed the fact that *E. coli* metabolises glucose via a phosphotransferase system (PTS) pathway whereas *Pseudomonas* utilises instead the Entner-Doudoroff pathway for glucose metabolism (Hoshino, 1998; Wang et al., 1959). The present study found that *S. aureus* degraded a smaller amount of sinigrin than the 16% that had been

found previously (Herzallah et al., 2011). Similarly, *S. aureus* does not utilise a PTS pathway for glucose metabolism; instead, central routes for glucose metabolism are the Embden-Meyerhof-Parnas pathway and the pentose phosphate cycle (Kumar et al., 2011). For pediococci, the same Embden-Meyerhof-Parnas pathway is used for glucose metabolism (Gasson, and DeVos, 1994). It was hypothesized by Witt and colleagues (1993) that glucosinolates, as β-glucosides, may need initial phosphorylation before they are hydrolysed by 6-phospho-β-glucosidases, suggesting that bacteria with PTS pathways may utilise glucosinolates more efficiently. Additionally, recent research showed that, in *E. coli* VL8, proteins involved in the PTS system (including sugar transport subunits and kinases, an N-acetyl glucosamine-specific PTS system component IIABC, and glucokinase) were upregulated upon sinigrin exposure (Luang-In et al., 2015). The authors also observed that more genes were upregulated in the presence of sinigrin in *L. agilis* R16 compared to *E. coli* VL8, indicating that metabolic pathways of sinigrin utilisation differ in each strain and that *E. coli* may be equipped to metabolise sinigrin more effectively. However, different protein expressions observed may have been influenced by the media used (MRS for *L. agilis* and NaCl in NB for *E. coli*). From these findings, it seems likely that an organism possessing a PTS system for sugar metabolism is able to utilise glucosinolates more effectively (as fewer genes were upregulated during sinigrin metabolism) than in other organisms.

For *E. coli* O157:H7 and *S. carnosus*, the extents of sinigrin degradation were lower than has previously been reported. Other Shiga toxin-producing *E. coli* strains were capable of hydrolysing up to 890.7 μM sinigrin at 35°C in 13 d (unpublished, this lab). In addition, *E. coli* O157:H7 has been reported to degrade 507.7 μM sinalbin at 25°C in 6 d, and *S. carnosus* has been shown to utilise 206.9 μM sinalbin under the same conditions (Luciano and Holley, 2011).

Lower values of sinigrin hydrolysis during the present work may have been due to the use of 21°C. It has been noted that the utilisation of sinigrin and its degradation by bacteria is affected by temperature, with higher temperatures yielding higher levels of hydrolysis (Olaimat et al., 2014). Recently, research by Cordeiro et al. (2015) indicated that 6-phospho-β-glucosidase encoded by genes *bglA* and *ascB* in *E. coli* O157:H7 seemed to play a large role in sinigrin degradation in the organism. The identification of these genes in other bacteria capable of hydrolysing sinigrin may help explain the myrosinase-like activity in these organisms.

The level of sinigrin degradation of these strains was correlated with the antibiotic MIC profiles of these organisms from Chapter 3 (Table 4.5). The bacterial MIC profile of AITC was poorly correlated with the ability to degrade sinigrin at -24.1%, whereas the MIC profile of azithromycin was the most highly correlated with ability to degrade sinigrin at 51.3%. This was taken to indicate that bacterial myrosinase-like activity was not related to AITC susceptibility, nor was it highly correlated with the antibiotic MIC profiles tested in the present study.

4.5.2 *Effect of sinigrin, glucose, and monosaccharide concentration on growth rate as measured by optical density in* E. coli *O157:H7*

In an environment containing more than one carbon source, the presence of a second peak in the growth curve of an organism indicates a shift in the activity of catabolic enzyme system(s) as the organism utilises a different energy source (Stülke and Hillen, 1999). The growth rates via change in optical density of *E. coli* O157:H7 cultures were monitored for the first three days of

the experiment for each of the glucose and three monosaccharide/sorbitol treatments. Comparison of the controls showed that the addition of 25 mM sinigrin caused a small significant increase in optical density for MHB and MHB. No difference in change in optical density was attained with the addition of sinigrin to 0.1 M glucose (Figs. 4.05-4.07). In all controls, only one exponential phase was observed, suggesting perhaps that the sinigrin utilised (approximately 0.3 mM) was not substantial enough to provide the organism with a second growth phase. As the degradation of glucosinolates results in the formation of glucose (among other secondary reaction products) (Mithen, 2001), the lack of second exponential phase observed in these results indicates that *E. coli* O157:H7 hydrolyzed the glycone moiety of the glucosinolate and metabolised the resulting glucose simultaneously with its consumption of glucose (Lara-Lledó et al., 2012; Cordeiro et al., 2015). Work by Combourieu and colleagues (2001) showed that the metabolism of sinigrin by human intestinal microflora yielded the same metabolites as those produced by glucose metabolism. Additionally, is also known that the metabolism of free glucose occurred at a more rapid rate than hydrolysis of glucosinolates (MacFarlane and Gibson, 1996). Luang-In and colleagues (2015) found that genes coding for phosphoglycerate phosphatase, glyceraldehyde-3-phosphate dehydrogenase, D-lactate dehydrogenase, and acetate kinases in *L. agilis* R16 and glucokinases, adenylate kinase, glucose-1-phosphatase, plus glucose-6-phosphate dehydrogenase in *E. coli* VL8 were upregulated upon addition of sinigrin to the growth medium. These enzymes are involved in sugar/glucose metabolism in both organisms, which suggests that the use of glucose as an initial trigger for the production of these enzymes may facilitate the breakdown and utilisation of sinigrin during metabolism of glucose without the observation of a second growth phase.

It was expected that the treatment involving addition of all three of sinigrin, glucose, and monosaccharide/sorbitol would result in the highest maximum optical density, but this was not observed in any of the treatments, likely due to the excess concentration of glucose. As reported by a multitude of studies, the addition of 2% glucose (~0.1 M) reduces the growth rate of *E. coli* O157:H7 (Stephenson and Gale, 1937; Epps and Gale, 1942; Boyd and Lichstein, 1951). Twenty-five mM is also considered an excess of glucose, therefore this concentration may also have contributed to a shortened stationary phase brought on by micronutrient depletion or low pH. It seemed that *E. coli* O157:H7 may have simultaneously metabolized both carbon sources present, which seems most plausible as the data convey this observation. As reported by Aidelberg et al. (2014), sugar promotor repression of non-PTS sugars is "soft" in that the promotors belonging to sugars lower on the hierarchy are not fully repressed in the presence of a preferred carbon source. Furthermore, studies detailing simultaneous utilisation of several carbon substrates have been demonstrated (Lendenmann et al., 1996). From the data, it seemed that *E. coli* O157:H7 simultaneously consumed glucose and hydrolysed sinigrin, suggesting that sinigrin degradation was constitutive to glucose metabolism. Further studies exploring the addition of sinigrin and glucose on *E. coli* O157:H7 growth rate in minimal media would be useful in clarifying these effects in the absence of other carbon sources. Also, exploration of glycoside hydrolase genes in *E. coli* O157:H7 and determining the gene expression during sinigrin breakdown and metabolism may help to understand this process by pinpointing genes responsible for sinigrin hydrolysis (Cordeiro, 2015).

4.5.3 Effects of sinigrin, glucose, and/or monosaccharide concentration on pH and E. coli *O157:H7 cell numbers*

The change in pH was monitored in tubes containing *E. coli* O157:H7 grown in pH 5.5 and 0.05 potassium phthalate-buffered MHB on days 0, 1, 3, 5, 7, and 13 (Fig. 4.08 and Table 4.7). Previous studies have indicated that the metabolism of peptides and amino acids by *E. coli* O157:H7 yields amine-containing compounds such as ammonium, which contribute to an increase in environmental alkalinity (Lazar et al., 1998; Sezonov et al., 2007). MHB contains beef infusion, casein acid hydrolysate, and starch as main sources of carbon and approximately 2 mM glucose (Cordeiro, unpublished; HiMedia, 2015). In non-supplemented MHB, after the organism's preferred carbon source of glucose has been utilised, the main available carbon sources are peptides and amino acids from beef infusion and casein acid hydrolysate, as *E. coli* O157:H7 is unable to hydrolyse the starch present (Rosales-Colunga and Martínez-Antonio, 2014). In the control tubes where only MHB was present, the pH was seen to rise to a final pH of 8.5 (Table 4.7), confirming that *E. coli* O157:H7 utlized the amino acids and peptides to form ammonia. In contrast, all tubes containing glucose dropped in pH and possessed final pH values of approximately 5.0 on day 13 of the experiment. *E. coli* utilizes sugars under anaerobic conditions via fermentation, resulting in the primary formation of acetate, ethanol, lactate, and formate (Clark, 1989). Aside from ethanol, these soluble fermentation products contribute to acidification of the cell's environment (Stancik et al., 2002). In the tubes containing glucose it was apparent that fermentation of the sugar present occurred, subsequently decreasing the pH of the growth medium by about 0.5 units. Interestingly, tube 2 (MHB and 2.5 mM sinigrin) resulted in a final pH of approximately 5.7, which may indicate that in this treatment the organism did not utilize the amino acids present in MHB to grow, at least not initially. The lack of pH change may also be attributed to the action of a 0.05 M potassium hydrogen phthalate buffer.

From Fig. 4.08, the pH of tube 2 was seen to drop very slightly at day 3 of the experiment before slowly rising for the rest of the analysis. This trend may indicate that the organism first metabolised sinigrin for growth before switching to using the amino acids present, as approximately 72 μM of sinigrin had been hydrolysed at this point (Fig. 4.10). A similar response was noted in Luria-Bertani Broth, another low-glucose, high-amino acid broth (Sezonov et al., 2007). The initial hydrolysis of sinigrin may have preserved the pH at close to 5.5 but perhaps the metabolism of 2.5 mM glucosinolate was insufficient to reduce the pH of the environment further, especially in the presence of a 0.05 M potassium hydrogen phthalate buffer. It is likely that *E. coli* O157:H7 first exhausted its supply of sinigrin before switching to amino acid metabolism closer to day 3, which may explain the minor increase in the pH. An experiment with more replicates and an increased time scale would help pinpoint whether or not the pH of the growth medium will continue to rise as the organism consumes the amino acids present.

The change in cell numbers of *E. coli* O157:H7 was monitored in tubes containing pH 5.5 and 0.05 M potassium phthalate-buffered MHB on days 0, 1, 3, 5, 7, and 13 (Fig. 4. 09 and Table 4.6). All tubes possessed an initial number of approximately 3.62 log CFU mL^{-1} and grew to a maximum number of approximately 8.50 log CFU mL^{-1} on day 3 of the experiment. There were no significant differences in day 13 cell populations, indicating that the amount of sinigrin transformed was not adequate to form sufficient amounts of AITC to inhibit the growth of *E. coli* O157:H7. This organism has been reported to be acid tolerant, and is able to grow over a wide range of pH values, from 4.0 – 9.2 (Presser et al., 1997; Stancik et al., 2002). Although it is able to survive acidic environments, it has also been shown to decrease its growth rate as a result of

pH reduction (Presser et al., 1997). In the present study, pH began to decrease at day 3 in all tubes containing glucose, and minor (0.60-0.85 log CFU mL^{-1}) decreases in cell numbers were noted by 13 d. Similar reductions after 14 d in *E. coli* O157:H7 populations have been observed with addition of lactic acid at pH 4.5-5.0 at 25°C, where cell numbers were decreased by approximately 0.5 log CFU mL^{-1} (Conner and Kotrola, 1995). Additionally, accumulation of metabolic by-products and depletion of nutrients in the media after 13 d may also have contributed to the population decline (Boyd and Lichstein, 1951), as it is known that high concentrations of major fermentative end products, acetate and formate, inhibit growth in *E. coli* O157:H7 (Roe et al., 1998; Russell and Diez-Gonzalez, 1998).

Previous work has indicated that small percentages (between 10 and 30% in individual strains) of sinigrin are successfully converted to AITC (Krul et al., 2002). Considering that the level of sinigrin utilised by *E. coli* O157:H7 in this work was minor (0.3 mM) likely due to the low temperature used, it is unlikely that the AITC produced by the degradation of sinigrin was adequate to inhibit the growth of the organism.

4.5.4 Effect of glucose and monosaccharide concentration on sinigrin degradation activity in E. coli *O157:H7*

Previous work described an increase in glucosinolate consumption in response to the addition of 25 mM glucose to the growth medium (Cordeiro, unpublished). In the present study, 25 mM glucose in addition to 25 mM of one of sorbitol, galactose, mannose, or ribose was not found to significantly change the level of sinigrin degradation by *E. coli* O157:H7 (Fig. 4.10), indicating

that sinigrin degradation may not be enhanced by addition of glucose as previously suggested (Cordeiro, unpublished; Olaimat, unpublished). With the addition of 0.1 M glucose to the growth medium (Fig. 4.11), the level of sinigrin degraded dropped by about 35-40% in all treatments, which suggested that suppression of the system *E. coli* O157:H7 used to hydrolyze glucosinolates was likely due to the high concentration of a preferred energy source (glucose) (Lara-Lledó et al., 2012); however, these results were not statistically significant. Previous work (Cordeiro, unpublished) has also indicated that the addition of $\geq$0.05 M glucose decreased the glucosinolate hydrolysis by *E. coli* O157:H7 (Cordeiro, unpublished). Additionally, Olaimat and colleagues (2014) described a similar decrease of myrosinase-like activity following the addition of high concentrations of glucose to *Salmonella* and *L. monocytogenes* cocktails. At 0.05 and 0.1% glucose (~25 and 50 mM, respectively), both mixed strain cocktails were able to degrade >80% sinigrin, but at 0.25 to 1.0% glucose, sinigrin hydrolysis was significantly reduced (Olaimat et al., 2014). Concentrations of glucose of approximately 2% (~0.1 M) have also been found to decrease deaminase activities and therefore slow down growth rate, which may in turn decrease sinigrin degradation activity of *E. coli* O157:H7 (Stephenson and Gale, 1937; Epps and Gale, 1942; Boyd and Lichstein, 1951). Conversely, Combourieu and colleagues (2001) found that the addition of 6 mM of glucose did not alter the rate at which human digestive microflora consumed sinigrin. No significant effects were found with the addition of 25 mM glucose and 0.1 M of one of sorbitol, galactose, mannose or ribose, suggesting that although 0.1 M of glucose seemed to reduce the sinigrin degradation activity of the organism, 0.1 M of these alternate sugars did not confer the same effect. Nevertheless, further studies examining smaller concentrations of glucose in conjunction with enhancement of sinigrin degradation are recommended. Additionally, experiments conducted at a higher temperature where myrosinase-

like activity is at a maximum may help identify whether glucose is enhancing sinigrin degradation, as sinigrin degradation levels are more pronounced at higher temperatures (Olaimat et al., 2014).

CHAPTER 5

5. Conclusions

Similarities among the antimicrobial activity profiles of AITC and the antibiotics ciprofloxacin and polymyxin B were discussed in Chapter 3. The ciprofloxacin MIC correlated highly with the MIC profile of AITC, and similarities in mechanisms of action were noted in the literature which included effects on enzymes involved with DNA synthesis. AITC, readily reactive with proteins and amino acids, was thought to be bactericidal by binding to amino acids needed for RNA replication, by breaking disulfide bonds in enzymes important to DNA folding, such as those in DNA polymerase I, or by attacking enzymes important in both of these processes. The AITC MIC also correlated highly with the MIC profile of polymyxin B, an antibiotic that attacks the outer cell membrane and affects enzymes important in cellular respiration, causing leakage of cellular metabolites and affecting the cellular respiration processes of the organism. Similarly, work has shown that AITC is capable of disrupting bacterial cellular membranes, and has indicated that ITCs cause changes in the synthesis of genes related to cellular respiration. The effect of AITC on the bacteria tested in this study was also discussed. It seems that AITC and other ITCs, as stated by Luciano and Holley (2009), likely possess multi-targeted antimicrobial activity, with studies indicating ITCs exert effects on a range of bacterial structures and processes, including the plasma membrane, bacterial respiration, and DNA/RNA replication. The effect of ITCs on membrane lipids and DNA/RNA replication should be investigated. To determine the effect of ITCs on bacterial respiration, evaluation of the genes upregulated and/or downregulated in the presence of ITCs may offer insight. In order to obtain a more accurate

correlation between AITC and antibiotics, concentration values above and below the concentration ranges of AITC and antibiotics tested in this work should be investigated further as changing these values will alter the level of correlation. Additionally, a wider range of bacteria should be examined in order to identify a stronger correlation between AITC and antibiotics. Once more focused values have been determined with a larger sample set of bacteria, a more confident relationship can be identified between AITC and antibiotic breakpoints, which may help in confirming a mechanism of antimicrobial action for AITC.

AITC was effective against *E. coli* O157:H7, *E. coli* K-12, *S. carnosus* and *S. aureus*. Against the wild type and mutant *Ps. aeruginosa* and *Ps. fluorescens*, the ITC was extremely effective, indicating that the use of efflux pumps was not a successful mechanism of resistance against the agent. However, the combination of low pH and low temperature may have contributed to the attenuated growth of the pseudomonads in this experiment. When used against the LAB, AITC was not inhibitory toward any of the strains, suggesting that Gram-positive organisms may be inherently resistant to the compound due to the need for AITC attachment to LPS, only found in Gram- negative organisms. However, both staphylococci were susceptible to the ITC, therefore investigating the method of resistance in LAB may help indicate the mechanism of action of AITC. In particular, investigating whether these organisms undergo any sort of internal change in response to AITC in comparison to the staphylococci may reveal differences in how ITCs enter each organism and may affect internal structures.

The ability of a range of bacteria to hydrolyse sinigrin was also explored. Interestingly, of the LAB, both a strain of *P. acidilactici* (UM 119P) and *L. plantarum* (UM 134L) were found to

possess myrosinase-like activity, while the other strains of the same species (UM 129P and UM 131L) did not. According to Cordeiro (2015), the genes *bglA* and *ascB* are responsible for the production of myrosinase-analogous enzymes in the organism. The presence or absence of these genes in those strains of LAB that do and do not hydrolyse sinigrin may help indicate the mechanism by which bacteria transform glucosinolates.

When the growth rate as indicated by the change in optical density of *E. coli* O157:H7 was monitored during the first three days in all three treatments, no second exponential phase of growth was observed, indicating that perhaps the organism was hydrolysing the glucosinolate simultaneusly during its growth on glucose. Growth during these experiments indicated that omission of glucose allowed *E. coli* O157:H7 to reach its highest maximum optical density across all treatments and monosaccharides/sorbitol, suggesting that 25 mM glucose may have already been in an excess concentration. Future research is required to identify genes important in bacterial utilisation of glucosinolates and to determine if genes involved in glucose metabolism play a role in the breakdown of glucosinolates in the organism or if the organism simultaneously hydrolyses sinigrin during its metabolism of glucose. Additionally, these experiments performed in a minimum medium would help to verify that growth occurred via metabolism of glucose, sinigrin, or sorbitol/monosaccharide instead of the amino acids and peptides present in MHB.

E. coli O157:H7 was examined for its ability to degrade sinigrin in the presence of 0.025 M or 0.1 M glucose plus 0.025 M or 0.1 M of sorbitol, galactose, mannose, or ribose in response to previous work showing that the organism's myrosinase-like activity was increased with the

addition of 0.025 M glucose and decreased with 0.1 M glucose (Cordeiro, unpublished). No significant increases or decreases in levels of sinigrin hydrolysed were observed within any of the three treatments. Despite the presence of glucose and another monosaccharide, sinigrin was observed to be consumed over the 13 d analysis.

pH change during 13 d sinigrin degradation trials was monitored and indicated that tubes containing just MHB and sinigrin maintained a pH of 5.5 throughout the analysis. As MHB contains a small concentration of glucose, *E. coli* O157:H7 likely hydrolysed the glucosinolate and utilised the resulting cleaved glucose as a carbon source after consuming what was present in the broth. Further research is needed to confirm the change in pH in a medium that does not contain other carbon sources, as well as to identify the enzymes responsible for sinigrin hydrolysis.

REFERENCES

Ahn, E.-S., Kim, Y.-S., Shin, D.-H. 2001. Observation of bactericidal effect of allyl isothiocyanate on *Listeria monocytogenes*. Food Sci. Biotechnol. 10, 31-35.

Aidelberg, G., Towbin, B. D., Rothschild, D., Dekel, E., Bren, A., Alon, U. 2014. Hierarchy of non-glucose sugars in *Escherichia coli*. BMC Syst. Biol. 8, 133.

Aldred, K. J., Kerns, R. J., Osheroff, N. 2014. Mechanism of quinolone action and resistance. Biochemistry, 53, 1565–1574.

Al-Nabulsi, A. A., Holley, R. A. 2007. Effects on *Escherichia coli* O157:H7 and meat starter cultures of bovine lactoferrin in broth and microencapsulated lactoferrin in dry sausage batters. Int. J. Food Microbiol. 113, 84–91.

Anderson, V. E., Gootz, T. D., Osheroff, N. 1998. Topoisomerase IV catalysis and the mechanism of quinolone action. J. Biol. Chem. 273, 17879–17885.

Arnold, K. W., Kaspar, C. W. 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 61, 2037-2039.

Aymerich, T., Martin, B., Garriga, M., Hugas, M. 2003. Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. Appl. Environ. Microbiol. 69, 4583-4594.

Azaiez, I., Meca, G., Manyes, L., Fernández-Franzón, M. 2013. Antifungal activity of gaseous allyl, benzyl and phenyl isothiocyanate *in vitro* and their use for fumonisins reduction in bread. Food Control, 32, 428–434.

Azimova, S. S., Glushenkova, A. I., Vinogradova, V. I. (Eds.). 2012. Lipids, lipophilic components and essential oils from plant sources. Springer, New York, NY, U.S.A.

Barry, A. L., Jones, R. N. 1987. *In vitro* activity of ciprofloxacin against Gram-positive cocci. Amer. J. Med. 82, 27-32.

Beisel, C. L., Storz, G. 2011. The base-pairing RNA spot 42 participates in a multioutput feedforward loop to help enact catabolite repression in *Escherichia coli*. Mol. Cell, 41, 286–297.

Berlanga, M., Montero, M. T., Hernández-Borrel, J., Viñas, M. 2004. Influence of the cell wall on ciprofloxacin susceptibility in selected wild-type Gram-negative and Gram-positive bacteria. Int. J. Antimicrob. Agents, 23, 627-630.

Bermingham, A., Derrick, J. P. 2002. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. BioEssays, 24, 637–648.

Birge, E. 2000. Bacterial and Bacteriophage Genetics. Springer, New York, NY, U.S.A.

Bjerg, B., Sorensen, H. 1987. Isolation of intact glucosinolates by column chromatography and determination of their purity, in: J.P. Wathelet (Ed.), World Crops: Production, Utilization, Description. Glucosinolates in Rapeseeds: Analytical Aspects. Martinus Nijhoff Publ., Boston, MA, U.S.A. pp. 59-75.

Blair, J. M., Piddock, L. J. 2009. Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Curr. Opin. Microbiol. 12, 512-519.

Bockstael, K., Aerschot, A. 2009. Antimicrobial resistance in bacteria. Cent. Eur. J. Med. 4, 141–155.

Bones, A. M. 1990. Distribution of β-thioglucosidase activity in intact plants, cell and tissue cultures and regenerated plants of *Brassica napus* L. J. Exp. Bot. 41, 737-744.

Bones, A. M., Rossiter, J. T. 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. Physiol. Plantarum, 97, 194–208.

Boyd, W. L., Lichstein, H. C. 1951. The inhibitory effect of glucose on certain amino acid deaminases. J. Bacteriol. 62, 711-715.

Brader, G., Mikkelsen, M. D., Halkier, B. A., Palva, E. T. 2006. Altering glucosinolate profiles modulates disease resistance in plants. Plant J. 46, 758-767.

Buchwaldt, L., Larsen, L. M., Plöger, A., Sørensen, H. 1986. Fast polymer liquid chromatography isolation and characterization of plant myrosinase, β-thioglucoside glucohydrolase, isoenzymes. J. Chromatogr. 363, 71-80.

Burden, D. W. 2012. Guide to the disruption of biological samples – 2012. Random Primers, 12, 1-25.

Canadian Food Inspection Agency (CFIA). 1999. Meat Hygiene Manual of Procedures. Chapter 4.10.15 (pp 48-72).

Camoez, M., Sierra, J. M., Pujol, M., Hornero, A., Martin, R. M., Domínguez, A. 2013. Prevalence and molecular characterization of methicillin-resistant *Staphylococcus aureus* ST398 resistant to tetracycline at a Spanish hospital over 12 Years. PLoS ONE 8: e72828.

Cejpek, K., Valušek, J., Velišek., J. 2000. Reactions of allyl isothiocyanate with alanine, glycine, and several peptides in model systems. J. Agric. Food Chem. 48, 3560-3565.

Cejpek, K., Urban, J., Velísek, J., Hrabcová, H. 1998. Effect of sulfite treatment on allyl isothiocyanate in mustard paste. Food Chem. 62, 53-57.

Chacon, P. A., Muthukumarasamy, P., Holley, R. A. 2006. Elimination of *Escherichia coli* O157:H7 from fermented dry sausages at an organoleptically acceptable level of microencapsulated allyl isothiocyanate. Appl. Environ. Microbiol. 75, 3096-3102.

Chan, A. C., Ager, D., Thompson, I. P. 2013. Resolving the mechanism of bacterial inhibition by plant secondary metabolites employing a combination of whole-cell biosensors. J. Microbiol. Methods, 93, 209–217.

Chen, C-W., Ho, C-T. 1998. Thermal degradation of allyl isothiocyanate in aqueous solution. J. Agric. Food Chem. 46, 220-223.

Chen, W., Jin, T. Z., Gurtler, J. B., Geveke, D. J., Fan, X. 2012. Inactivation of *Salmonella* on whole cantaloupe by application of an antimicrobial coating containing chitosan and allyl isothiocyanate. Int. J. Food Microbiol. 155, 165-170.

Cheng, D-L., Hashimoto, K., Uda, Y. 2004. *In vitro* digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. Food Chem. Toxicol. 42, 351-357.

Chopra, I., Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. R. 65, 232–260.

Choubdar, N., Li, S., Holley, R.A. 2010. Supercritical fluid chromatography of myrosinase reaction products in ground yellow mustard seed oil. J. Food Sci. 75, C341-C345.

Clark, D. P. 1989. The fermentation pathways of *Escherichia coli*. FEMS Microbiol. Rev. 5, 223-234.

Clarke, D. B. 2010. Glucosinolates, structures and analysis in food. Anal. Methods, 2, 310-325.

Clinical and Laboratory Standards Institute (CLSI). 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard – ninth edition M07-09, 1-68. NCCLS, Wayne, PA.

Combourieu, B., Elfoul, L., Delort, A. M., Rabot, S. 2001. Identification of new derivatives of sinigrin and glucotropaeolin produced by the human digestive microflora using ^{1}H NMR spectroscopy analysis of in vitro incubations. Drug Metab. Dispos. 29, 1440-1445.

Conceição Gonçalves, M. P. J., Dos Santos Pires, A. C., Soares, N. de F. F., Araújo, E. A. 2009. Use of allyl isothiocyanate sachet to preserve cottage cheese. Journal of Foodservice, 20, 275–279.

Conner, D. E., Kotrola, J. S. 1995. Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. Appl. Environ. Microbiol. 61, 382-385.

Cordeiro, R. P., Wu, C., Holley, R. A. 2014. Contribution of endogenous plant myrosinase to the antimicrobial activity of deodorized mustard against *Escherichia coli* O157:H7 in fermented dry sausage. Int. J. Food Microbiol. 189, 132–138.

Cordeiro, R. P. 2015. Genetic basis for glucosinolate hydrolysis in *E. coli* O157:H7 by glycoside hydrolase action and nature of its adaptation to isothiocyanate toxicity. (Doctoral dissertation.) Retrieved from University of Manitoba Libraries MSpace. (http://hdl.handle.net/1993/30599)

Cordeiro, R. P., Doria, J. H., Zhanel, G. G., Sparling, R., Holley, R. A. 2015. Role of glycoside hydrolase genes in sinigrin degradation by *E. coli* O157:H7. Int. J. Food Microbiol. 205, 105-111.

Cui, W., Eskin, N. A. M. 1998. Process and properties of mustard products and components, in: Mazza, G., Shi, J. and Le Maguer, M. Functional foods: biochemical and processing aspects CRC Press. Boca Raton, FL, U.S.A. pp. 235-245.

D'Aimmo, M. R., Modesto, M., Biavati, B. 2007. Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. Int. J. Food Microbiol. 115, 35-42.

Danielsen, M., Simpson, P. J., O'Connor, E., Ross, R. P., Stanton, C. 2007. Susceptibility of *Pediococcus* spp. to antimicrobial agents. J. Appl. Microbiol. 102, 384-389.

Delaquis, P., Sholberg, P. 1997. Antimicrobial activity of gaseous allyl isothiocyanate. J. Food Prot. 71, 943-947.

Delaquis, P. J., Mazza, G. 1995. Antimicrobial properties of isothiocyanates in food preservation. Food Technol. 49, 73-84.

Delcour, A. H. 2009. Outer membrane permeability and antibiotic resistance. Biochimica et Biophysica Acta, 1794, 808–816.

Deris, Z. Z., Akter, J., Sivanesan, S., Roberts, K. D., Thompson, P. E., Nation, R. L., Li, J., Velkov, T. 2014. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. J. Antibiot. (Tokyo), 67, 147-151.

Desai, T. A., Rao, C. V. 2010. Regulation of arabinose and xylose metabolism in *Escherichia coli*. Appl. Environ. Microbiol. 76, 1524–1532.

Dever, L. A., Dermody, T. S. 1991. Mechanisms of bacterial resistance to antibiotics. Arch. Intern. Med. 151, 886–895.

Dias, C., Aires, A., Saavedra, M. J. 2014. Antimicrobial activity of isothiocyanates from cruciferous plants against methicillin-resistant *Staphylococcus aureus* (MRSA). Int. J. Mol. Sci. 15, 19552-19561.

Dias, M. V., Soares Nde, F., Borges, S. V., de Sousa, M. M., Nunes, C. A., de Oliveira, I. R., Medeiros, E. A. 2013. Use of allyl isothiocyanate and carbon nanotubes in an antimicrobial film to package shredded, cooked chicken meat. Food Chem. 141, 3160-3166.

Domagala, J. M., Hanna, L. D., Heifetz, C. L., Hutt, M. P., Mic, T. F., Sanchez, J. P., Solomon, M. 1986. New structure-activity relationships of the quinolone antibacterials using the target enzyme. The development and application of a DNA gyrase assay. J. Med. Chem. 29, 394-404.

Drobnica, L., Zemanová, M. 1967. Antifungal activity of isothiocyanates and related compounds. II. Mononuclear aromatic isothiocyanates. Appl. Microbiol. 15, 710-717.

Dufour, V., Alazzam, B., Ermel, G., Thepaut, M., Rossero, A., Tresse, O., Baysse, C. 2012. Antimicrobial activities of isothiocyanates against *Campylobacter jejuni* isolates. Front. Cell Infect. Microbiol. 53, 1-13.

Dufour, V., Stahl, M., Rosenfeld, E., Stintzi, A., Baysse, C. 2013. Insights into the mode of action of benzyl isothiocyanate on *Campylobacter jejuni*. Appl. Environ. Microbiol. 79, 6958–6968.

EFSA (European Food Safety Authority). 2009. Analysis of the baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in holdings with breeding pigs, in the EU, 2008 - Part A: MRSA prevalence estimates on request from the European Commission. The EFSA Journal, 7, 1376-1458.

Ekanayake, A., Kester, J. J., Li, J. J., Zehentbauer, G. N., Bunke, P. R., Zent, J. B., 2006. Isogard™: a natural anti-microbial agent derived from white mustard seed. Acta Horticulturae, 709, 101–108.

Eloff, J. N. 1988. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med. 64, 711-713.

Epps, H. M. R., Gale, E. F. 1942. The influence of the presence of glucose during growth on the enzymic activities of *Escherichia coli*: comparison of the effect with that produced by fermentation acids. Biochem. J. 36, 619-623.

Evans, G., de Challemaison, B., Cox, D. N. 2010. Consumers' ratings of the natural and unnatural qualities of foods. Appetite, 54, 557–563.

Fahey, J. W., Zalcmann, A. T., Talalay, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry, 56, 5–51.

Fan, J., Crooks, C., Creissen, G., Hill, L., Fairhurst, S., Doerner, P., Lamb, C. 2011. *Pseudomonas* sax genes overcome aliphatic isothiocyanate–mediated non-host resistance in *Arabidopsis*. Science, 331, 1185–1188.

Fenwick, G. R., Heaney, R. K., Mullin, W. J. 1983. Glucosinolates and their breakdown products in food and food plants. Crit. Rev. Food Sci. Nutr. 18, 123-201.

Foter, M. J. 1940 . Bactericidal properties of allyl isothiocyanate and related oils. Food Res. 5, 147-161.

Foter, M. J., Golick, A. M . 1938. Inhibitory properties of horseradish vapors. Food Res. 3, 609-613.

Fourmy, D., Recht, M. I., Blanchard, S. C., Puglisi, J. D. 1996. Structure of the A-site of *Escherichia coli* 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science, 274, 1367–1371.

Fratamico, P. M., Yan, X., Caprioli, A., Esposito, G., Needleman, D. S., Pepe, T., Tozzoli, R., Cortesi, M. L., Morabito, S. 2011. The complete DNA sequence and analysis of the

virulence plasmid and of five additional plasmids carried by Shiga toxin-producing *Escherichia coli* O26:H11 strain H30. Int. J. Med. Microbiol. 301, 192-203.

Gale, E. F. 1963. Mechanisms of antibiotic action. Pharmacol. Rev. 15, 481–530.

Ganin, H., Rayo, J., Amara, N., Levy, N., Krief, P., Meijler, M. M. 2013. Sulforaphane and erucin, natural isothiocyanates from broccoli, inhibit bacterial quorum sensing. Med. Chem. Comm. 4, 175–179.

Gasson, M. J., DeVos, W. M. (eds.). 1994. Genetics and biotechnology of lactic acid bacteria. Blackie Academic & Professional, Glasgow, UK.

Getty, K. J. K., Phebus, R. K., Marsden, J. L., Fung, D. Y. C., Kastner, C. L. 2000. *Escherichia coli* O157:H7 and fermented sausages: a review. J. Rapid Meth. Autom. Microbiol. 8: 141-170.

Graumann, G. H., Holley, R. A. 2008. Inhibition of *Escherichia coli* O157:H7 in ripening dry fermented sausage by ground yellow mustard. J. Food Prot. 71, 486–493.

Graumann, G. H., Holley, R. A. 2009. Survival of *E. coli* O157:H7 during manufacture of dry-cured Westphalian ham surface-treated with allyl isothiocyanate or hot mustard powder. J. Sci. Food Agr. 89, 617–624.

Green, D. W. 2002. The bacterial cell wall as a source of antibacterial targets. Expert Opin. Ther. Targets, 6, 1-19.

Griffiths, A. J., Miller, J. H., Suzuki, D. T., Lewontin, R. C., Gelbart, W. M. 2000. Catabolite repression of the lac operon: positive control, in: An Introduction to Genetic Analysis. 7th edition. New York: W. H. Freeman [Online]. Retrieved June 5, 2014, from http://www.ncbi.nlm.nih.gov/books/NBK22065/

Guo, M., Jin, T. Z., Scullen, O. J., Sommers, C. H. 2013. Effects of antimicrobial coatings and cryogenic freezing on survival and growth of *Listeria innocua* on frozen ready-to-eat shrimp during thawing. J. Food Sci. 78, M1195–M1200.

Hammes, W. P., Hertel, C. 1998. New developments in meat starter cultures. Meat Sci. 49, S125-S138.

Hamouda, T., Baker, J. R. 2000. Antimicrobial mechanism of action of surfactant lipid preparations in enteric Gram-negative bacilli. J. Appl. Microbiol. 89, 397-403.

Hancock, R. E. W. 1997. Peptide antibiotics. Lancet 349, 418-422.

Hancock, D. D., Rice, D. H., Thomas, L. A., Dargatz, D. A., Besser, T. E. 1997. Epidemiology of *Escherichia coli* O157 in feedlot cattle. J. Food Prot. 60, 462–465.

Hansen, M., Møller, P., Sørensen, H., de Trejo, M. C. 1995. Glucosinolates in broccoli stored under controlled atmosphere. J. Am. Soc. Horticult. Sci. 120, 1069-1074.

Hasegawa, N., Matsumoto, Y., Hoshino, A., Iwashita, K. 1999. Comparison of effects of *Wasabia japonica* and allyl isothiocyanate on the growth of four strains of *Vibrio parahaemolyticus* in lean and fatty tuna meat suspensions. Int. J. Food Microbiol. 49, 7–34.

Henwood, C. J., Livermore, D. M., James, D., Warner, M. 2001. Antimicrobial susceptibility of *Pseudomonas aeruginosa*: results of a UK survey and evaluation of the British Society for Antimicrobial Chemotherapy disc susceptibility test. J. Antimicrob. Chemother. 47, 789–799.

Hermsen, E. D., Sullivan, C. J., Rotschafer, J. C. 2003. Polymyxins: pharmacology, pharmacokinetics, pharmacodynamics, and clinical applications. Infect. Dis. Clin. N. Am. 17, 545–562.

Herzallah, S., Lledó, M. L., Holley, R. 2011. Influence of NaCl and $NaNO_3$ on sinigrin hydrolysis by foodborne bacteria. J. Food Prot. 74, 2162–2168.

Hill, D., Rose, B., Pajkos, A., Robinson, M., Bye, P., Bell, S., Elkins, M., Thompson, B., MacLeod, C., Aaron, S. D., Harbour, C. 2005. Antibiotic susceptibilities of *Pseudomonas aeruginosa* isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions. J. Clin. Microbiol. 43, 5085–5090.

HiMedia Laboratories. 2015. Technical Data: Mueller Hinton Broth. Retrieved from http://www.himedialabs.com/TD/M391.pdf

Hinkens, J. C., Faith, N. G., Lorang, T. D., Bailey, P., Buege, D., Kaspar, C. W., Luchansky, J. B. 1996. Validation of pepperoni processes for control of *Escherichia coli* O157:H7. J. Food Prot. 59, 1260-1266.

Hirani, A. H., Li, G., Zelmer, C. D., McVetty, P. B. E., Asif, M., Goyal, A. 2012. Molecular genetics of glucosinolate biosynthesis in *Brassicas*: Genetic manipulation and application aspects, in: Goyal A. (ed.) Crop Plant [Online]. Available from: http://www.intechopen.com/books/crop-plant/molecular-genetics-of-glucosinolate-biosynthesis-in-brassicas10.5772/45646

Hodges, N. 2011. Antibiotics and synthetic antimicrobial agents: their properties and uses, in: Hugo and Russell's Pharmaceutical Microbiology 8th ed. Denyer, S. P., Hodges, N., Gorman, S. P., Gilmore, B. F. (eds.). Blackwell Publishing Ltd., West Sussex, pp. 169-199.

Hogema, B. M., Arents, J. C., Bader, R., Eijkemans, K., Inada, T., Aiba, H., Postma, P. W. 1998. Inducer exclusion by glucose 6-phosphate in *Escherichia coli*. Mol. Microbiol. 28, 755–765.

Holck, A. L., Axelsson, L., Rode, T. M., Høy, M., Måge, I., Alvseike, O., L'abée-Lund, T.M., Omer, M.K., Granum, P.E., Heir, E. 2011. Reduction of verotoxigenic *Escherichia coli* in production of fermented sausages. Meat Sci. 89, 286–295.

Hontanaya, C., Meca, G., Luciano, F. B., Mañes, J., Font, G. 2014. Inhibition of aflatoxin B1, B2, G1 and G2 production by *Aspergillus parasiticus* in nuts using yellow and oriental mustard flours. Food Control, 47, 154–160.

Horáková, K., Greifová, M., Seemannová, Z., Gondová, Wyatt, G. M. 2004. A comparison of the traditional method of counting viable cells and a quick microplate method for monitoring the growth characteristics of *Listeria monocytogenes*. Lett. Appl. Microbiol. 38, 181-184.

Hoshino, T. 1998. Transport Systems in *Pseudomonas*, in *Pseudomonas*. Montie, T. (ed.). Springer, New York, NY, U.S.A., pp 171-172.

Hugas, M., Monfort, J. M. (1997). Bacterial starter cultures for meat fermentation. Food Chem. 59, 547–554.

Hummel, A. S., Hertel, C., Holzapfel, W. H.,Franz, C. M. A. P. 2006. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl. Environ. Microbiol. 73, 730-739.

Inatsu, Y., Bari, M. L., Kawasaki, S., Kawamoto, S. 2005. Effectiveness of some natural antimicrobial compounds in controlling pathogen or spoilage bacteria in lightly fermented Chinese cabbage. J. Food Sci. 70, M393-M397.

Isshiki, K, Tokuora, K, Mori, R., Chiba, S. 1992. Preliminary examination of allyl isothiocyanate vapor for food preservation. Biosci. Biotech. Biochem. 56, 1476-1477.

Jacob F, Monod J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3, 318-356.

Jacoby, G.A. 2005. Mechanisms of resistance to quinolones. Clin. Infect. Dis. 41, S120-S126.

Jakobsen, T. H., Bragason, S. K., Phipps, R. K., Christensen, L. D., van Gennip, M., Alhede, M., Skindersoe, M., Larsen, T. O., Høiby, N., Bjarnsholt, T., Givskov, M. 2012. Food as a source for quorum sensing inhibitors: iberin from horseradish revealed as a quorum sensing inhibitor of *Pseudomonas aeruginosa*. Appl. Environ. Microb. 78, 2410–2421.

Jin, T., Gurtler, J. B. 2012. Inactivation of *Salmonella* on tomato stem scars by edible chitosan and organic acid coatings. J. Food Prot. 75, 1368-1372.

Johnson, T. J., Nolan, L. K. 2009. Pathogenomics of the virulence plasmids of *Escherichia coli*. Microbiol. Mol. Biol. Rev. 73, 750-774.

Jones-Mortimer, M. C., Kornberg, H. L. 1974. Genetic control of inducer exclusion by *Escherichia coli*. FEBS Letters, 48: 93-95.

Jovin, T. M., Englund, P. T., and Bertsch, L. L. 1969. Enzymatic synthesis of deoxyribonucleic acid: XXCI. Physical and chemical studies of a homogeneous deoxyribonucleic acid polymerase. J. Biol. Chem. 244, 2996-3008.

Kaban, G., Kaya, M. 2008. Identification of lactic acid bacteria and Gram-positive catalase-positive cocci isolated from naturally fermented sausage (sucuk). J. Food Sci. 73, M385–M388.

Kamen, B. 1997. Folate and antifolate pharmacology. Semin. Oncol. 24, S1830-S1839.

Kamii, E., Isshiki, K. 2009. Antimicrobial efficacy of benzyl isothiocyanate. Shokuhin Eiseigaku Zasshi, 50, 311-314.

Kang, D. H. Fung, D. Y. C. 1999. Effect of diacetyl on controlling *Escherichia coli* O157:H7 and *Salmonella typhimurium* in the presence of starter cultures in a laboratory medium and during meat fermentation. J. Food Prot. 62, 975–979.

Kanoh, S., Rubin, B. K. 2010. Mechanisms of action and clinical application of macrolides as immunomodulatory medications. Clin. Microbiol. Rev. 23, 590–615.

Kassie, F., Knasmüller, S. 2000. Genotoxic effects of allyl isothiocyanate (AITC) and phenethyl isothiocyanate (PEITC). Chem. Biol. Interact. 127, 163-180.

Kassie, F., Laky, B., Nobis, E., Kundi, M., Knasmüller, S. 2001. Genotoxic effects of methyl isothiocyanate. Mut. Res./Gen. Toxicol. Environ. Mutagen. 490, 1-9.

Kawakishi, S., Kaneko, T. 1985. Interaction of oxidized glutathione with allyl isothiocyanate. Phytochemistry, 24, 715–718.

Kawakishi, S., Kaneko, T. 1987. Interaction of proteins with allyl isothiocyanate. J. Agr. Food Chem. 35, 85–88.

Kawakishi, S., Namiki, M. 1969. Decomposition of allyl isothiocyanate in aqueous solution. Agr. Biol. Chem. 33, 452–459.

Kim, Y. S., Ahn, E. S., Shin, D. H. 2002. Extension of shelf life by treatment with allyl isothiocyanate in combination with acetic acid on cooked rice. J. Food Sci. 67, 274–279.

Klein, G., Hallmann, C., Casas, I. A., Abad, J., Louwers, J., Reuter, G. 2000. Exclusion of vanA, vanB and vanC type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. J. Appl. Microbiol. 89, 815–824.

Kliebenstein, D. J., Kroymann, J., Mitchell-Olds, T. 2005. The glucosinolate-myrosinase system in an ecological and evolutionary context. Curr. Opin. Plant Biol. 8, 264–271.

Ko, J. A., Kim, W. Y., Park, H. J. 2012. Effects of microencapsulated allyl isothiocyanate (AITC) on the extension of the shelf-life of kimchi. Int. J. Food Microbiol. 153, 92-98.

Kojima, M. Ogawa, K. 1971. Studies on effects of isothiocyanates and their analogs on microorganisms. I. Effect of isothiocyanates on oxygen-uptake of yeasts. J. Ferment. Bioeng. 49, 740-746.

Kolm, R. H., Danielson, U. H., Zhang, Y., Talalay, P., Mannervik, B. 1995. Isothiocyanates as substrates for human glutathione transferases: structure-activity studies. Biochem. J. 211 pt. 2, 453–459.

Kotra, L. P., Haddad, J., Mobashery, S. 2000. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob. Agents Chemother. 44, 3249–3256.

Krul, C., Humblot, C., Philippe, C., Vermeulen, M., van Neunen, M., Havenaar, R., Rabot, S. 2002. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic *in vitro* large-intestinal model. Carcinogenesis 23, 1009-1016.

Kumar, A., Chua, K.-L., Schweizer, H. P. 2006. Method for regulated expression of single-copy efflux pump genes in a surrogate *Pseudomonas aeruginosa* strain: identification of the *BpeEF-OprC* chloramphenicol and trimethoprim efflux pump of *Burkholderia pseudomallei* 1026b. Antimicrob. Agents Chemother. 50, 3460–3463.

Kumar, P. S., Kumar, Y. N., Prasad, U. V., Yeswanth, S., Swarupa, V., Vasu, D., Venkatesh, K., Srikanth, L., Rao, V.K., Sarma, P.V.G.K. 2014. Comparative structural and functional analysis of *Staphylococcus aureus* glucokinase with other bacterial glucokinases. Indian J. Pharm. Sci. 76, 430-436.

Lambert, P. A. 2005. Bacterial resistance to antibiotics: modified target sites. Adv. Drug Del. Rev. 57, 1471-1485.

Lambert, P. 2011. Mechanisms of action of antibiotics and synthetic anti-infective agents, in: Hugo and Russell's Pharmaceutical Microbiology 8th ed. Denyer, S. P., Hodges, N., Gorman, S. P., Gilmore, B. F. (eds.). Blackwell Publishing Ltd., West Sussex, pp. 200-216.

Lancini, G., Parenti, F., Gallo, G. G. 1995. Antibiotics. Springer Science & Business Media, New York, NY, U.S.A.

Lara-Lledó, M., Olaimat, A., Holley, R. A. 2012. Inhibition of *Listeria monocytogenes* on bologna sausages by an antimicrobial film containing mustard extract or sinigrin. Int. J. Food Microbiol. 156, 25-31.

Lazar, S. W., Almirón, M., Tormo, A., and Kolter, R. 1998. Role of the *Escherichia coli* SurA protein in stationary-phase survival. J. Bacteriol. 180, 5704-5711.

Leclercq, R. 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. 15, 482-492.

Lendenmann, U., Snozzi, M., Egli, T. 1996. Kinetics of the simultaneous utilization of sugar mixtures by *Escherichia coli* in continuous culture. Appl. Environ. Microbiol. 62, 1493-1499.

Li, S., Aliani, M., Holley, R.A. 2013. Sensory evaluation of dry-fermented sausage containing ground deodorized yellow mustard. J. Food Sci. 78, S1595-1601.

Li, S., Li, Z., Wei, W., Ma, C., Song, X., Li, S., He, W., Tian, J., Huo, X. 2015. Association of mutation patterns in *gyrA* and *parC* genes with quinolone resistance levels in lactic acid bacteria. J. Antibiot. 68, 81-87.

Li, X.-Z., Mehrotra, M., Ghimire, S., Adewoye, L. 2007. β-Lactam resistance and β-lactamases in bacteria of animal origin. Vet. Microbiol. 121, 197–214.

Lim, J. Y., La, H. J., Sheng, H, Forney, L. J., Hovde, C. J. 2010. Influence of plasmid pO157 on *Escherichia coli* O157:H7 Sakai biofilm formation. Appl. Environ. Microbiol. 76, 963-966.

Lim, J. Y., Yoon, J. W., Hovde, C. J. 2010. A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. J. Microbiol. Biotechnol. 20, 5-14.

Lin, C. M., Preston, J. F. 3rd, Wei, C. I. 2000a. Antibacterial mechanism of allyl isothiocyanate. J. Food Prot. 63, 727-734.

Lin, C. M., Kim, J. M., Du, W. X. Wei, C. I. 2000b. Bactericidal activity of isothiocyanate against pathogens on fresh produce. J. Food Prot. 62, 5100-5107.

Lin, J., Smith, M. P., Chapin, K. C., Baik, H. S., Bennet, G. N., Foster, J. W. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. 62, 3094-3100.

Lina, G., Quaglia, A., Reverdy, M. E., Leclercq, R., Vandenesch, F, Etienne, J. 1999. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrob. Agents Chemother. 43, 1062-1066.

Loomis Jr., W. F., Magasanik, B. 1965. Genetic control of catabolite repression of the lac operon in *Escherichia coli*. Biochem. Biophys. Res. Commun. 20, 230–234.

Luang-In, V., Narbad, A., Cebeci, F., Bennett, M., Rossiter, J. T. 2015. Identification of proteins possibly involved in glucosinolate metabolism in *L. agilis* R16 and *E. coli* VL8. Protein J. 34, 135-146.

Luciano, F. B. Holley, R. A. 2010. Bacterial degradation of glucosinolates and its influence on the growth of *E. coli* O157:H7 in a dry fermented sausage model – Part I. Fleischwirtschaft Int. 25, 67-70.

Luciano, F. B., Holley, R. A. 2009. Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. Int. J. Food Microbiol. 131, 240–245.

Luciano, F. B., Belland, J., Holley, R.A. 2011. Microbial and chemical origins of the bactericidal activity of thermally treated yellow mustard powder toward *Escherichia coli* O157:H7 during dry sausage ripening. Int. J. Food Microbiol. 145, 69-74.

Luciano, F.B., Holley, R.A., 2011. Bacterial degradation of glucosinolates and its influence on the growth of *E. coli* O157:H7 - Part 2. Fleishwirtschaft Int. 25, 67-70.

Luciano, F. B., Hosseinian, F. S., Beta, T. Holley, R. A., 2008. Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7. J. Food Sci. 73, M214–M220.

MacDonald, D. M., Fyfe, M., Paccagnella, A., Trinidad, A., Louie, K., Patrick, D. 2004. *Escherichia coli* O157:H7 outbreak linked to salami, British Columbia, Canada, 1999. Epidemiol. Infect. 132, 283-289.

MacFarlane, G. T., Gibson, G. 1996. Carbohydrate fermentation, energy transduction and gas metabolism in the human large intestine, in Ecology and Physiology of Gastrointestinal Microbes. Mackie, R. J., White, B. A. Chapman and Hall (eds). New York, NY, U.S.A. pp 269-318.

Makino, K., Ishii, K., Yasunaga, T., Hattori, M., Yokoyama, K., Yutsudo, C.H., Kubota, Y., Yamaichi, Y., Iida, T., Yamamoto, K., Honda, T., Han, C.G., Ohtsubo, E., Kasamatsu, M., Hayashi, T., Kuhara, S., Shinagawa, H., 1998. Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic *Escherichia coli* O157:H7 derived from Sakai outbreak. DNA Res. 5, 1–9.

Mari, M., Leoni, O., Bernardi, R., Neri, F., Palmieri, S. 2008. Control of brown rot on stonefruit by synthetic and glucosinolate-derived isothiocyanates. Postharvest Biol. Technol. 47, 61–67.

Mathur, S., Singh, R. 2005. Antibiotic resistance in food lactic acid bacteria – a review. Int. J. Food Microbiol. 105, 281-295.

McKay, A. F., Garmaise, D. L., Gaudry, R., Baker, H. A., Paris, G. Y., Kay, R. W., Just, G. E., Schwartz, R. 1959. Bacteriostats. II. The chemical and bacteriostatic properties of isothiocyanates and their derivatives. J. Am. Chem. Soc. 81, 4328–4335.

Microsoft. 2007. Excel statistical functions: Pearson. Retrieved from https://support.microsoft.com/en-us/kb/828129.

Mingeot-Leclercq, M.-P., Glupczynski, Y., Tulkens, P. M. 1999. Aminoglycosides: activity and resistance. Antimicrob. Agents Chemother. 43, 727–737.

Mitchell, A., Romano, G. H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., Dahan, O., Pilpel, Y. 2009. Adaptive prediction of environmental changes by microorganisms. Nature, 460, 220-224.

Mithen, R. F., Dekker, M., Verkerk, R., Rabot, S. Johnson, I. T. 2000. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. J. Sci. Food Agri. 80, 967-984.

Mithen, R. 2006. Sulfur-containing compounds, in Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet. Crozier et al. (eds.). Blackwell Publishing, Ames, Iowa, pp. 25-46.

Mitscher, L. A. 2005. Bacterial topoisomerase inhibitors: Quinolone and pyridone antibacterial agents. Chem. Rev. 105, 559−592.

Monod, J. 1942. Recherches sur la croissance des cultures bactériennes. Hermann and Cie, Paris, France.

Morcillo, A., Castro, B., Rodríguez-Álvarez, C., Abreu, R., Aguirre-Jaime, A., Arias, A. 2015. Descriptive analysis of antibiotic-resistant patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) st398 isolated from healthy swine. Int. J. Environ. Res. Public Health, 12, 611-622.

Nadarajah, D., Han, J. H., Holley, R. A. 2005. Use of mustard flour to inactivate *Escherichia coli* O157:H7 in ground beef under nitrogen flushed packaging. Int. J. Food Microbiol. 99, 257–267.

Nakamura, Y., Kawakami, M., Yoshihiro, A., Miyoshi, N., Ohigashi, H., Kawai, K., Osawa, T., Uchida, K. 2002. Involvement of the mitochondrial death pathway in chemopreventive benzyl isothiocyanate-induced apoptosis. J. Biol. Chem. 277, 8492–8499.

Newton, B.A. 1956. The properties and mode of action of the polymyxins. Bacteriol Rev. 20, 14-27.

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol. Biol. R. 67, 593–656.

Nilson, A. M., Holley, R.A., 2012. Use of deodorised yellow mustard powder to control *Escherichia coli* O157:H7 in dry cured Westphalian ham. Food Microbiol. 30, 400-407.

Noller, H. F. 1991. Ribosomal RNA and translation. Ann. Rev. Biochem. 60, 191–227.

Nowicki, D., Maciq-Dorszyńska, M., Kobiela, W., Herman-Antosiewicz, A., Weqrzyn, A., Szalewska-Pałasz, A., Węqrzyn, G. 2014. Phenethyl isothiocyanate inhibits shiga toxin production in enterohemorrhagic *Escherichia coli* by stringent response induction. Antimicrob. Agents Chemother. 58, 2304-2315.

O'Loughlin, C. T., Miller, L. C., Siryaporn, A., Drescher, K., Semmelhack, M. F., Bassler, B. L. 2013. A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. P. Natl. Acad. Sci. US. 110, 17981–17986.

Obaidat, M. M., Frank, J. F. 2009a. Inactivation of *Escherichia coli* O157:H7 on the intact and damaged portions of lettuce and spinach leaves by using allyl isothiocyanate, carvacrol, and cinnamaldehyde in vapor phase. J. Food Prot. 72, 2046–2055.

Obaidat, M. M., Frank, J. F. 2009b. Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on sliced and whole tomatoes by allyl isothiocyanate, carvacrol, and cinnamaldehyde in vapor phase. J. Food Prot. 72, 315-324.

Ohta, Y., Takatani, K., Kawakishi, S. 1995. Decomposition rate of allyl isothiocyanate in aqueous solution. Biosci. Biotech. Biochem. 59, 102-103.

Ohtsuru M., Tsuruo I., Hata T. 1969. Studies of fungous myrosinase part I: production, purification and some characteristics. Agric. Biol. Chem. 33, 1309-1314.

Olaimat, A. N., Fang, Y., Holley, R. A. 2014. Inhibition of *Campylobacter jejuni* on fresh chicken breasts by κ-carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized oriental mustard extract. Int. J. Food Microbiol. 187, 77–82.

Olaimat, A. N., Sobhi, B, Holley, R. A. 2014. Influence of temperature, glucose, and iron on sinigrin degradation by *Salmonella* and *Listeria monocytogenes*. J. Food Prot. 77, 2133-2138.

Otoni, C. G., Soares, N. de F. F., da Silva, W. A., Medeiros, E. A. A., Baffa Junior, J. C. 2014. Use of allyl isothiocyanate-containing sachets to reduce *Aspergillus flavus* sporulation in peanuts. Package Technol. Sci. 27, 549–558.

Palop M. L., Smiths J. P., Brink B. T. 1995. Degradation of sinigrin by *Lactobacillus agilis* strain R16. Int. J. Food Microbiol., 26, 219-229.

Pang, Y.-H., Sheen, S., Zhou, S., Liu, L., Yam, K. L. 2013. Antimicrobial effects of allyl isothiocyanate and modified atmosphere on *Pseudomonas aeruginosa* in fresh catfish fillet under abuse temperatures. J. Food Sci. 78, M555–559.

Pearson, K. 1896. Mathematical contributions to the theory of evolution, III: regression, heredity and panmixia. Philos. Trans. Ryoal. Soc. London 187, 253-318. Reprinted in *Karl Pearson's Early Statistical Papers* (1956) 113-178. Cambridge University Press, Cambridge, United Kingdom.

Pérez-Alfaro, R. S., Santillan, M., Galan-Vasquez, E. 2014. Regulatory switches for hierarchical use of carbon sources in *E. coli*. Network Biology. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=CN2014200051

Pérez-Díaz, I. M., McFeeters, R. F. 2010. Preservation of acidified cucumbers with a natural preservative combination of fumaric acid and allyl isothiocyanate that target lactic acid bacteria and yeasts. J. Food Sci. 75, M204–M208.

Pestka, S. 1969. Studies on the formation of transfer ribonucleic acid-ribosome complexes X. phenylalanyl-oligonucleotide binding to ribosomes and the mechanism of chloramphenicol action. Biochem. Biophys. Res. Commun. 36, 589–595.

Piercey, M. J., Mazzanti, G., Budge, S. M., Delaquis, P. J., Paulson, A. T., Truelstrup Hansen, L. 2012. Antimicrobial activity of cyclodextrin entrapped allyl isothiocyanate in a model system and packaged fresh-cut onions. Food Microbiol. 30, 213–218.

Poole, K. 2004. Resistance to beta-lactam antibiotics, Cell Mol. Life Sci. 61, 2200-2223.

Poole, K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microb. Biotech. 3, 255–264.

Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Yamasaki, T., Neshat, S., Yamagishi, J., Li, X. Z., Nishino, T. 1996. Overexpression of the mexC–mexD–oprJ efflux operon in nfxB-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. 21, 713–725.

Presser, K. A., Ratkowsky, D.A., Ross, T. 1997. Modelling the growth rate of *Escherichia coli* as a function of pH and lactic acid concentration. Appl. Environ. Microbiol. 63, 2355*2360.

Radojčić Redovniković, I., Glivetić, T., Delonga, K., Vorkapić-Furač, J. 2008. Glucosinolates and their potential role in plants. Periodicum Biologorum, 110, 297-309.

Rakariyatham N., Sakorn P. 2002. Biodegradation of glucosinolates in brown mustard seed meal (*Brassica juncea*) by *Aspergillus* sp. NR-4201 in liquid and solid-state cultures. Biodegradation, 13, 395-399.

Raney, J. P., Rakov, G. F. 2007. Seeds, oils and seed meals produced from *Sinapis alba*. US Patent No. US 9,279,618 B2. United States Patent and Trademark Office.

Rawel, H. M., Kroll, J. 1995. Some aspects of reactions of benzyl isothiocyanate with bovine sarcoplasmic proteins. Die Nahrung, 39, 465-474.

Reed, C. 1995. Challenge study – *Escherichia coli* O157:H7 in fermented sausages. U.S. Department of Agriculture, Food Safety and Inspection Service, Washington, D.C. (Letter to plant managers).

Resch, M., Nagel, V., Hertel, C. 2008. Antibiotic resistance of coagulase-negative staphylococci associated with food and used in starter cultures. Int. J. Food Microbiol. 127, 99-104.

Reynolds, P. 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur. J. Clin. Microbiol. 8, 943–950.

Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargrett, N. T., Black, P. A., Cohen, M. L. 1983.

Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. 308, 681-685.

Rode, T. M., Holck, A., Axelsson, L., Høy, M., Heir, E. 2012. Shiga toxigenic *Escherichia coli* show strain dependent reductions under dry-fermented sausage production and post-processing conditions. Int. J. Food Microbiol. 155, 227–233.

Rodríguez-Rojas, A., Rodríguez-Beltrán, J., Couce, A., Blázquez, J. 2013. Antibiotics and antibiotic resistance: a bitter fight against evolution. Int. J. Med. Microbiol. 303, 293–297.

Roe, A. J., McLaggan, D., Davidson, I., O'Byrne, C., Booth, I. R. 1998. Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. J. Bacteriol. 180, 767-772.

Rosales-Colunga, L. M., Martínez-Antonio, A. 2014. Engineering *Escherichia coli* K12 MG1655 to use starch. Microb. Cell Fact. 13, 74.

Rosenstein, R., and Götz, F. 2010. Genomic differences between the food-grade *Staphylococcus carnosus* and pathogenic staphylococcal species. Int. J. Med Microbiol. 300, 104-108.

Rosenstein, R., Nerz, C., Biswas, L., Resche, A., Raddatz, G., Schuster, S. C., Götz, F. 2009. Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. Appl. Environ. Microbiol. 75, 811-822.

Ruíz, F. O., Gerbaldo, G., García, M. J., Giordano, W., Pascual, L., Barberis, I. L. 2012. Synergistic effect between two bacteriocin-like inhibitory substances produced by lactobacilli strains with inhibitory activity for *Streptococcus agalactiae*. Curr. Microbiol. 64, 349–356.

Russell, J. B., Diez-Gonzalez, F. 1998. The effects of fermentation acids on bacterial growth. Adv. Microb. Physiol. 39, 205-234.

Sanders, C. C. 1988. Ciprofloxacin: *in vitro* activity, mechanism of action, and resistance. Rev. Infect. Dis. 10, 516–527.

Sang, J., Minchinton, I., Johnstone, P. Truscott, R. 1984. Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. Can. J. Plant Sci. 64, 77-93.

Sartz, L., De Jong, B., Hjertqvist, M., Plym-Forshell, L., Alsterlund, R., Lofdahl, S., Osterman, B., Stahl, A., Eriksson, E., Hansson, H. B., and Karpman, D. 2008. An outbreak of *Escherichia coli* O157:H7 infection in southern Sweden associated with consumption of fermented sausage; aspects of sausage production that increase the risk of contamination. Epidemiol. Infect. 136, 370–380.

Sellitti, M. A., Pavco, P. A., Steege, D. A. 1987. Lac repressor blocks *in vivo* transcription of lac control region DNA. P. Natl. Acad. Sci. US. 84, 3199-3203.

Seo, H.-S., Bang, J., Kim, H., Beuchat, L. R., Cho, S. Y., Ryu, J.-H. 2012. Development of an antimicrobial sachet containing encapsulated allyl isothiocyanate to inactivate *Escherichia coli* O157:H7 on spinach leaves. Int. J. Food Microbiol. 159, 136–143.

Sezonov, G., Joseleau-Petit, D., D'Ari, R. 2007. *Escherichia coli* physiology in Luria-Bertani broth. J. Bacteriol. 189, 8746-8749.

Sharma, L., Verma, A. K., Kumar, A., Rahat, A., Nigam, N., Nigam, R. 2015. Incidence and pattern of antibiotic resistance of *Staphylococcus aureus* isolated from clinical and subclinical mastitis in cattle buffaloes. Asian Journal of Animal Sciences 9, 100-109.

Shibata, T., Kimura, Y., Mukai, A., Mori, H., Ito, S., Asaka, Y., Oe, S., Tanaka, H., Takahashi, T., Uchida, K. 2011. Transthiocarbamoylation of proteins by thiolated isothiocyanates. J. Biol. Chem. 286, 42150–42161.

Shin, I.-S., Han, J.-S., Choi, K.-D., Chung, D.-H., Choi, G.-P., Ahn, J. 2010a. Effect of isothiocyanates from horseradish (*Armoracia rusticana*) on the quality and shelf life of tofu. Food Control, 21, 1081–1086.

Shin, J., Harte, B., Ryser, E., Selke, S. 2010b. Active packaging of fresh chicken breast, with allyl isothiocyanate (AITC) in combination with modified atmosphere packaging (MAP) to control the growth of pathogens. J. Food Sci. 75, M65–71.

Simpson, C. A., Geornaras, I., Yoon, Y., Scanga, J. A., Kendall, P. A., Sofos, J.N. 2008. Effect of inoculum preparation procedure and storage time and temperature on the fate of *Listeria monocytogenes* on inoculated salami. J. Food Prot. 71, 494-501.

Sofrata, A., Santangelo, E. M., Azeem, M., Borg-Karlson, A.-K., Gustafsson, A., Putsep, K. 2011. Benzyl isothiocyanate, a major component from the roots of *Salvadora persica* is highly active against Gram-negative bacteria. PLoS One 6, e23045. Available from: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0023045

Sozzi, T., Smiley, M. B., 1980. Antibiotic resistances of yoghurt starter cultures *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Appl. Environ. Microbiol. 40, 862–865.

Stancik, L. M., Stancik, D. M., Schmidt, B., Barnhard, D. M., Yoncheva, Y. N., Slonczewski, J. L. 2002. pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. J. Bacteriol. 184, 4246-4258.

Stephenson, M., Gale, E. F. 1937. Factors influencing bacterial deamination. Biochem. J. 31, 1316-1322.

Stewart, E.J., Åslund, F., Beckwith, J. 1998. Disulfide bond formation in the *Escherichia coli* cytoplasm: an *in vivo* role reversal for the thioredoxins. The EMBO Journal 17, 5543-5550.

Stülke, J., Hillen, W. 1999. Carbon catabolite repression in bacteria. Curr. Opin. Microbiol. 2, 195–201.

Tan, S. Y., Liu, Y., Chua, S. L., Vejborg, R. M, Jakobsen, T. H., Chew, S. C., Li, Y., Nielsen, T. E., Tolker-Nielsen, T., Yang, L., Givskov, M. 2014. Comparative systems biology analysis to study the mode of action of the isothiocyanate compound iberin on *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 58, 6648-6659.

Tang, L., Zhang, Y. 2005. Mitochondria are the primary target in isothiocyanate-induced apoptosis in human bladder cancer cells. Mol. Cancer Therapy, 4, 1250-1259.

Tilden, J., Jr, Young, W., McNamara, A. M., Custer, C., Boesel, B., Lambert-Fair, M. A., Majkowski, J., Vugia, D., Werner, S.B., Hollingsworth, J., Morris, J. G., Jr. 1996. A new route of transmission for *Escherichia coli*: infection from dry fermented salami. Am. J. Public Health, 86, 1142–1145.

Tille, P. 2013. Bailey and Scott's Diagnostic Microbiology. Mosby, Maryland Heights, MO, U.S.A.

Trachtman, H., Christen, E. 1999. Pathogenesis, treatment, and therapeutic trials in hemolytic uremic syndrome. Curr. Opin. Pediatr. 11, 162-168.

Tuttle, J., Gomez, T., Doyle, M. P., Wells, J. G., Zhao, T., Tauxe, R. V., Griffin, P. M. 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. Epidemiol. Infect. 122, 185-192.

Uda, Y., Hayashi, H., Takahashi, A., Shimizu, A. 2000. Mutagenic and antimutagenic property of 3-hydroxymethylene-2-thioxopyrrolidine, a major product generating from pungent principle of radish. Lebensm. Wiss. Technol. 33, 37-43.

Uda, Y., Matsuoka, H., Kumagami, H., Shims, H., Meede, Y. 1993. Stability and antimicrobial property of 4-methylthio-8-butenyl isothiocyanate, the pungent principle in radish. Nippon Shokuhin Kogyo Gakkaishi 40, 743-746.

Vaara M, Vaara T, Sarvas M. 1979. Decreased binding of polymyxin by polymyxin-resistant mutants of *Salmonella typhimurium*. J. Bacteriol. 139, 664–667.

Vanderhaeghen, W., Hermans, K., Haesebrouck, F., Butaye, P. 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) in food production animals. Epidem. Infect. 138, 606-625.

Velebit, B., Fetsch, A., Milorad, M., Teodorovic, V., Jovanovic, M. 2010. MRSA in pigs in Serbia. Vet. Rec. 167, 183-184.

Verhegghe, M., Pletinckx, L.J., Crombé, F., Vandersmissen, T., Haesebrouck, F., Butaye, P., Heyndrickx, M., Rasschaert, G. 2013. Methicillin-resistant *Stapylococcus aureus* (MRSA) ST398 in pig farms and multispecies farms. Zoonoses Public Health, 60, 366-374.

Vidal, C. A., Collins-Thompson, D. L. 1987. Sensitivity of meat lactic acid bacteria to antibiotics. J. Food. Prot. 50, 737-740.

Virtanen, A. I. 1965. Studies on organic sulfur containing compounds and other labile substances in plants. Phytochemistry, 4, 207-228.

Wang, C. H., Stern, J., Gilmour, C. M. 1959. The catabolism of glucose and gluconate in *Pseudomonas* species. Arch. Biochem. Biophys 81, 489-492.

Wang, S. Y., Chen, C.-T., Yin, J.-J. 2010. Effect of allyl isothiocyanate on antioxidants and fruit decay of blueberries. Food Chem. 120, 199–204.

Ward, S. M., Delaquis, P. J., Holley, R. A., Mazza, G. 1998. Inhibition of spoilage and pathogenic bacteria on agar and pre-cooked roast beef by volatile horseradish distillates. Food Res. Int. 31, 19–26.

Wells, J. G., Davis, B. R., Wachsmuth, I. K., Riley, L. W., Remis, R. S., Sokolow, R., Morris, G. K. 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. J. Clin. Microbiol. 18, 512-520.

Wiktelius, E., Stenberg, G. 2007. Novel class of glutathione transferases from cyanobacteria exhibit high catalytic activities towards naturally occurring isothiocyanates. Biochem. J. 406, 115-123.

Williams, R. C., Isaacs, S., Decou, M. L., Richardson, E. A., Buffet, M. C., Slinger, R. W., Brodsky, M. H., Ciebin, B. W., Ellis, A., Hockin, J. 2000. Illness outbreak associated with *Escherichia coli* O157:H7 in Genoa salami. Can. Med. Assoc. J. 162, 1409-1413.

Wilson, A. E., Bergaentzle, M., Bindler, F., Marchioni, E., Lintz, A., Ennahar, S. 2013. *In vitro* efficacies of various isothiocyanates from cruciferous vegetables as antimicrobial agents against foodborne pathogens and spoilage bacteria. Food Control, 30, 318-324.

Witt, E., Frank, R., Hengstenberg, W. 1993. 6-phospho-beta-galactosideases of Gram-positive and 6-phospho-beta-glucosidase-b of Gram-negative bacteria – comparison of structure and function by kinetic and immunological methods and mutagenesis of the lacG gene of *Staphylococcus aureus*. Protein Eng. 6, 913-920.

Wittstock, U., Halkier, B. A. 2002. Glucosinolate research in the *Arabidopsis* era. Trends Plant Sci. 7, 263–270.

Woodin, K. A., Morrison, S. H. 1994. Antibiotics: mechanisms of action. Pediatr. Rev. 15, 440-447.

Wright, P. R., Richter, A. S., Papenfort, K., Mann, M., Vogel, J., Hess, W. R., Backofen, R., Georg, J. 2013. Comparative genomics boosts target prediction for bacterial small RNAs. P. Natl. Acad. Sci. US. 110, E3487–E3496.

Wu, H., Zhang, X., Zhang, G.-A., Zeng, S.-Y., Lin, K.-C. 2011. Antifungal vapour-phase activity of a combination of allyl isothiocyanate and ethyl isothiocyanate against *Botrytis cinerea* and *Penicillium expansum* infection on apples. J. Phytopathol. 159, 450–455.

Wu, X., Zhou, Q., Xu, K. 2009. Are isothiocyanates potential anti-cancer drugs? Acta. Pharm. Sinica, 30, 501–512.

Zavascki, A. P., Goldani, L. Z., Li, J., Nation, R. L. 2007. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. J. Antimicrob. Chemother. 60, 1206–1215.

Zhang, Y., Talalay, P. 1994. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. Cancer Res. 54, 1976-1981.

Zoetendal, E. G., Smith, A. H., Sundset, M. A., Mackie, R. I., 2008. The *BaeSR* two component regulatory system mediates resistance to condensed tannins in *Escherichia coli*. Appl. Environ. Microbiol. 74, 535-539.

Zrybko, C. L., Fukuda, E. K., Rosen, R. T. 1997. Determination of glucosinolates in domestic and wild mustard by high-performance liquid chromatography with confirmation by electrospray mass spectrometry and photodiode-array detection. J. Chromatogr. A. 767, 43-52.

Zsolnai, T. 1966. Antimicrobial activity of potential isothiocyanate producers. Arzei-Forschung, 16, 1092-1099.