Identification and Optimization of the Antagonistic Potential of Native Spinach Microbiota towards *Escherichia coli* O157:H7

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Abstract

Leafy greens such as spinach have been the object of several recent food-borne pathogen outbreaks. The purpose of this study was to isolate bacteria spinach epiphytic bacteria that inhibit growth of *E. coli* O157:H7, which we describe as antagonism. The mechanism of antagonism was investigated and we attempted to improve the antagonistic potential *in vitro* and on spinach leaves when cellobiose, a carbon source utilized by the antagonists but not *E. coli* O157:H7, was added.

There were larger culturable populations of bacteria on the leaves of savoyed cultivars compared to flat. From the isolated colonies, 47 displayed antagonism towards *E.coli* O157:H7, and were identified as members of 11 different genera and sixteen species. A representative isolate from each species was evaluated for three possible mechanisms of antagonism: acid production, secretion of an inhibitory compounds or secreted protease. The majority (14/16) produced at least a moderate level of acid. Two of these strains, *Paenibacillus polymyxa* and *Pseudomonas espejiana*, were found to secrete a non- protease antagonistic compound.

These antagonists varied in their reduction of *E.coli* O157:H7 numbers *in vitro,* but all significantly reduced numbers in 48 hours of co-culturing in nutrient rich media. Five antagonists resulted in a significant reduction in *E.coli* O157:H7 populations when co-cultured on spinach leaves. Application of cellobiose did not improve the amount of antagonism *in vitro* or on the leaf surface after 24 hours.

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Chapter 1

Introduction and Justification

Recent years have seen an increase in the number of food-borne pathogen outbreaks associated with fresh produce such as leafy greens (5). This is partially a result of Americans consuming increased amounts of fresh, minimally processed produce (4). In many cases, consumers eat the produce without further preparation, such as cooking, that could kill any harmful microorganisms. Therefore, if a food-borne pathogen is present on the produce at the time that it is purchased, there is a high probability, depending upon the concentration of the pathogen and how the produce is prepared, that the consumer who purchased it will become ill (6). One notable recent produce outbreak of *Escherichia coli* O157:H7 was traced back to fresh, minimally processed spinach in 2006 (3, 4).

Spinach leaves are populated by epiphytic microbiota including bacteria, fungi and yeast (59). When a food-borne pathogen such as *E.coli* O157:H7 is introduced onto a spinach leaf, it can interact with the epiphytic microbiota that is already present on the leaf (7). In some cases, it is proposed that the epiphytic microbiota can improve the epiphytic fitness of the food-borne pathogen, such as through the formation of a biofilm (7). In other cases, the epiphytic microbiota can prevent the growth and/or establishment of the food-borne pathogens, through competition for resources or secreting an antagonistic compound (2).

This project identifies native bacteria which prevent the growth of *E.coli* O157:H7, and seeks to identify the mechanism used to inhibit growth, such as the production of acid or a secreted molecule, which could be a protease. Growth inhibition of *E.coli* will be referred to as antagonism throughout this document. The study also examined if the addition of a carbon

source, cellobiose, that could be metabolized by the antagonistic isolates but not by *E.coli* O157:H7 reduced the growth of the pathogen while increasing the growth of the isolated antagonists. This information could be useful in development of pre or post harvest intervention for preventing the growth of *E.coli* O157:H7 on produce. This may decrease the number of outbreaks that occur as a result of the pathogen, thereby increasing product safety and consumer confidence in the product.

Objectives and Hypothesis

- **Objective 1: Isolate bacteria from spinach leaves which inhibit growth of *E.coli* O157:H7*in vitro***

  Hypothesis: After isolating bacteria from the surface of spinach leaves, some will exhibit the potential to inhibit the growth of *E.coli* O157:H7*in vitro*

- **Objective 2: Determine if a secreted metabolite is the mechanism through which spinach bacterial isolates display antagonism and inhibit the growth of *E.coli* O157:H7 *in vitro*.**

  Hypothesis: After screening all of the spinach isolates that have displayed antagonism towards *E.coli* O157:H7, at least one will produce a secreted molecule responsible for preventing *E.coli* O157:H7 from growing *in vitro*.

- **Objective 3: Determine if excess acid byproducts of metabolism of select epiphytic bacteria results in growth inhibition of *E.coli* O157:H7 *in vitro*. The majority of bacteria produce acid as an end-product of various metabolic pathways. Therefore, acid production is a likely mechanism of antagonism.**

  Hypothesis: The majority of spinach isolates that display antagonism towards *E.coli* O157:H7 will produce excessive acid as a metabolic byproduct.

- **Objective 4: Characterize the growth rate of both antagonistic spinach isolates and *E.coli* O157:H7 *in vitro* in the presence of cellobiose as the sole carbon source.**

1st Hypothesis: Based on literature studies, *E.coli* O157:H7 will not be able to utilize cellobiose, therefore no growth will be observed.

2nd Hypothesis: Other bacteria which are known to inhabit the phyllosphere including members of the *Enterobacteriacae and Pseudomonas* will be able to use cellobiose as a sole carbon source for growth *in vitro*

- **Objective 5: Identify if the growth of spinach epiphytic bacteria on cellobiose as the sole carbon source *in vitro* has either a synergestic or antagonistic effect on the growth of *E.coli* O157:H7.**

1st Hypothesis: It is unknown if bacteria previously described as antagonists on a complex carbon source will also prevent the growth of *E.coli* O157:H7 when cellobiose is the sole carbon source.

- **Objective 6: Determine if the application of a cellobiose dip onto a spinach leaf increases the population of the antagonistic bacteria either found naturally on the leaf or additionally added.**

Hypothesis: The application of cellobiose in a dip onto a spinach leaf may decrease the population of *E.coli* O157:H7 that was additionally added onto the leaf along with one of the isolated antagonists.

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Chapter 2

Literature Review

Food-borne pathogenic outbreaks on fresh produce

It is estimated that approximately 76 million people become sick and 5,000 people die from food-borne pathogens or related illnesses annually (14). Although most people associate food-borne pathogenic outbreaks with improperly cooked meat, the United States Food and Drug Administration (FDA) reports that in the late 1990s an average of 16 food-borne outbreaks have been attributed to a produce vehicle each year (14). This number has increased over the past four decades, where food-borne pathogenic outbreaks on produce consisted of less than 1% of all total outbreaks in the 1970s, but increased to 6% by the end of the 1990s (59). In recent years, food-borne outbreaks attributed to fresh produce have included *Salmonella* on peppers and tomatoes and *E.coli* O157:H7 on spinach leaves (29). In the United States, leafy greens including spinach account for around 30% of these food-borne outbreaks attributed to produce (41). Between 1996-2006, consumption of leafy greens increased by 9%, while food-borne outbreaks on leafy greens increased by 39% (41). Outbreaks of food-borne disease can have a significant impact upon agriculture, health care and business sectors. It is estimated by the United States Department of Agriculture (USDA) Economic Research Service that food-borne pathogen outbreaks result in ~17.1 million dollars in health care costs (74). This number however, does not include the cases that are not reported or diagnosed as being caused by a food-borne pathogen. Along with the costs of health care, food-borne pathogen outbreaks also can have a large economic impact on producers, packagers and retailers. It is estimated that California producers and processors lost 74 million dollars as a result of the 2006 spinach associated *E.coli* O157:H7

outbreak (61). In addition, a food-borne outbreak often creates a negative product image, in which a consumer associates a product with the outbreak and is less likely to purchase it (12, 48). Therefore, it is important to examine the reason behind these outbreaks and identify steps that can be taken to help reduce the probability of them occurring in the future.

As a result of the recent trend in the United States towards healthier eating, Americans are consuming a greater amount of fresh, minimally processed produce. Due to the increase in demand of fresh produce, many types of produce are grown in areas that can support year round production and then the produce is shipped to other areas where it is no longer in season (40). This allows for a potential wider distribution of contaminated produce, since in many cases produce that is grown in one area is shipped across the country or worldwide (68). Along with this, it has become common to have animals located in close proximity to agricultural fields. This increases the probability that a pathogen residing in the animal as a host could come into contact with the produce, either directly, such as contaminated manure, or indirectly, such as in irrigation water contaminated with feces (64). This is problematic because in many cases consumers eat the fresh produce without further cleaning or preparation, relying upon the fact that the fresh produce has already been washed and bagged. Therefore, it is important to explore new ways that food-borne outbreaks on fresh produce can be prevented.

Background on the vegetable crop spinach

Spinacia oleracea is the scientific name for spinach, a leafy green vegetable that is a cool weather crop able to survive frosts and light freezes. Although spinach can be grown in warmer climates, the increased temperature can cause it to produce a seed stalk, or bolt, earlier than cool temperatures can, a problem since spinach that has bolted is not considered to be sellable (19).

The United States is the second largest producer of spinach in the world, producing around 3 percent of the total spinach produced globally. Domestically, California is the largest producing state, producing about 73% of the domestic spinach crop, and Arizona and New Jersey are the second and third highest producers (40). Spinach is usually harvested at around 45 days and from there can either be preserved, such as through canning or freezing, or stored in cool, humid conditions so that it can be sold as fresh produce on the market (40). Fresh spinach can lose nutritional components only a few days after harvest, so fresh spinach must either be consumed soon after harvest or stored in conditions, such as modified atmosphere, that reduces the nutritional loss (40). In regards to food-borne pathogens, it is important to understand the respiration rate of spinach because excess respiration can cause spinach leaves to deteriorate in quality and secrete excess nutrients, a condition that makes the leaves more suitable for pathogenic growth (40). As a result, it is important when studying the ability of *E.coli* O157:H7 to survive and grown on spinach leaves to make sure that the rate of respiration for spinach leaves is kept low so that the conditions are not made more suitable for the survival of *E.coli* O157:H7.

Although canned and frozen spinach used to be the primary type of spinach that was consumed, recent years have seen an increase in the fresh minimally processed spinach. In 2007, around 75% of spinach consumed in the United States was fresh, a level of fresh spinach consumption that has not been seen since the 1940s (Figure 1) (40). The increase in consumption of fresh spinach is attributed to the creation of pre-washed bagged spinach that make fresh spinach consumption more convenient and an increased awareness about healthy eating among the American public. The average value of the United States spinach crop has been around 175 million dollars, making it an important crop to the farm industry (40).

Spinach is divided into three main cultivars that are characterized by different appearance and color. Savoy spinach consists of cultivars that are deep green in color and contain deep wrinkles all over the spinach leaves. Savoy cultivars tend to produce larger leaves, and due to the deep wrinkles are not preferred for processing or fresh minimally processed spinach since the wrinkles make it difficult to effectively clean the spinach. However, due to the leaf's vein having a longer period of growth, savoy spinach is considered to have larger surface area and more nutritional components than the other cultivars of spinach (26). This type of spinach is most commonly sold in high end salad mixes and as a novelty produce item. Types of savoy spinach include Bloomsdale Long Standing, Regiment and Menorca (19). The second type of spinach is the flat leaf spinach. This type has flat leaves with few wrinkles and crevices on them, making them ideal for use in processed spinach such as frozen or canned. Also, flat leaf spinach that is harvested early is often marketed under the name of baby spinach, which has increased in popularity due to it being more tender and having a milder flavor than mature spinach leaves (26). Types of spinach that are flat leaf cultivars include Springfield, Avenger and Monza (19). The third type of spinach is semi-savoy, which is a hybrid formed by crossing savoy and flat leaf spinach. Semi-savoy spinach is lighter in green color and has wrinkles, but not as deep as the ones in the savoy cultivar. Semi-savoy spinach is often used in order to have a product that has a similar texture as savoy, but is easier to clean. Examples of semi-savoy spinach cultivars are Correnta, Cherokee and Unipack (19). The difference in spinach cultivars is not only important for spinach processing however, with differences in cultivars also potentially having an impact upon the composition of the epiphytic microbiota on the spinach leaves. These variations can be due to differences in the surface area and structure of the spinach leaves (72). Lopez-Velasco *et al.* has shown that the savoy cultivar Menorca has a significantly larger surface area and more

stomata when compared to Monza, a flat leaf cultivar. The increased surface area of the savoy cultivar led to the leaf being more wrinkled with deeper ridges, producing both more space for the epiphytic bacteria to colonize and areas of the leaf where water could collect (87). The increased number of stomata present in the savoy cultivars could also impact the epiphytic microbial population, since stomata have been shown to secrete both water and nutrients onto the surface of the plant (10). As a result, the different spinach cultivars could potentially have different amounts and compositions of epiphytic microbiota (19).

Various factors could affect the nutritional components of the spinach leaf exudates including the age of the leaf, the environmental conditions that the spinach was grown in and if the leaf tissue of the spinach had been damaged. Older spinach leaves have a greater concentration of nutrients, such as amino acid and sugars, than younger leaves (62). The presence of food-borne pathogens on different ages of leaves has also been shown to impact the amount of damage that occurs on the leaf. Older leaves inoculated with a pathogen have been shown to be more susceptible to damage than younger leaves that have been inoculated with a pathogen (34). This is important for the ability of food-borne pathogens to survive on the surface of spinach leaves since an increase in the nutrient levels of the exudates, as occurs in the older plants, would be more beneficial to the growth of the food-borne pathogen on the surface of the leaf than on the younger leaves, which have fewer nutrients in their exudates.

One food-borne pathogen that is increasingly associated with fresh produce such as leafy greens is *E.coli* O157:H7, a food-borne pathogen that until recent years was most commonly associated with undercooked meat products. It was first associated with an outbreak in hamburger sold at Jack in the Box restaurants located in Washington state in 1993 (13). However, outbreaks of *E.coli* O157:H7 have also been associated with fresh produce including

spinach, lettuce and unpasteurized apple cider (16).One of the most publicized recent produce borne outbreaks was attributed to baby spinach distributed by Dole. Although the source of contamination on the spinach was never determined, it is believed to have been the result of either irrigation water that had been contaminated with wild pig or cattle feces or the presence of wild pigs in the field (35). The outbreak resulted in 205 reported cases of *E.coli* O157:H7 illness, with 31 people developing hemolytic uremic syndrome resulting in three deaths (35). This outbreak of *E.coli* O157:H7 occurred despite the fact that the conditions found on produce, such as varying temperatures, amount of water and UV light, are not consistent with the conditions that favor the growth of *E.coli* O157:H7 (3). Despite these limitations, *E.coli* O157:H7 is still able to survive on the produce for a long enough period of time to make people sick. One possible explanation for this is that the *E.coli* O157:H7 is interacting with the epiphytic microbiota that is found on plant such as spinach leaves.

The human food-borne pathogen *E. coli* O157:H7

Escherichia coli is a Gram negative bacterium that is adapted for life in the small and large intestines of animals. One serotype of *E.coli* that is commonly associated with food-borne disease is *E.coli* O157:H7, which produces a toxin know as a vero cytotoxin (11). Although *E. coli* O157:H7 can be transmitted through other routes, 61% of cases involving *E.coli* O157:H7 have food as the method of transmission (Figure 2) (53). It has been proposed by scientists that the infectious dose of *E.coli* O157:H7 is only between 10 to 100 cells (51). *E.coli* O157:H7 can produce severe gastrointestinal distress in people, with symptoms including bloody diarrhea, vomiting and abdominal cramps. In addition, between 5-10% of people who become ill with *E.coli* O157:H7 develop hemolytic uremic syndrome (HUS) as a result of the vero cytotoxin, which can result in kidney failure and death (14). The population that is at the greatest risk for

developing HUS after they have contracted *E.coli* O157:H7 are young children and the elderly (14). In the United States, it is predicted that around 73,000 people become ill from *E.coli* O157:H7 every year, with the number dramatically increasing since the 1990s (53). The first reported case of *E.coli* O157:H7 occurred in 1982 and involved the consumption of contaminated hamburger and the first report of an *E.coli* O157:H7 outbreak involving produce occurred in 1991 (53, 54). Since then, the number of *E.coli* O157:H7 outbreaks occurring on produce has increased to 34% of all food-borne outbreaks of *E.coli* O157:H7 (Figure 3) (53).

Although food-borne pathogenic outbreaks on produce are increasing, environmental conditions on produce are not optimal for the growth of food-borne pathogens. *E.coli* O157:H7 is commonly associated with the gastrointestinal tract of mammals where they have adapted to increased temperature of around 37-40˚C and a rich supply of nutrients (3). In the case of *E.coli* O157:H7, the host animal is usually live stock (cattle), wild animals (deer or wild pigs), or humans (14). On the surface of a leafy green such as spinach, *E.coli* O157:H7 is not presented with the same constant environment. Instead, it has to deal with dramatic changes in environmental conditions such as varying temperatures, availability of water and presence of damaging UV radiation.

Preharvest contamination of leafy greens

Contamination of the produce can occur at several different stages in production and subsequent processing. In some cases, produce becomes contaminated while it is still growing in the field. This can occur by irrigating the crops with water that has been contaminated with the feces of nearby livestock, raw sewage or wild animals that are shedding *E.coli* O157:H7 (6). Surface water should not be used for irrigation of produce since it can easily become

contaminated with a food-borne pathogen (73). Instead, irrigation is supposed to occur with ground or municipal water, which is generally considered to be safer and less likely to have a food-borne pathogen. However, ground and municipal water can still become contaminated. This could occur if the water used to recharge the well is from a poorly managed and allowed to come into contact with sources of contamination, such as livestock or wild animals (27). Transmission of *E.coli* O157:H7 through irrigation water was replicated in a study by Johannessen *et. al*.,with crisp head lettuce. It was shown that *E.coli* O157:H7 remained in reduced, but significant number in the soil up to eight weeks after inoculation (36).

Contamination may occur if the produce came into direct contact with the feces of an infected animal, if for example livestock or a wild animal infected with *E.coli* O157:H7 got into the field where the produce was being grown (4). Produce contact with contaminated feces could also occur if the fields were fertilized with improperly composted animal manure. Islam *et. al.* showed that onions and carrots that had been fertilized with bovine or poultry manure inoculated with *E.coli* O157:H7 became contaminated with the pathogen (33). *E.coli* O157:H7 was found to survive in the soil fertilized with all of the manure for at least 154 days. In soil fertilized with manure inoculated with 7.0 $\log_{10}$ CFU/g *E.coli* O157:H7, there was a retention of 1.5–2.0 $\log_{10}$ CFU/g on the carrots on day 126, when carrots are usually harvested. *E.coli* O157:H7 was able to on the onions survive for 74 days but were not detected on day 126, the time period when onions are usually harvested. (33). Although all animal manure that is used to fertilize produce fields is required to be treated prior to application, such as by being stored and composted for at least six months, outbreaks of food-borne pathogens attributed to contaminated manure have occurred. One such outbreak occurred in Ontario Canada in 2000, where improperly treated cattle manure that was contaminated with *E.coli* O157:H7 entered into a

municipal water supply and resulted in over 1,300 reported cases of illness (27). Soil infected with *E.coli* O157:H7 can also lead to produce becoming contaminated with the food-borne pathogen. It has been shown by Warriner *et al.* that uninfected spinach seedlings that were transferred to soil inoculated with *E.coli* O157:H7 had the food-borne pathogen present on both the surface and internalized in the leaves after 42 days (79). Also, it has been proposed that insects such as house flies could potentially play a role in spreading *E. coli* O157:H7 (1). A study by Janisiewicz *et al.* showed that fruit flies that had been inoculated with a GFP tagged strain of *E.coli* O157:H7 were able to spread the *E.coli* O157:H7 to apples that contained lesions on their skin (34).

Post-harvest contamination of leafy greens

After harvest, leafy greens can become contaminated at the processing facility as they are being prepared for sale (85). Contamination can occur at this stage through contact with machinery that has not been properly sanitized or workers that are not following proper handling or sanitation regulations (6). Contamination of leafy greens during processing is of special importance due to the fact that 80% of outbreaks involving *E.coli* O157:H7 on leafy greens have been traced to processed, bagged salad mixes (50). During the processing of products like bagged salads, leafy greens from multiple fields are mixed together, resulting in cross contamination of other leaves, thereby effectively spreading out the number of bags that can contain the contaminated produce (6). Companies producing minimally processed bagged leafy greens do take measures to help prevent food-borne pathogenic outbreaks on the product by employing practices such as triple washing the leafy greens and using chlorinated water. However, chlorinated water is not able to completely kill *E.coli* O157:H7, which is able to internalize within cut leaves after they have been cut, although there is a reduction in its numbers

(67). The washing step is therefore more useful in removing dirt and insects that could be on the leaves and not killing food-borne pathogens. Along with this, using disinfectors on produce may cause more harm than good. Instead of killing off the food-borne pathogen, the disinfectant also kills off the native microbiota on the leaf, thereby removing microorganism that could potentially compete with the pathogen (25).

The storage conditions of fresh produce is another area that can impact the ability of food-borne pathogens to survive after the produce is harvested and until it is sold to the consumer. Following harvest, leafy greens continue to respire, a process through which organic material stored within the plant is broken down into energy, thereby damaging the leaf and reducing the shelf life of the item. Since damaged leaves are more prone to colonization by food-borne pathogens, it is important maximize the quality and shelf life of a product (40). This can be done by reducing the respiration of the produce to 6.8 mg kg^{-1} h^{-1}, in comparison to 242.0 mg kg^{-1} h^{-1}, the normal respiration rate of spinach growing in the field (52). Spinach is considered to have a very high respiration rate, and as a result must be cooled immediately after harvest (52). Following harvest, spinach is often stored in modified atmosphere packaging that consists of a high amount of carbon dioxide and low levels of oxygen in order to reduce respiration.

Finally, leafy greens can become contaminated after they have left the processing facility in the store, consumer's home or restaurant where they are consumed. In these cases, contamination is usually the result of the produce coming into contact with the food-borne pathogen due to improper sanitation or cross contamination, such as placing the leafy greens on a surface that raw meat came into contact with or a person handling the leafy green without washing their hands (80). For the food safety industry, various methods have been utilized to

help prevent outbreaks of food-borne diseases including using Good Agricultural Practices, Good Manufacturing Practices, disinfectants and irradiation.

Epiphytic microbiota on leafy greens

One of the areas that is currently being examined as an intervention strategy is the use of competition between epiphytic microbiota and food-borne pathogens. Epiphytic microbiota are microorganism that have naturally found a niche on the surface of the leaves. Known as the phyllosphere, this is the area where the waxy cuticle of the plant is in contact with both the environment and the surface of the leaf (5). In order to survive in this area, the microbiota have undergone adaptations in order to survive the harsh conditions that are present on the leaves including changes in available water, temperature and UV light (31). The population of culturable epiphytic microbiota on the surface of leaves range from 10^5 to 10^7 CFU/g (86). The majority of culturable epiphytic microbiota are Gram-negative bacteria, belonging to the genera *Erwinia, Pseudomonas, Enterobacter* and *Bacillus* (66). Although there can be upwards of 34 different genera of culturable bacteria found in the phyllosphere, the majority of these tend to consist of a few genera (69). In a previous study, it was found that out of the bacterial strains identified on surface of the beet leaves, over 41% belonged to either the genera *Erwinia* or *Pseudomonas* (69).

Despite the large number of different species that can be found on the leaf's surface, the number is actually small in comparison to the number of species found in the soil. A previous study examining the bacteria found on the surface of produce and the soil surrounding it found that while on average there where thirteen culturable bacteria species found on the surface of leaves, there were over 40 culturable species of bacteria present in the surrounding soil (69).

These results are in accordance with the information available on the type and amount of nutrients that are present in both the soil and on leaf surfaces, with there being generally a greater number of nutrients and in greater concentration in the soil than on the leaf's surface. Microbes are not distributed evenly across the leaf surface. Bacteria, including human pathogens, tend to congregate around the leaf's veins, hydathodes, stomata and trichomes (9, 10). *E.coli* O57:H7 inoculated onto leaf surfaces achieves larger populations in areas surrounding the veins found on the leaves of the Arabidopsis plant (9). This occurs because these are the areas on the leaves where the majority of the molecules are secreted. It would therefore be more beneficial for the epiphytic microbiota and any food-borne pathogens present on the leaf to be located as close as possible to the location where the carbon source was being secreted. (5).

Epiphytic bacteria require carbon, nitrogen and inorganic molecules in order to survive on the phyllosphere(44). Plants secrete sugars, amino acids and salts into the phyllopshere (44). The type and composition of the secreted molecules onto the leaf surface varies based on the type of plant, the growing season and the environmental conditions (37). Environmental conditions can have an impact upon not only the plant's ability to grow but the composition and density of the epiphytic microbiota. It has been found that there can be a 10-fold difference in microbial composition between different leaves of the same type of plant that are grown under the same environmental conditions (37). However, even in conditions where the plant is secreting the maximum amount of molecules onto the leaf's surface, there will still be competition between the epiphytic microbiota for the molecules that are necessary for growth. This competition between microbiota for carbon sources and other molecules is one explanation for the variety in both number and diversity of microbiota that are found on the leaf's surface (37). In bean plants the majority of the secreted molecules consisted of fructose, sucrose and

glucose (44). This is of special importance in regards to the survival of *E.coli* O157:H7 on the leaf's surface since the pathogen is able to readily utilize all three of the carbon sources as a source of energy (44). However, it was also noted that even on leaf surfaces that had high populations of microbiota, there were still carbon sources present on the surface of the leaf that could not be utilized (44). The presence of unused secreted molecules could be the result of the microbiota not have the proper mechanisms to utilize the carbon sources or because the structure of the plant prevented the microbiota from reaching the molecules (44).

Carbon sources alone do not determine the population of epiphytic microbiota present on the leaf. The age of the leaf can have an impact upon the amount of nutrients that are secreted on the leaf and the ability of the pathogen to survive on the leaf as well. It has been found that on Romaine leaf lettuce inoculated with *E.coli* O157:H7 the population size of the food-borne pathogen is 10-fold higher on the younger leaves than it was on the older leaves. This difference in population size is due, at least in part, to larger concentrations of nitrogen on young leaves in comparison to older and middle aged leaves (9).

The physical condition of the plant leaf can impact the ability of a food-borne pathogen to survive. Tears or plant lesions may supply food-borne pathogens with an entry into the plant, which is more hospitable for growth than the intact surface of the leaf (45). Damaged areas of leaf tissue may also provide higher levels of nutrients that could promote the growth of food-borne pathogens (45). It is therefore important that when the leafy produce is being harvested and processed that it remain as undamaged as possible, since the presence of damaged leaves can help support the survival and growth of food-borne pathogens.

In addition to the previously discussed plant controlled factors, there are additional external factors that affect the diversity and richness of the phyllosphere bacterial community. A study conducted on sugar beets found that the greatest amount of diversity in the microbiota in the phyllosphere occurred during the autumn in a Mediterranean climate, in contrast the lowest diversity was observed in winter and during mid-summer, the periods of the year that generally have the lowest amount of precipitation in this climate (69). Other studies on bean leaves have shown decreases in diversity were associated with lower availability of water (30).

The interaction between the phyllosphere of the leaf's surface and the microbiota that reside there is not one way. Epiphytic bacteria alter the surface of the leaf, altering the water permeability of leaf's surface. This is important because the permeability of the phyllosphere affects the type and concentration of molecules that are secreted onto the leaf's surface, thus impacting what microbiota can survive on the leaf's surface (58). The alteration of the phyllosphere by microbiota can also be seen in the ability of the bacteria *Pseudomonas aeruginosa* and others to produces excess salicylic acid as it grows on the leaf. This excess acid is not just a byproduct of its metabolism, but also a method through which it prevents the growth of *Botrytis cinere,* a plant pathogen (21). Altering the conditions on the leaf is not just a practice that is restricted to epiphytic microbiota however. Food-borne pathogens such as *E.coli* O157:H7 were also shown to increase the permeability of the leaf's membranes, allowing for more nutrients to leach from the plant onto the leaf's surface (58).

Interactions between epiphytic microbiota and food-borne pathogens

Microbiota found in the phyllosphere serve important functions for the health of the plant by impacting the conditions on the surface of the leaves. Along with playing an important role in

the health of the plant, the epiphytic microbiota could potentially interact with food-borne pathogens (80). In some circumstances, the epiphytic microbiota can create antagonism, a process in which the growth of one bacteria is inhibited by another bacteriums's growth or production of an antimicrobial compound. The ability of epiphytic microbiota found on spinach leaves to display antagonism towards a food-borne pathogen could be the result of several different factors. In some cases, the displayed antagonism is the result of the epiphytic microbiota being able to simply out compete the *E.coli* O157:H7 on the leaf's surface for nutrients and surface areas. The epiphytic microbiota has evolved for survival on the leaf's surface and the harsh environmental conditions that accompany it. Therefore, in some cases these adaptations allow the native microbiota to be more competitive in obtaining nutrients than pathogens like *E.coli* O157:H7 (74). This can be seen with the bacterium *Pseudomonas,* which has been found to be able to out compete plant pathogenic species of *Fusarium* and *Pythium* for iron. This ability of *Pseudomonas* is especially useful since various species of *Fusarium* and *Pythium* can either be toxic to humans or damaging to plants (82). In other cases, the antagonism is the result of the epiphytic microbiota secreting a compound that prevents the growth of the pathogen. This can be seen in the case of a strain of *Bacillus subtillus* IFS-01. *In vitro* studies indicate antagonism towards food-borne pathogens *Listeria monocytogenes* and *Staphylococcus aureus* through a secreted antimicrobial compound (24). These antagonistic microbiota are seen as a potential method through which food-borne pathogens could be prevented from colonizing the leaf. Used as a natural bio-control method, antagonistic microbiota could be applied either in the field or post harvest as a spray or a dip that could make it more difficult for a food-borne pathogen to colonize the leaf (25). However, when studying these antagonists it is important to examine if increasing their number would have a detrimental effect upon the shelf life of the

product, such as the bacteria producing acid, which has a negative effect on the flavor and appearance of the spinach leaves (6).

Methods of potentially improving antagonism towards *E.coli* O157:H7

Potentially microorganisms or microbial compounds found to be antagonistic to *E.coli* O157:H7 could be added to leaves to prevent the growth of *E.coli* O157:H7. Along with this, compounds could be added to the spinach that improves the growing conditions for the antagonistic microorganisms over the *E.coli* O157:H7. One method of changing the conditions on the leaves could consist of adding to the leaf a carbon source that can be used by the antagonistic microorganism found on the leaf, but not by *E.coli* O157:H7. One potential carbon source of interest is cellobiose, a reducing disaccharide that is naturally found on many different types of plants and fungi (20). Cellobiose is derived from the breakdown of cellulose, one of the most common organic compounds. Commonly found in the cell walls of plants, cellulose is made up of multiple cellobiose units that are joined together (15). Cellobiose is formed in a catalyzed reaction in the breakdown of cellulose, when cellulose in hydrolyzed by bacteria and broken down into cellobiose. Like cellulose, cellobiose can be found in many types of plant and plant products such as paper and cotton (60). Cellobiose is commonly found in the environment in several types of wood and various species of fungi, such as *Phanerochaete chrysosporium* (76). Research has shown that cellobiose is commonly found in apples and products that are made from processed apples, such as apple juice or apple sauce (43). As a result of being naturally found in plants, cellobiose is considered to be generally recognized as safe (GRAS) and therefore able to be used in food processing, such as the preparation of fresh produce (43).

Since it is a disaccharide, cellobiose can be degraded either through phosphorylation or hydrolysis, resulting in glucose molecules (60). The amount of glucose molecules that are produced can differ, ranging from trace to substantial amounts, and vary by the bacteria and the method through which cellobiose is metabolized (60). Research has shown that *E.coli* O157:H7 is not able to metabolize cellobiose as a carbon source (4). However, many of the microbiota found on spinach leaves such as those belonging to *Enterobacteriacae, Bacillus* and *Pseudomonas* are reported to be able to metabolize it as a carbon source. In the case of *Bacillus subtillus*, the bacterium is able to utilize cellobiose and produces the byproduct L-lactate, which has been shown to display an antimicrobial effect towards *E.coli* O157:H7 (55).Therefore, it becomes important to figure out a way to add an additional amount of a compound such as cellobiose that could increase the growth of antagonists on the spinach leaf.

One potential way of adding additional compounds to produce is dissolving it into a liquid and then either spraying it onto the leaf or dipping the leaf into the solution. A very common application of this method in the produce processing industry is using a dip or a spray to apply sanitizers such as chlorine to produce while it is being processed. The addition of a dip or spay has also been used to add compounds other than sanitizers as well, such as compounds that can help increase the shelf life and quality of the product. This can be seen in the case of sliced fresh pears, where it was found that the addition of 4-hexylresorcinol, ascorbic acid and calcium lactate in dip form can prevent the pears from turning brown and increase their shelf life (85).

The use of a spray or dip has also been examined as a potential way of adding a substance, such as additional concentration of a GRAS microorganism, onto fresh produce so that it can prevent the growth of a hazardous pathogen or fungi. This can be seen in the method

of controlling *Pennicillium expansum* on apples. Apples can be dipped into a solution containing *Pseudomonas* spp., which had been isolated from the apples and found to display antagonism towards *Pennicillium expansum.* The apples dipped into the antagonist inoculum were found to have a reduced amount of lesions on the apple surface associated with *Pennicillium expansum* (23). Another example can be seen in *Burkholderia cepacia* that was isolated from the microbiota of the banana. When bananas were dipped into an inoculum consisting of the bacteria, it was found prevent the growth pathogens crown rot and anthracnose (70). Along with this, if the inoculum also includes a carbon or nitrogen source that can be utilized by the antagonists but not the food-borne pathogen, it increases the probability that the antagonist may be able to out compete the pathogen on the surface of the produce (31). Therefore, the addition in the form of a dip of antagonistic bacteria and chemical compounds that could help increase the antagonists growth have been shown to be a useful method of post-harvest control of food-borne pathogens. However, in order to be able to utilize this method, the *E.coli* O157:H7 and isolated antagonists must be tagged in some form so that their presence and concentration can be determined.

Use of the green fluorescent protein as a marker

One fluorescent marker that is commonly used with *E.coli* O157: H7 is green fluorescent protein (GFP). The marker GFP is a protein that was isolated from the *Aequorea victoria* species of jellyfish (71). In the case of *E.coli* O157:H7, the GFP marker can be transformed into the cell in the form of a plasmid. This plasmid then causes the *E.coli* O157:H7 to fluoresce green under UV light, allowing it to be observed and counted using microscopy. To transform *E.coli* O157:H7 with a GFP marker, the protein must by imbedded into a plasmid vectors that can be transformed into the *E.coli* O157:H7 cell (71). *E.coli* O157:H7 is considered to be an ideal

bacteria to be tagged with a GFP since it will readily uptake the marker. It has been shown that strains of *E.coli* O157:H7 that have been transformed with GFP exhibit the same characteristics as non-transformed strains (77). Also, a previous study by Ajjarapu and Shelef has shown that the GFP tagged *E.coli* O157:H7 is able to display fluorescence even in the presence of native microbiota (2). A study involving applying manure containing GFP tagged *E.coli* O157:H7 to lettuce plants showed that the *E.coil* O157:H7 was able to be transferred to the lettuce from the manure and that it retained the ability to fluoresce, thereby demonstrating that the microbiota of leafy greens do not prevent GFP tagged *E.coli* O157:H7 from fluorescing (78). Along with being used to tag *E.coli* O157:H7, GFP markers have also been shown to be successfully utilized in other bacteria as well.

Figures and Tables

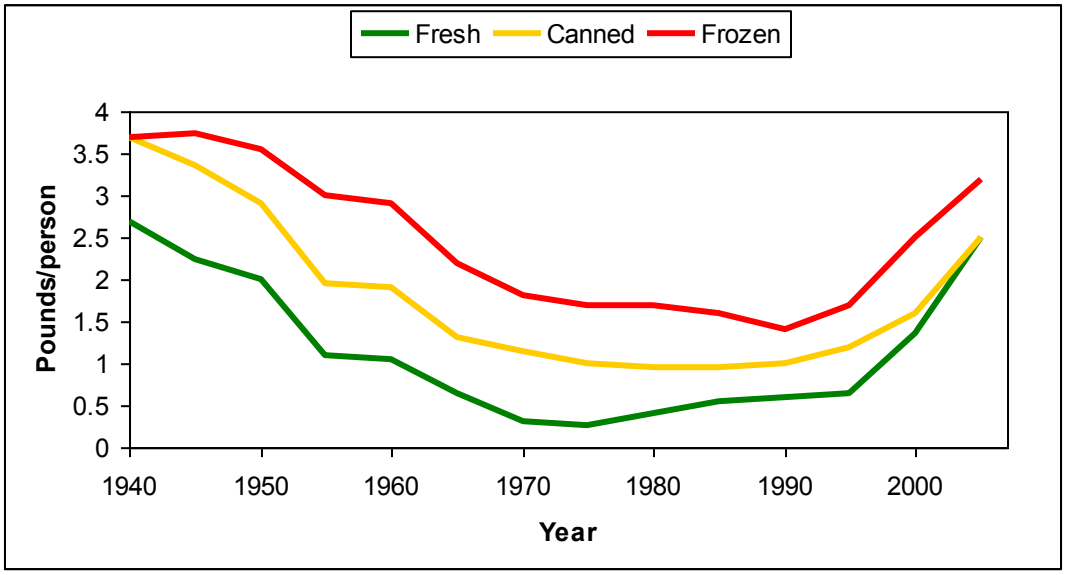

Figure 1: The annual per capita use of spinach grown in the United States between 1940 and 2007. Adapted from (Lucier, G., 2007)

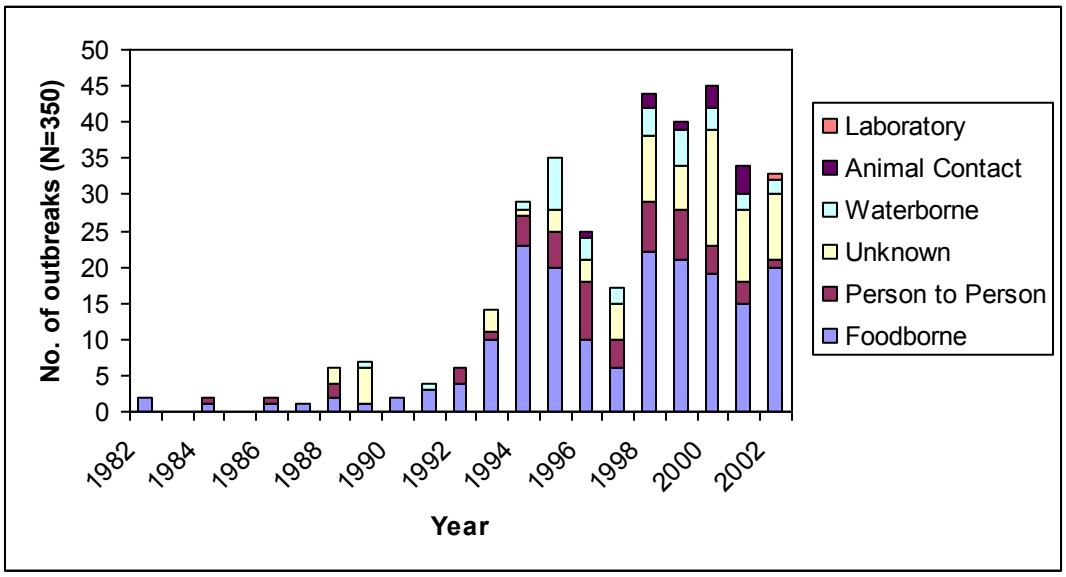

Figure 2. Sources of *Escherichia coli* O157:H7 outbreaks by year from between 1982 to 2002. Adapted from (Rangel, J.M *et al.,* 2005)

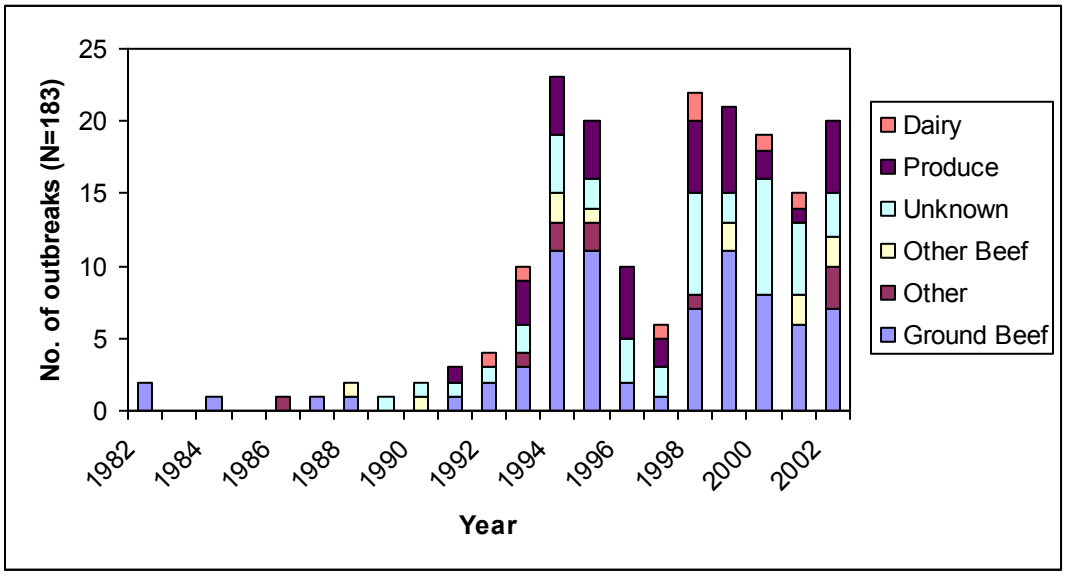

Figure 3. The Vehicles of Food-borne *Escherichia coli* O157:H7 Outbreaks by Year from 1982 to 2002. Adapted from (Rangel, J.M *et al.,* 2005)

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Chapter 3

Isolation and identification of epiphytic microorganisms isolated from spinach leaves which inhibit growth of *E.coli* O157:H7 *in vitro*

Introduction

Epiphytic microbiota that are naturally present on the surface of the leaves, have evolved to survive the harsh environmental conditions that are present on the surface of spinach leaves. These bacteria may compete for nutrients with food-borne pathogens that may lead to inhibition of the growth of food-borne pathogens, such as *E.coli* O157:H7. The purpose of this study was to isolate epiphytic microorganisms from three different spinach cultivars, Monza, Menorca and Unipack. The isolated microorganisms were then tested identify bacteria which inhibit the growth of *E.coli* O157:H7 *in vitro*, which is characterized as antagonism.

Materials and Methods:

Microbial Counts for Different Cultivars and Isolation of Bacteria from Spinach Leaves

In the spring 2008 spinach, cultivars Monza, Unipack and Menorca, were grown using conventional agricultural practices, as previously described (6). When plants reached a 6-8 leaf stage of maturity, the leaves were harvested and placed into sterile plastic bags. The spinach was transported in a cooler that had a temperature of around 10˚C. Within an hour of harvest, the leaves were rinsed with sterile water to remove any dirt and soil and microbial counts were determined. Microbes were removed from the leaf surface by combining 10g of spinach leaves

with 90ml of sterile peptone water that contained 1% Tween 80 in a sterile stomacher bag and then mixing it in a BagMixer (InterScience Labs) for five minutes. The solution was serially diluted and plated onto R2A, plate count agar (PCA) and potato dextrose agar (PDA). All plating was completed in triplicate. The plates were incubated for 16 days at 25˚C and counts were performed after 16 days.

Additional plates were also made using the same procedure and incubated at 25˚C and at 4˚C and the colonies were counted after days 2, 5, 7, 10 and 15 days of incubation. Plating for each temperature of incubation was completed in triplicate. At each interval, new colonies were transferred to an individual square in a 36 grid plate containing R2A. A total of 1,062 colonies were transferred.

Preparation of Inoculum for Antagonist Test and Screening of Isolates for Antagonism towards *E.coli* O157:H7

The inoculum used in the test for antagonism was prepared by inoculating 10mL of ½ strength tryptic soy broth (TSB) with 1mL of *E.coli* O157:H7 culture and incubating it at 35˚C for 24 hours. A 1mL aliquot of the culture was placed in a flask containing 100mL of sterile ½ TSB and incubated at 35˚C shaking at 120 RPM for 48 hours to achieve a density of 10^8 CFU/ml in stationary phase. The inoculum was then prepared by taking 25mL of the culture and placing it in a tube containing 25mL of ½ TSB, creating a final density of 10^4 CFU/ml.

One hundred microliters of the mixture was then spread plated onto ½ R2A and allowed to dry for 3 minutes in a Bio-safety cabinet to create a lawn of *E,coli* O157:H7. Isolated colonies were transferred with a sterile toothpick and transferred to a plate containing the lawn of *E.coli* O157:H7. A total of seven colonies were transferred to each plate, with each plate being

replicated in triplicate. The plates were incubated at 25˚C and checked every 24 hours for 72 hours for signs of antagonism, which was indicated by a clear halo surrounding the spinach bacterial isolate where the pathogen was not able to grow.

Identification of Isolated Antagonists

The bacteria that were determined to be antagonists were identified at the taxonomic level by extracting the bacterial DNA using the Puregene genomic DNA purification kit and amplifying the 16S rRNA gene. The amplified 16S rDNA was then sequenced at Virginia Bioinformatics Institute. Sequences were analyzed using the Ribosomal Database Project, version 10, by comparing the similarities of the sequences to the database in order to determine taxonomic identity (5).

Growth Curves of Antagonists and *E.coli* O157:H7

Growth curves were made for the identified antagonists and *E.coli* O157:H7 strain H1730 to determine each bacterium's growth rate individually. A GFP tagged strain of *E.coli* was utilized for this portion of the experiment. Tubes containing 9mL of ½ TSB were inoculated with either one of the antagonists or *E.coli* O157:H7 and incubated at 120 rpm at 25˚C to a density of OD_{600} corresponding to 10^8 cells/ml in stationary phase. In triplicate, 270µl of TSB was placed into each well of the 100 well Bioscreen Honeycomb Plates along with either 30µl of an individual antagonist or *E.coli* O157:H7. The lid was then placed onto the plate and it was incubated in a Microbiological Growth Reader Bioscreen C Version2.1.2 (Oy Growth Curves Ab Ltd) for 72 hours at 25˚C with measurements being taken every 15 minutes. The data was then graphed using Microsoft Excel to create growth curves for each antagonist and *E.coli* O157:H7. Growth rates and time in log phase was calculated for each antagonist and *E.coli* O157:H7

Results and Discussion

Total culturable bacterial populations ranged from 5.52 log CFU/g on Monza cultivar to over 6.5 log for the savoyed cultivars (Figure 1). Numbers of total culturable bacteria were significantly smaller for the flat cultivar, Monza in comparison to savoyed cultivars Menorca and Unipack (Figure 1). This may be due to differences in the surface area and/or structure of their leaves. As a flat leaf cultivar, Monza leaves have less surface area and the fewer ridges and crevices (6). By increasing the surface area the number of ridges and crevices present on the spinach leaves may increase, potentially providing protection from environmental factors such as UV light. Crevices may also collect nutrients and water, two factors that often limit a bacterium's growth on the phyllosphere (1, 3). Bacteria are often found in higher numbers around the stomata, veins and trichomes of leaves (7). Stomata can secrete water and nutrients that bacteria can utilize, offering a possible explanation for why bacterial populations are higher around them (4). However, it should be noted that the differences in surface area, leaf thickness and amount of crevices were not measured in this study.

Individual colonies with different colony morphologies and pigmentation were tested for antagonism towards *E.coli* O157:H7. Antagonism was defined as inhibition of the growth of *E.coli* O157:H7 surrounding the spinach bacterial isolate. Colonies were randomly chosen from each cultivar and screened for antagonism. A total of 49 isolates were identified that displayed antagonism toward *E.coli* O157:H7 (Table 3). These bacteria represent 7.4%, 1.8%, 1.4% of the randomly chosen bacteria isolated from Monza, Unipack and Menorca cultivars (Table 2). These bacteria belonged to fifteen different species. Two species, *Ps. sp.* and *Flav. sp.,* were isolated from two or more cultivars (Table 3). Bacteria belonging to these genera, specifically *Pseudomonas* and *Erwinia,* are frequently isolated from the surfaces of leaves (2,8,9). A study

conducted by Randazzo *et al.* with fresh salad mixes containing leafy greens such as endive, chicory and sugar loaf found that bacteria from the genera *Acintinobacter, Enterobacter, Erwinia, Pseudomonas* and *Pantoea* were commonly found on the surface of the leaves (8). Rudi *et al.* found high populations of *Pseudomonas* and *Enterobacteriaceae* in the microbial community of crisp head lettuce (9)

The growth rates, time in lag phase and number of hours before entering stationary phase were calculated for a representative isolate from each species (Table 4). Of the screened antagonists the majority entered log phase around the same time as *E.coli* O157:H7. However, three isolates entered log phase before *E.coli* O157:H7, suggesting they may use nutrients faster that *E.coli* O157:H7. In contrast, four bacteria took significantly longer to enter log phase (Table 4). The majority of antagonists grew at a significantly slower rate in comparison to *E.coli* O157:H7 (Table 4). The amount of time in log phase before entering stationary phase was significantly different from *E.coli* O157:H7 for all isolates except, *Erw. persicina, Ps. sp., Ent. sp*. and *Pan. ananatis.* Out of the sixteen different species, 4 of the isolated antagonists, *Erw. persicina, Ps. sp., Ent. sp*. and *Pan. ananatis,* overall had similar growth characteristics as *E.coli* O157:H7 *in vitro* (Appendix A). Five of the isolated antagonists, *Erw. pantoea, Flav. sp., Ps. espejiana, B. cereus* and *S. sp.*, had a growth curve that overall followed a similar trend, but was not identical to *E.coli* O157:H7 and 7 antagonists, *Brev. vesicularis, Paen. polymyxa, Micro. sp., Frigo. actinobacterium, B. pumilus, Acidovorax sp., S. maltophilia,* had an overall growth curve different from *E.coli* O157:H7. (Appendix A). These antagonists generally remained in lag phase for a longer period of time and did not have as steep of a log phase as *E.coli* O157:H7. Because of this, these antagonists did not start exponentially increasing as fast or at as high of a rate as *E.coli* O157:H7, allowing the *E.coli* O157:H7 to grow faster than the antagonists. This is

significant because it shows that not all of the antagonists have the same growth characteristics and that a majority of them display growth curves that are different from the one displayed by *E.coli* O157:H7. As a result of this, it can be proposed that since the growth curves by the antagonists are different, all of the antagonists do not display antagonism towards *E.coli* O157:H7 by the same mechanism. For example, the antagonists such as the *Ent. sp.* that have growth curves very similar to the one displayed by *E.coli* O157:H7 might create antagonism by out competing *E.coli* O157:H7 for resources such as carbon sources (Appendix A). Antagonists such as *Frigo. actinobacterium* have growth curves different from *E.coli* O157:H7 by remaining in stationary phase for longer and not having as steep of a log phase (Appendix A). These antagonists therefore are probably not able to out compete *E.coli* O157:H7 for resources that are utilized by both bacteria and instead more than likely must utilize other mechanisms to display antagonism, such as secreting an antagonistic molecule or utilizing carbon sources that *E.coli* O157:H7 cannot use.

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Tables

Table 1: Total bacterial counts from different spinach cultivars after 16 days incubated at 25˚C on R2A.

Cultivar	Microbial Count (log CFU/g)
Monza	$5.52 + 3.3 \times 10^5$ (A)
Menorca	$6.88 + 7.5 \times 10^6$ (B)
Unipack	$6.63 + 4.3 \times 10^6$ (B)

Samples with different letters (A, B) were statistically different (p=0.0005) by Tukeys comparison.
Normalized to numbers/g

Table 2: Numbers and identities of bacterial isolates that displayed antagonism towards *E.coli* O157:H7 from three spinach cultivars.

Cultivar	Number of Isolates Screened	Number of Antagonists (%)	Species Identified
Monza	317	10 (3.15%)	*Bacillus cereus, Enterobacter sp., Erwinia sp., Erwinia persicina, Flavobacterium sp., Pseudomonas espejiana, Pseudomonas sp., Stenotrophomonas maltophilia and Stenotrophomonas sp.*
Unipack	450	8 (1.78%)	*Acidovorax sp., Bacillus pumilus, Brevundimonas vesicularis, Enterobacter sp., Frigoribacterium actinobacterium, Microbacterium sp.* and *Stenotrophomonas sp.*
Menorca	295	5 (1.69%)	*Flavobacterium sp.* and *Pseudomonas sp.*

Table 3: Species designations of spinach bacterial isolates that display antagonism towards *E.coli* O157:H7.

Genus	Species	Monza	Unipack	Menorca
Acidovorax	*Acidovorax sp.*		(+)	
Bacillus	*B. cereus*	(+)		
	B. pumilus		(+)	
Brevundimonas	*Brev. vesicularis*		(+)	
Enterobacter	*Ent. sp.*	(+)	(+)	
Erwinia	*Erw. pantoea*	(+)		
	Erw. persicina	(+)		
Flavobacterium	*Flav. sp.*	(+)		(+)
Frigoribacterium	*Frigo. actinobacterium*		(+)	
Microbacterium	*Micro. sp.*		(+)	
Paenibacillus	*Paen. polymyxa*			
Pantoea	*Pan. ananatis*			
Pseudomonas	*Ps. espejiana*	(+)		
	Ps. sp.	(+)		(+)
Stenotrophomonas	*S. sp.*	(+)	(+)	
	S. maltophilia	(+)		

A (+) means that an antagonist of this species was isolated from the cultivar.

Table 4: Time entering into log phase, growth rate (replications per hour) and time entering into stationary phase for representative antagonists and *E.coli* O157:H7 at 25°C in ½ TSB.

Isolate	Time Entering into Log Phase (hours)	Growth Rate Constant ($hour^{-1}$)	Time Entering into Stationary Phase (hours)
E.coli O157:H7	12.45	1.93	49.30
Acidovorax sp.	15.30*	1.16*	24.15*
B. cereus	11.45	0.78*	54.45*
B. pumilus	12.15	1.39*	31.00*
Brev. vesicularis	21.30*	1.28*	39.00*
Ent. sp.	12.15	1.81	46.45
Erw. pantoea	12.15	1. 67	35.00*
Erw. persicina	9.45*	1. 88	37.15*
Flav. sp.	14.15	0.85*	68.30*
Frigo. actinobacterium	25.00*	0.58*	42.30*
Micro. sp.	10.30*	1.99	28.15*
Paen. polymyxa	15.30*	1.06*	40.30*
Pan. ananatis	10.45	1.53	40.30*
Ps. espejiana	10.00*	1.98	19.15*
Ps. sp.	12.30	1.43	37.45*
S. sp.	12.30	0.91*	65.00*
S. maltophilia	21.00*	0.87*	35.00*

A * means that the value was found to be statistically significant when compared to *E.coli* O157:H7 alone.

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Chapter 4

Mechanism of growth inhibition of *E.coli* O157:H7 by select spinach epiphytic bacteria

Introduction

As the previous chapter has shown, some epiphytic bacteria isolated from spinach leaves inhibit the growth of *E.coli* O157:H7 *in vitro*, a phenomenon which we describe as antagonism. The growth rates, time in lag and stationary phase, collectively referred to as the growth pattern, for these antagonists vary, suggesting that direct competition is not solely responsible for the antagonism. Microorganisms found on the surface of plants and leaves have been shown to display antagonism towards other microorganisms by production of acid, secretion of inhibitory molecules such as antibiotics or siderophores, or out competing the other microorganisms for space and nutrients (1, 12, 18, 20, 21, 22). Secreted molecules could play an important role in preventing the growth of a food-borne pathogen on the surface of fresh produce since the application of these molecules could have negative minimal impact upon the quality of the produce compared to the direct application of the microorganism. The purpose of this study is to examine the mechanism through which the select spinach epiphytic bacteria inhibit growth of *E.coli* O157:H7 *in vitro*.

Materials and Methods

Bacterial cultures

Bacteria isolated from spinach leaves which were shown to inhibit growth of *E.coli* O157:H7 *in vitro* (Chapter 2) were grown shaking (120 rpm) to stationary phase in ½ TSB media at 25°C, achieving a density of 10^8 cells/ml, which was diluted in ½ TSB to a final density of 10^4 cells/ml.

Supernatant inhibition assay

Twenty antagonists, corresponding to one isolate per species, were screened to determine if a secreted molecule was responsible for the antagonism towards *E.coli* O157:H7. When there was more than one antagonist of the same species, the antagonist that displayed the largest zone of inhibition was selected. Cells were collected from 10 mL ½ TSB by centrifugation for 15 minutes at 4,500 RPMs at 4° C. The resulting supernatant was then filtered through a 0.2µm PTFE filter to remove any remaining cells. The filtered supernatant was then spotted (10µL) onto a plate where a lawn of *E.coli* O157:H7 was just applied to on ½ R2A as described in Chapter 2. Each supernatant was spotted in triplicate with eight supernatants spotted per plate. The plates were incubated at 25˚C and checked every 24 hours for 72 hours for signs of antagonism, which were indicated by a clear halo surrounding the supernatant where the pathogen was not able to grow. The diameters of the zones of inhibition were measured for comparison to the size of the inhibition zones obtained from the bacterial colonies.

Protease Activity Assay

The protease activity assay was carried out on the isolated antagonists that tested positive in the supernatant inhibition assay by following the protocol described by Chavira *et. al.* (27). Briefly, 5mg of Azocoll (Sigma, St. Louis, MO) was combined with 4.5mL of 10mM potassium phosphate buffer and inoculated with ~ 1.0 x 10^4 cells/ml in stationary phase. A negative control was also made by adding 0.5mL of sterile water to a tube. The tubes were immediately incubated at 30˚C water in a shaking water bath at 85 rpm for fifteen minutes then chilled on ice before filtering through Whatman No. 4 filter paper into spectrometer cuvettes. The absorbance of the filtrate was measured at 520nm against the water blank, which was the negative control. The

amount of Azocoll hydrolyzed by each isolate was calculated based on the absorbance of 1mg/ml Azocoll at OD_{520nm} which was found to be 0.593 (27). For this study, the OD_{520} was multiplied by 2.965.

Acid Production Assay

Phenol red agar plates were prepared as previously described (5). Each plate was spread with 10^7 *E.coli* O157:H7 stationary phase cells in 100µL to form a lawn. The antagonists were then inoculated onto the lawn in a concentration of 2.0×10^5 stationary phase cells in a 2 µl droplet. There was one 2 µl droplet placed onto the center of each plate and each plate was replicated in triplicate. Each plate also contained a negative control consisting of a 2 µl droplet of sterile DI water. The plates were then incubated at 25˚C and checked every 24 hours for 72 hours for signs of antagonism, which was apparent by a clear halo surrounding the isolated bacteria. A change in color from red to yellow signified that the pH had fallen below 6.6 indicated acid was produced by the antagonist.

Results

Supernatant Inhibition and Protease Activity Assay

The supernatant of two spinach isolated bacteria, *Paen. polymyxa* and *Ps.espejiana,* resulted in growth inhibition of *E.coli* O157:H7 *in vitro* (Table 1). The zone of inhibitions for the supernatants of the two antagonists were 5mm and 6.5mm in diameter for *Paen. polymyxa* and *Ps. espejiana* , respectively. Colonies of these antagonists resulted in larger zones of inhibition; 13mm diameter for *Paen. polymyxa* and 9mm diameter for *Ps. espejiana*. Azocoll hydrolysis was not observed for supernatants of *Paen. polymyxa* or *Ps. espejiana*, indicating secreted proteases are not responsible for the inhibition (Results not shown).

Acid Production Assay

Acid production was determined by inoculating the antagonistic bacteria onto a lawn of *E.coli* O157:H7 and examining the phenol red agar plate for acid production after 48 hours. The production of acid, denoted by + + +, describes color change of the media to a bright yellow, while + + acid production describes a pale yellow media and a + acid production describes a orange color production (Figure 1). From the twenty antagonistic bacteria that were examined, six were found to produce acid (+ + +) , eight were found to + + produce acid, 2 were found to have + acid production and 4 were found to have no acid production when grown on phenol red agar (Table 2).

Discussion

When bacteria like *E. coli* O157:H7 land on edible plants, they will compete for resources with native members of the phyllosphere. Native members are well adapted to nutrient limitation, low water activity, and harsh environmental (4). It is likely that interactions with the native microorganisms have a role in the persistence and establishment of pathogenic bacteria. In this study, *in vitro* interactions of *E. coli* O157:H7 with the phylloepiphytic bacteria isolated from spinach leaves were studied under conditions of low nutrients. The objectives were to identify bacteria that can potentially inhibit (negative interactions) growth of *E.coli* O157:H7 *in vitro* and describe the mechanisms used for this antagonism. Mechanisms of growth inhibition observed in this study include: alteration of the microenvironment due to acid production, secretion of a soluble molecule and likely competition for nutrients.

The filtered supernatants of two isolates, *Paen. polymyxa* and *Pseudo.espejiana*, resulted in growth inhibition; however the amount of growth inhibition was smaller compared to the inhibition zone observed in the presence of the bacteria itself (Table 1). This suggests that while a secreted molecule plays a role in the displaying antagonism of *E.coli* O157:H7, the actual

bacteria is more important. These bacteria may out compete the *E.coli* O157:H7 for essential carbon, nitrogen, iron and other nutrients that are necessary for its growth (20, 25). Previous work in this laboratory has shown a strong correlation between carbon sources used by *E.coli* O157:H7 and a strain of *Pseudomonas* closely related to *Ps. espejiana* (20). This suggests that *Ps. espejiana* may antagonize growth of *E.coli* O157:H7 through a combined mechanism of competition for nutrients and secretion of an inhibitory molecule. The closely related *Pseudomonas syringae* secretes large numbers of siderophores that allows for the *P. syringae* to outcompete other plant epiphytic bacteria for iron and thereby limit its growth (25). It is unlikely that the secreted inhibitory molecules in this study for *Paen. polymyxa* and *Ps. espejiana* are siderophores, as no azocoll hydrolysis occurred, indicating no protease compounds were present in the supernatant. Another secreted molecule that could be responsible for the antagonism is one that alters the ability of the pathogen to survive in the environment. It was found that compounds isolated during an acid-challenge test were found to alter the acid resistance pathways of food-borne pathogens *E. coli* O157:H7, *Salmonella enterica* or *Listeria monocytogenes* so that they were more susceptible to lower acid levels. This could also offer an explanation for why many of the antagonists displayed at least minimal acid production while they were displaying antagonism, with the acid production not being the primary mechanism of antagonism but still playing a role (2). A secreted antagonistic molecule offers the possibility of adding an antagonistic compound without adding bacteria that could be harmful to the plant or consumer. This could be useful in pre-harvest management of food-borne pathogens, where the application of a compound would be more useful than applying a live bacterium.

Epiphytic bacteria found on plants have been shown to utilize non-proteinaceous secondary metabolites as a method to inhibit growth of other bacteria. A *Pseudomonas sp*. CHA0

has been shown to produce hydrogen cyanide, a highly poisonous chemical compound. The production of this chemical allow it to suppress the growth of *Thielaviopsis basicola*, a bacteria that is responsible for black root rot on tobacco. In another case several strains of pseudomonads have been shown to produce 2,4-diacetylphloroglucinol (DAPG), which can reduce growth of food-borne pathogens such as *Staphylococcus aureus* (10, 20). Other strains of *Pseudomonas* sp. produce pyridine-2,6-dithiocarboxylic acid (PDTC), a metal chelator, which degrades carbon tetrochloride and is associated with growth inhibition of *E.coli* (20). In these cases, antagonistic secondary metabolites produced by bacteria are only partially the mechanism through which they display antagonism (1). This supports the results found in this study, where the sterile supernatant of *Paen. polymyxa* and *Ps. espejiana*, was able to display antagonism towards *E.coli* O157:H7, but not at as effectively as when the bacteria was present. It could also be that for these antagonists the method of antagonism involves the production of cell signals that interfere with functions in the pathogen's cells. It has been found that *Bac. thuringiensis* is able to limit the virulence of *Erw. carotovora* through signal interference. In this case, the *Bac. thuringiensis* produces an acyl-homoserine lactone-lactonase that degrades AHL quorum-sensing signals that are necessary for *Erw. carotovora* to be growth and virulence (6). In another case, probiotic microorganism that were co-cultured with *Clostridium difficile* were found to alter the surrounding environment so that the signal pathways necessary for *C. difficile* toxin were no longer as active (29)

The majority of antagonist isolates did not secrete a soluble inhibitory molecule. These isolates may inhibit growth by competing for carbon sources or acidification of the environment as a by-product of its metabolism as shown by Johnston *et al. Enterobacter asburiae* inhibits growth of *E.coli* O157:H7 by out competing it for nitrogen and carbon sources (4, 11). This is

significant because one of the isolated antagonists identified in this study was *Ent. sp.* KFSNO1E4. This strain of *Enterobacter* had a similar growth rate to *E.coli* O157:H7, suggesting that it also uses competition as a mechanism of antagonism towards *E.coli* O157:H7. Acid production by bacteria is also a potential mechanism of antagonism. Lactic acid bacteria on sprouts inhibited growth of *E. coli* O157:H7, *Salmonella enterica* or *Listeria monocytogenes* (28). While presence of acid alone resulted in a reduction in all three food-borne pathogens, it was necessary for the bacteria to be present on the sprouts for growth inhibition, presumably because the bacteria were able to maintain a suboptimal pH by continually produce the acid over an extended period of time (28). In this study *Frigo. actinobacterium, Pan. ananatis, Ps. espejiana, B. pumilus, Ps. sp* KF21A5. and *S. maltophilia* resulted in production of the most acid, as determined through colorimetric change of the phenol red dye, in the presence of *E.coli* O157:H7. Also, the production of acid by the bacterium could be potentially problematic if the antagonistic bacteria are going to be applied to spinach leaves, as the excessive acid production could negatively affect the appearance and flavor of the spinach. Application of organic acids, such as lactic or acetic acid, can have a negative impact upon the flavor and texture of fresh produce and accelerates browning, where the spinach loses its green color and loses its nutritional value, texture and flavor (15, 17).

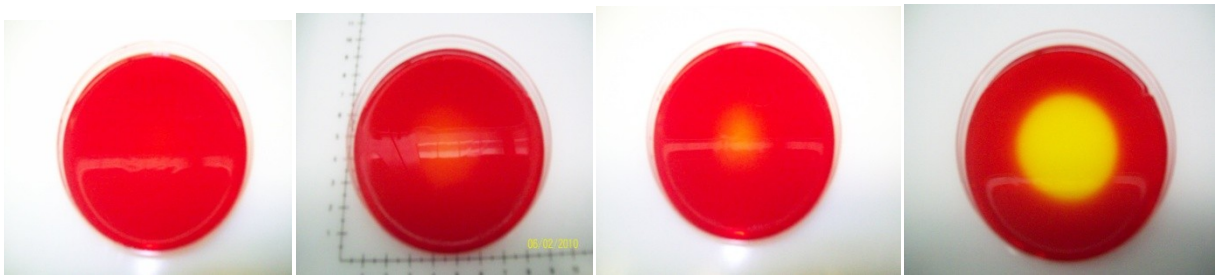
Antagonism of growth of *E.coli* O157:H7 by several of these isolates cannot be explained by any methods tested in this paper. In these cases the isolates may be out competing the pathogen for the limited amount of space that is available, in a process called “metabolic crowding”. The food-borne pathogen *Yersinia enterocolitica* is out-competed by other Gram-negative bacteria. By growing faster than the *Y. enterocolitica* and reaching stationary phase first, the other Gram-negative bacteria limit the level of growth that *Y. enterocolitica* can

achieve, possibly due to nutrient exhaustion (19). This could possibly be the mechanism of antagonism for isolates such as *Ent. sp.,* where the growth rates, time entering into log and stationary phase is comparable to *E.coli* O157:H7. Another possibility is that the antagonists produce molecules that give them a competitive advantage over *E.coli* O157:H7 in the environment, such as a sideophore (23).

The antagonistic bacteria isolated from the surface of spinach leaves have therefore been shown to have different mechanisms of displaying antagonism towards *E.coli* O157:H7. This mechanism could consist of the secretion of an antagonistic molecule, competition for nutrients and space or acid production. Due to the differences in the mechanism of antagonism, it important to examine how the antagonist and *E.coli* O157:H7 behave in co-culture both *in vitro* and on the surface of a spinach leaf. Along with this, several of the mechanism of antagonism, including nutrient competition and production of an antagonistic compound, can be impacted by the carbon sources present. Therefore, it is important to study the impact of adding a carbon source utilized by the isolated antagonists but not *E.coli* O157:H7 could have on the rate of antagonism.

Tables and Figures

Figure 1: Key for phenol red media color change in acid production assay



No acid production + ++ +++

Table 1: The size of the zones of inhibition of growth of *E.coli* O157:H7 surrounding select spinach bacterial epiphytes on ½ R2A incubated at 25ºC for 72 hours.

Bacteria	Supernatant Inhibition (Diameter of Zone of Inhibition (mm))
Acidovorax sp.	-
B. cereus	-
B. pumilus	-
Bac. sp.	-
Brev. vesicularis	-
Ent. sp.	-
Erw. pantoea	-
Erw. persicina	-
Flav. sp.	-
Frigo. actinobacterium	-
Micro. sp.	-
Paen. polymyxa	(5.0)
Pan. ananatis	-
Ps. espejiana	(6.5)
Ps. sp. KF20F6	-
Ps. sp. KFNO2D2A	-
Ps. sp. KF21A5	-
Ps. sp. KF2C3	-
S. sp.	-
S. maltophilia	-

A (-) indicates that no zone of inhibition was observed

Table 2: Relative amounts of acid produced by select spinach bacterial epiphytes when inoculated onto a lawn of *E.coli* O157:H7 on phenol red agar when incubates at 25ºC for 72 hours

Bacteria	Acid production
Acidovorax sp.	+ +
B. cereus	+ +
B. pumilus	+ + +
Bac. sp.	+ +
Brev. vesicularis	+ +
Ent. sp.	-
Erw. pantoea	-
Erw. persicina	+ +
Flav. sp.	+ +
Frigo. actinobacterium	+ + +
Micro. sp.	+ +
Paen. polymyxa	+ +
Pan. ananatis	+ + +
Ps. espejiana	+ + +
Ps. sp. KF20F6	+
Ps. sp. KFNO2D2A	-
Ps. sp. KF21A5	+ + +
Ps. sp. KF2C3	-
S. sp.	+
S. maltophilia	+ + +

The presence of a (-) indicates that no inhibition occurred while the presence of a (+) indicates that inhibition did occur. See Figure 1 for depiction of the levels of acid production.

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Chapter 5

Effect of Cellobiose as the Sole Carbon Source on the Growth Rate of *E.coli* O157:H7 and Antagonistic Isolates *in vitro* and on Spinach Leaves

Introduction

After examining the potential mechanisms used by spinach phylloepiphytic bacteria to antagonize growth of *E.coli* O157:H7, it was important to look at potential factors that could improve the rate of antagonism, especially on the leaf's surface. Environmental conditions on leaf surfaces fluctuate in temperature, water and nutrient availability. Within the field it may be possible to manipulate the amount and type of nutrients available in the leaf surface. By amending the leaves with a carbon source that is metabolized by spinach epiphytic bacteria but not by *E.coli* O157:H7, it may be possible to decrease populations of *E.coli* O157:H7 (13). One carbon source that cannot be utilized by *E.coli* O157:H7 and is naturally found in plants is cellobiose (1). Epiphytic bacteria are adapted to use carbon sources naturally found on the surface of leaves, such as fructose or cellobiose. Since it can be utilized by many of the antagonistic microorganism found on the surface of the spinach leaves, the presence of cellobiose as the sole carbon source could potentially improve the level of antagonism displayed towards *E.coli* O157:H7. Also, since it is naturally found in trace amounts on plants, cellobiose is considered by the FDA to be Generally Regarded as Safe and therefore could be applied to spinach, such as in the form of a spray or a dip (1).

The purpose of this portion of the study was to determine if the presence of cellobiose as the sole carbon source improved the rate of antagonism towards *E.coli* O157:H7. *In vitro* assays examined the ability of the antagonist to reduce the numbers of *E.coli* O157:H7 when co-cultured in minimal media containing cellobiose as the sole carbon source. *In vivo* assays were

also performed to determine the effect of cellobiose introduction on a leaf surface when co-inoculated with *E.coli* O157:H7 and one of the antagonists.

Materials and Methods

Spinach Used for Study

The spinach used for this portion of the study was Dole Brand Baby Spinach.

Preparation of Inoculum

Due to similarities of colonial morphology of *E.coli* and some epiphytic bacteria a GFP tagged strain of *E.coli* O157:H7 strain H1730^{GFP} that was kanamycin resistant was used for this portion of the study. All cultures were grown shaking at 120 rpm in ½ TSB at 25˚C to stationary phase. The cells were then collected by centrifugation and re-suspended in 9mL of 0.8% sodium chloride solution. The process was repeated three times so that all residual nutrients would be removed. All cultures were re-suspended to a density of ~4 log CFU/ml.

Co-culture plate counts of *E.coli* O157:H7 and the isolated antagonists on TSA and M9 Minimal Media with 20% cellobiose as the sole carbon source

E.coli O157:H7 and bacterial antagonists identified in Chapter 2 were co-cultured in a rich media (½ TSB) or a minimal media containing 20% cellobiose as the sole carbon source (1). This was done to determine the effect of cell-cell contact on the growth of *E.coli* O157:H7 *in vitro*. Co-cultures were prepared as described by Leroi *et al*. (4). Briefly, 9mL of ½ TSB or M9 Minimal Media with 20% cellobiose were inoculated with 10^4 stationary phase cells of the antagonist and *E.coli* O157:H7 H1730^{GFP}. The tubes were then incubated while shaking at 120 rpm at 25˚C for 48 hours. Bacterial numbers were determined by serial dilution and plating onto TSA or M9 cellobiose agar, depending on the starting media. The plates were then incubated for

48 hours at 25˚C. Colonies which fluoresced under UV light were counted as *E.coli* O157:H7, as the isolated antagonists did not fluoresce. This was based upon a control plate, where E. *coli* O157:H7 colonies were observed to fluoresce under UV light. Data was normalized by conversion to $\log_{10}$ CFU/g^{-1} of spinach. The results were assessed for statistical significance using PROC Mixed function of SAS (SAS Institute Inc., Cary, NC). Bacterial enumeration experiments were repeated with spinach leaves (18-29) totaling 10g per treatment with duplicate plate counts. In order to minimize error from the differences in sizes of the leaves all samples were processed with 10g of fresh weight. This resulted in varying numbers of spinach leaves due to natural variation in sizes of the leaves

Growth rates for antagonists and *E.coli* O157:H7 with cellobiose as the sole carbon source and the presence of spinach extract

E.coli O157:H7 and each antagonist were grown individually and in co-culture to determine growth rates. Microwell plates (96) were filled according to the grouping described in Table 1, with each well containing a total of 300µl. Growth measurements were completed in triplicate for each group described in Table 1. Growth rates at 25°C for select antagonists and *E.coli* O157:H7 were calculated based on increases in optical density $OD_{450\text{-}600nm}$ for 48 hours with readings performed every 15 minutes.

Population sizes of *E.coli* O157:H7 on spinach leaves in co-culture with an antagonist provided cellobiose as a carbon source

Spinach leaves were surface disinfected by soaking in a solution of 70% ethanol for 30 seconds, and subsequently allowed to air dry in a biosafety cabinet for one hour (17). Approximately 18-29 of surface disinfected spinach leaves with a total weight of 10g were selected for each group. The spinach leaves dipped into a solution of 20% cellobiose or into

sterile water for 5 seconds and subsequently air dried in the biosafety cabinet for 30 minutes. After drying, the leaves were inoculated with *E.coli* O157:H7 and/or one of the antagonists, as described in Table 2 that had a final concentration of ~10^6 cells/ml in stationary phase. The leaves were subsequently air dried in the biosafety cabinet for 30 minutes. The leaves were incubated at 25°C for 24 hours inside of plastic bags to reduce wilting of the spinach leaves. After incubation, inoculated cells were dissociated from the leaves by stomaching 10g of the leaves with 90mL of sterile peptone water with 1% Tween in a stomacher (BagMixer, Interscience). Serial dilutions were performed and plated in triplicate onto TSA media with kanamycin (25mg per 500ml) to select for only the inoculated *E.coli* O157:H7. After 24 hours incubation at 25°C all colonies were counted. Data was normalized by conversion to $\log_{10}$ CFU/g^{-1} of spinach. Statistical significance of the carbon source and presence of the antagonist on the counts of *E.coli* O157:H7 were determined using the PROC mixed function of SAS.

Results and Discussion

Growth rates for antagonists and *E.coli* O157:H7 with cellobiose as the sole carbon source and the presence of spinach slurry

The growth rates and final yield were calculated for *E.coli* O157:H7 in pure culture in different media types. The growth rates and yields of co-cultures of *E.coli* O157:H7 with *B. pumilis, Paen. polymyxa* and *Ps. espejiana* were reduced compared to *E.coli* O157:H7 alone but increased in comparison to the antagonist alone (Table 3). The final yield of the *E.coli* O157:H7 in the pure culture was however found to be significantly higher than the final yield of all of the co-cultures grown in ½ TSB and ½ TSB with spinach extract. In comparison to the pure antagonist cultures, *Bac. sp., Paen.polymyxa* and *Ps. espejiana* were found to have significantly lower yields than the co-cultures when both grown in ½ TSB and ½ TSB with spinach extract

(Appendix B). As the growth curves were calculated based on a change in $OD_{450-600}$, it is not possible to determine which bacteria contributes to the change in growth rates in co-culture reported in Table 3. Attempts to quantify *E.coli* O157:H7 by measuring the absorbance at $OD_{420-520}$, which corresponds to the emitting wavelength of GFP, were unsuccessful. No difference in optical density could be measured when plate counts indicated an increase in numbers (results not shown).

E.coli O157:H7 did not grow in M9 minimal media with 20% cellobiose with or without spinach extract when incubated at 25°C for 72 hours. This suggests that alterations to the growth rates observed in the M9 minimal media were due to the presence of the antagonist. There were significantly higher growth rates for all of the co-cultures grown in M9 Minimal Media when compared to the growth of the pure *E.coli* O157:H7 in M9 Minimal Media (Table 3). The growth rates of both *Bacillus* isolates co-cultured with *E.coli* O157:H7 increased in M9 media with 20% cellobiose, while the rest of the antagonists did not significantly increase the growth rate compared to the pure culture (Table 3).

For the time that the cultures entered into log phase, all of the strains except *Erw. persicina, Pan. ananatis* and *Ps. sp.* grown in pure culture and *Bac. sp*., *Erw. persicina, Pan. ananatis, Ps. espejiana* and *Ps. sp.* grown in co-culture with *E.coli* O157:H7 the time entering into log phase for the cultures grown in ½ TSB and ½ TSB with spinach extract was significantly different from the cultures grown in M9 Minimal Media with 20% cellobiose and M9 Minimal Media with 20% cellobiose and spinach extract (Table 4). For *Pan. ananatis* and *Ps. sp.* in pure culture there was a significant difference in the time entering into log phase when grown in ½ TSB and ½ TSB with spinach extract when compared to only cultures grown in M9 Minimal Media with 20% cellobiose (Table 4).

The addition of spinach extract to the ½ TSB did not increase the growth rate, final yield or time entering into log of *E.coli* O157:H7. This is due to the fact that the TSB media contains a large amount of dextrose, which *E.coli* O157:H7 prefers to use as a carbon source, which is not exhausted within 24 hours of growth (12). Although the *E.coli* O157:H7 could possibly utilize nutrients found in the spinach extract, the spinach extract is lower in nutrients than the TSB, making its addition to the media not significant. Addition of spinach extract to the M9 media allowed the growth of *E.coli* O157:H7 when co-cultured with all antagonists, indicating that *E.coli* O157:H7 can use nutrients in spinach extract as a nutrient source. The spinach extract provided additional nutrients extending the amount of time in log phase for all co-cultures with the exception of *E.coli* O157:H7 in cultures with *Bac. sp.* and *Pan. ananatis*. For the antagonist, *Ps. sp.* it is possible that its lower growth rate in the co-culture than in the pure culture was due to its ability to out compete the *E.coli* O157:H7 for the nutrients in the spinach extract, giving it a competitive advantage (15). Although the antagonists *Paen. polymyxa* and *Ps. espejiana* have been shown to secrete an antagonistic compound, in the M9 Minimal Media with spinach slurry these antagonists were unable to significantly reduce the growth of *E.coli* O157:H7. It is possible that in the cellobiose media necessary nutritional components were not present for the two isolated antagonists to produce the secreted molecule. This has been seen in *Ps. fluorescens* isolated from the sugar beet rhizosphere that produces an antibiotic antagonistic toward *Pythium ultimum* and *Rhizoctonia solani*. It was found that *P. fluorescens* only produced the antibiotic 2,4- in a medium that was rich in glucose (15). Therefore, it could be that *Paen. polymyxa* and *Ps. espejiana* need a carbon source other than cellobiose to produce their antagonistic secreted molecule.

When the cultures entered into stationary phase, the time for all of the cultures grown in ½ TSB and ½ TSB with spinach extract were significantly lower from the culture grown in M9 Minimal Media with 20% cellobiose and M9 Minimal Media with 20% cellobiose and spinach extract (Table 5). For *Bac. sp., Ent. sp., Erw. persicina* and *Pan. ananatis* in pure culture and *Paen. polymyxa, Pan. ananatis*, *Ps. espejiana* and *Ps. sp.* KF2C3 in co-culture with *E.coli* O157:H7 there was a significant difference in the time entering into stationary phase when grown in M9 Minimal Media with 20% cellobiose when compared to cultures grown in M9 Minimal Media with 20% cellobiose and spinach extract (Table 5). It took significantly less time for co-cultures of *E.coli* O157:H7 with *Paen. polymyxa* and *Ps. espejiana* to reach stationary phase compared to the pure antagonist culture in M9 media with 20% cellobiose and spinach extract (Table 5). This suggests that *E.coli* O157:H7 and the antagonist were competing for some essential nutrient in the spinach extract that was exhausted faster in the presence of the pathogen. In contrast, it took significantly more time to reach stationary phase for *Erw. persicina* co-cultured with *E.coli* O157:H7 in M9 media with 20% cellobiose and spinach extract (Table 5), suggesting that presence of the pathogen may have provided additional nutrients that could be used or that *E.coli* O157:H7 may have reduced the growth rate of the antagonist.

Effect of cellobiose as a carbon source and presence of an antagonist on numbers of *E.coli* O157:H7 *in vitro*

The presence of an antagonist in co-culture with *E.coli* O157:H7 significantly reduced the numbers of culturable *E.coli* O157:H7 after 12 hours in TSB media for five antagonists, by 24 hours all antagonists resulted in smaller populations of the pathogen (Table 6). Growth was completely inhibited when co-cultured with one of the bacteria isolates, *Ps. espejiana* for 12

hours. However, after 24 hours *E.coli* O157:H7 populations began to increase but were still reduced in numbers compared to *E.coli* O157:H7 alone. *E.coli* O157:H7 continued to grow achieving large populations in 48 hours. Significant decreases in the population of *E.coli* O157:H7 were detected for all antagonists for 48 hours when compared to *E.coli* O157:H7 grown alone (Table 6).

The co-culture tests were also carried out on a media containing 20% cellobiose, a carbon source that can support only limited or no growth of *E.coli* O157:H7. Growth of *E.coli* O157:H7 was significantly reduced in cellobiose media compared to TSB (Table 6). No growth of *E.coli* O157:H7 occurred in cellobiose media when incubated alone for up to 36 hours, indicating that *E.coli* O157:H7 could not use cellobiose as a carbon source (Table 6). After 36 hours a limited amount of growth occurred, presumably due to nutrients becoming available as cells began to die. Although its level of growth would be less than the *E.coli* O157:H7 that was co-cultured in TSB, the nutrients provided by the lysed cells have been shown to be significant enough to allow *E.coli* cells to grow in what would be otherwise a low nutrient environment (4). Two antagonists, *Erw. pescinia* and *Pan. ananatis*, supported the growth of low levels of *E.coli* O157:H7 in cellobiose media (Table 6). The reduction of cellobiose results in production of glucose which can then be used by *E.coli* O157:H7 as a carbon source (1). After 48 hours only the bacteria *B. pumilus, Paen. polymyxa* and *Ps. espejiana* significantly reduced the counts of *E.coli* O157:H7 in co-culture in cellobiose media, suggesting these bacteria out competed the *E.coli* O157:H7 for the glucose by-product or that a secreted molecule prevents the *E.coli* O157:H7 from growing (10). These isolates could only inhibit growth of *E. coli* O157:H7 completely *in vitro* in cellobiose media. In the richer TSB media the numbers of *E.coli* O157:H7 were reduced but not inhibited.

Three isolates, *Paen. polymyxa*, *Ps.* sp. KF2C3, and *Ps. espejiana*, reduced growth of *E.coli* O157:H7 within 12 hours of inoculation, while both bacteria were in logarithmic stage of growth. The other strains needed to reach stationary phase before significant reductions in *E.coli* O157:H7 numbers occurred (Table 6). Previous studies have also shown that an acclimation period is necessary before an optimal level of inhibition is produced since it can take the bacteria time to produce any molecules that it might be utilizing as a source of its antagonism (9,11). Stationary phase cultures of two of these isolates, *Paen. polymyxa* and *Ps. espejiana*, were found to secrete a soluble molecule that antagonized growth of *E.coli* O157:H7 in vitro (Chapter 4). For the 48 hour time period the log reduction rate was in general lower than what was expressed at 24 hours. By 48 hours the log reduction in *E.coli* O157:H7 began to decline, which may be due to exhaustion of nutrients and/or death of the antagonists, allowing the remaining *E.coli* O157:H7 cells to grow (4).

Effect of immersion in inoculum consisting of cellobiose on the growth of the isolated antagonists and *E.coli* O157:H7 on spinach leaves

Populations of *E.coli* O157:H7 increased one log on spinach leaves that were dipped into water or a cellobiose solution (Table 7). A statistically significant decrease in the numbers of *E.coli* O157:H7were recovered from leaves inoculated with *E.coli* O157:H7 and the antagonists *Ps. sp.* KF2C3*, Erw. perscina, Pan. ananatis, Ent. sp.* and *B. pumilus* (Table 7). No difference in numbers of *E.coli* O157:H7 recovered were determined when a solution of 20% cellobiose was applied to the leaf surface prior to inoculation compared to when water was applied (Table 7). No colonies were recovered on TSA-KAN plates for the groups that did not include the kanamycin resistant *E.coli* O157:H7, indicating that epiphytic bacteria did not contribute to these counts.

The population decreases of *E.coli* O157:H7 on the leaves in the presence of the antagonist were significantly smaller than observed for the *in vitro* studies. In addition growth of *E.coli* O157:H7 occurred on the leaf surface in the presence of *Ps. sp* KF2C3*., Ps. espejiana, Paen. polymyxa, Ent. sp., Bac. sp.,* and *B. pumilus* that inhibited growth of *E.coli* O157:H7 on the cellobiose media *in vitro*. These differences in growth inhibition are likely due to the presence of additional carbon sources on the surface of the leaf surface that can be used by the pathogen as an energy source (13). Since cellobiose would no longer be the sole carbon source for the bacteria to utilize, the antagonists would no longer have as great a competitive advantage over the *E.coli* O157:H7. Also, although the spinach leaves were surface disinfected, there were still residual bacteria that remained on the leaf. These bacteria could also have impacted the ability of *E.coli* O157:H7 to survive on the leaf (15). It is possible that for the leaves dipped into cellobiose, the other bacteria present on the leaf's surface were able to utilize the carbon source. This could both create competition over the carbon source with the antagonists and allow for the bacteria on the leaf's surface to break down the cellobiose into a compound that could be utilized by the pathogen (1). It is also possible that the remaining bacteria on the surface of the spinach leaves were able to secrete compounds that could enhance the ability of *E.coli* O157:H7 to grow. This has been seen with *Wasteria paucula*, an epiphytic bacterium that has been isolated from lettuce leaves. This bacterium has been shown to be able to significantly increase the growth of *E.coli* O157:H7 that had been inoculated onto the surface of the leaves (2). Other studies have also shown an inability for antagonism co-culture tests performed *in vitro* to transfer to *in vivo* studies. This has been seen with a study that used *Lactobacillus sakei* to inhibit the growth of *E.coli* O157:H7 on ground beef. It was found that although the *L. sakei* was able to statistically inhibit the growth of *E.coli* O157:H7 *in vitro,* when the experiment was performed on ground

beef no significant reduction in *E.coli* O157:H7 was found and the presence of the antagonist negatively impacted the quality of the beef (11).

Conclusions

In this study, the effect of co-culturing *E.*coli O157:H7 with isolated antagonists both *in vitro* and *in viva* was studied. It was first observed that the growth rate of a co-culture of *E.coli* O157:H7 and select antagonists were reduced when compared to *E.coli* O157:H7 alone but greater than the antagonist alone, suggesting that two bacteria interact, even compete *in vitro*. Co-culture plate studies confirmed that the presence of select antagonists did reduce populations of *E.coli* O157:H7 when incubated in the presence of the antagonists both *in vitro* and on the leaf surfaces. Strains of *Pseudomonas,* closely related to the strains isolated in this study, have been shown as able to successfully out compete other bacteria for nutrients and space. A study conducted by Simons *et al.* found that in the rhizosphere of tomatoes roots inoculated with *Pseudomonas fluorescens* strain WCS365, an effective root colonizing pseudomonad, the *Ps. fluorescens* WCS365 was able to out compete other strains of *Pseudomonas* and *Rhizobium* (12*)*. Isolates of *Ps. sp.* have been shown to reduce the numbers of *Salmonella typhimurium,* in vitro (8).

This study showed that while all eight of the isolated antagonists that were co-cultured *in vitro* with *E.coli* O157:H7 were able to significantly inhibit the growth of the pathogen, when the antagonists were added to the surface of the spinach leaves only *Ps. sp., Erw. perscina, Paen. polymyxa, Pan. ananatis, Ent. sp.* and *B. pumilus* were able to significantly inhibit the pathogen's growth. It was also demonstrated that while the presence of cellobiose is able to increase the inhibition of isolated antagonists towards *E.coli* O157:H7 *in vitro*, when added as a dip onto the

surface of spinach leaves it does not improve the level of inhibition displayed by an added antagonist towards *E.coli* O157:H7.

Tables and Figures

Table 1: Composition of the groups for the growth curves.

	TSB	Cellobiose Media	Type of Inoculum
Group A	270µl	0µl	30µl of either one of the antagonists or *E.coli* O157:H7
Group B	0µl	270µl	30µl of either one of the antagonists or *E.coli* O157:H7
Group C	240µl	0µl	30µl of one of the antagonists and 30µl of *E.coli* O157:H7
Group D	0µl	240µl	30µl of one of the antagonists and 30µl of *E.coli* O157:H7
Group E	240µl	0µl	30µl of spinach slurry and 30µl of *E.coli* O157:H7
Group F	0µl	240µl	30µl of spinach slurry and 30µl of *E.coli* O157:H7
Group G	240µl	0µl	30µl of spinach slurry and 30µl of one of the antagonists
Group H	0µl	240µl	30µl of spinach slurry and 30µl of one of the antagonists
Group I	210µl	0µl	30µl of spinach slurry, 30µl of one of the antagonists and 30µl of *E.coli* O157:H7
Group J	0µl	210µl	30µl of spinach slurry, 30µl of one of the antagonists and 30µl of *E.coli* O157:H7
Group K	300µl	0µl	No Inoculum
Group L	0µl	300µl	No Inoculum
Group M	0µl	0µl	300µl of spinach slurry

Table 2: Composition of groups for spinach leaf inoculation.

	Dip Solution	Type of Inoculum
Group A	Cellobiose Solution	100µl of *E.coli* O157:H7
Group B	Sterile Water	100µl of *E.coli* O157:H7
Group C	Cellobiose Solution	100µl of one of the antagonists and 100µl of *E.coli* O157:H7
Group D	Sterile Water	100µl of one of the antagonists and 100µl of *E.coli* O157:H7
Group E	Cellobiose Solution	No Inoculum
Group F	Sterile Water	No Inoculum

Table 3: Growth rate (hours^{-1}) determined for selected antagonists individually in ½ TSB or M9 Minimal Media or in co-culture with *E.coli* O157:H7 as determined by $OD_{450-600}$

	Isolate	TSB	TSB with Spinach Extract	M9 Minimal Media with 20% Cellobiose	M9 Minimal Media with 20% Cellobiose and Spinach Extract
Individual	*E.coli* O157:H7	0.75 [1]	0.68 [1]	0.00 [2]	0.00 [2]
	Bac. sp.	0.53 [A1]	0.56 [A1]	0.01* [A2]	0.00 [A2]
	B. pumilus	0.17* [A1]	0.18 [A1]	0.01 * [A1]	0.01 * [A1]
	Ent. sp.	0.53 [A1]	0.56 [A1]	0.01 * [A2]	0.03 * [A2]
	Erw. persicina	0.60 [A1]	0.52 [A1]	0.03 * [A2]	0.02 * [A2]
	Paen. polymyxa	0.35* [A1]	0.15 [A2]	0.03 * [A2]	0.02 * [A2]
	Pan. ananatis	0.78 [A1]	0.72 [A1]	0.00 [A2]	0.02 * [A2]
	Ps. espejiana	0.17* [A1]	0.15 [A1]	0.30 * [A2]	0.02 * [A1]
	Ps. sp. KF2C3	0.66 [A1]	0.19 [A2]	0.02* [A3]	0.03* [A3]
Co-culture with *E.coli* O157:H7	*Bac. sp.*	0.65 [A1]	0.64 [A1]	0.18 [*A2]	0.01* [A2]
	B.pumilus	0.55 [B1]	0.50* [B1]	0.18 [*A2]	0.14 [*A2]
	Ent. sp.	0.58 [A1]	0.62 [A1]	0.03* [A2]	0.03 [*A2]
	Erw. persicina	0.57 [A1]	0.46 [*A1]	0.02 [*A2]	0.01 [*A2]
	Paen. polymyxa	0.56 [B1]	0.46* [B1]	0.09 [*A2]	0.02 [*A2]
	Pan. ananatis	0.69 [A1]	0.69 [A1]	0.02* [A2]	0.01 [*A2]
	Ps. espejiana	0.64 [B1]	0.68 [B1]	0.21 [*A2]	0.01* [A2]
	Ps. sp. KF2C3	0.70 [A1]	0.69 [A1]	0.02* [A2]	0.01 [B2]

Number with a * next to them were found to be statistically significant when compared to *E.coli* O157:H7 with $p<0.05$.

A different letter within each column is significantly different from the same antagonist in the same media with $p<0.05$.

A different number within each row is significantly different from other values in the same row with $p<0.05$.

Initial incoulum density of 1 x 10^4

Table 4: Amount of time (hours) entering into log determined for selected antagonists individually in ½ TSB or M9 Minimal Media or in co-culture with *E.coli* O157:H7 as determined by $OD_{450-600}$

	Isolate	TSB	TSB with Spinach Extract	M9 Minimal Media with 20% Cellobiose	M9 Minimal Media with 20% Cellobiose and Spinach Extract
Individual	*E.coli* O157:H7	0.50[1]	0.50[1]	NG[2]	NG[2]
	Bac. sp.	0.50 [A1]	0.50 [A1]	27.5* [A2]	NG[A]
	B. pumilus	0.50 [A1]	0.50 [A1]	20.0 [*A2]	23.5* [A2]
	Ent. sp.	0.50 [A1]	0.50 [A1]	5.00 [*A2]	2.00* [A2]
	Erw. persicina	1.00[A1]	1.00[A1]	2.50* [A1]	0.50 [A1]
	Paen. polymyxa	0.50 [A1]	0.50 [A1]	24.5* [A2]	30.0* [A3]
	Pan. ananatis	1.00[A1]	1.00[A1]	35.5 [*A2]	1.50* [A1]
	Ps. espejiana	0.50 [A1]	0.50 [A1]	27.5* [A2]	27.5* [A2]
	Ps. sp. KF2C3	1.00[A1]	1.00[A1]	15.0 [*A2]	0.50* [A1]
Co-culture with *E.coli* O157:H7	*Bac. sp.*	0.50 [A1]	0.50 [A1]	19.5*[B2]	26.5* [B3]
	B.pumilus	0.50 [A1]	0.50 [A1]	18.5 [*A2]	18.5* [B2]
	Ent. sp.	0.50 [A1]	0.50 [A1]	19.5*[B2]	21.5* [B2]
	Erw. persicina	1.00[A1]	1.00[A1]	6.50 [*A2]	13.5* [B3]
	Paen. polymyxa	0.50 [A1]	0.50 [A1]	11.0*[B2]	4.50* [B3]
	Pan. ananatis	1.00[A1]	1.00[A1]	3.50*[B1]	10.0* [B2]
	Ps. espejiana	0.50 [A1]	0.50 [A1]	15.5*[B2]	6.00* [B3]
	Ps. sp. KF2C3	1.00[A1]	1.00[A1]	15.0 [*A2]	4.00* [B3]

Number with a * next to them were found to be statistically significant when compared to *E.coli* O157:H7 with $p<0.05$.

A different letter within each column is significantly different from the same antagonist in the same media with $p<0.05$.

A different number within each row is significantly different from other values in the same row with $p<0.05$.

Initial incoulum density of 1×10^4.

NG designates that no growth on the plate was observed.

Table 5: Time (hours) entering into stationary phase determined for selected antagonists individually in ½ TSB or M9 minimal media or in co-culture with *E.coli* O157:H7 as determined by $OD_{450-600}$

	Isolate	TSB	TSB with Spinach Extract	M9 Minimal Media with 20% Cellobiose	M9 Minimal Media with 20% Cellobiose and Spinach Extract
Individual	*E.coli* O157:H7	7.00[1]	7.00[1]	NG[2]	NG[2]
	Bac. sp.	6.50 A1	6.50 A1	44.5* A2	NG*A3
	B .pumilus	8.50 A1	8.00 A1	41.5* A2	44.0* A2
	Ent. sp.	4.50 A1	4.50 A1	26.5* A2	48.0* A3
	Erw. persicina	5.50 A1	50.0 A1	30.0* A2	14.0* A3
	Paen. polymyxa	6.00 A1	7.50 A1	46.0* A2	45.5* A2
	Pan. ananatis	6.50 A1	6.50 A1	48.0* A2	30.0* A3
	Ps. espejiana	6.50 A1	6.00 A1	45.5* A2	45.5* A2
	Ps. sp. KF2C3	9.00 A1	8.50 A1	48.0* A2	48.0* A2
Co-culture with *E.coli* O157:H7	*Bac. sp.*	6.50 A1	6.50 A1	46.0* A2	42.0* B2
	B.pumilus	8.00 A1	8.00 A1	45.5* A2	41.0* A2
	Ent. sp.	4.50 A1	4.50 A1	45.5* B2	47.5* A2
	Erw. persicina	5.00 A1	5.50 A1	29.5* A2	33.0* B2
	Paen. polymyxa	7.00 A1	7.00 A1	48.0* A2	36.5*B3
	Pan. ananatis	6.50 A1	6.50 A1	17.0* B2	28.5* A3
	Ps. espejiana	6.00 A 1	6.00 A1	48.0* A2	38.5* B3
	Ps. sp. KF2C3	8.50 A1	8.50 A1	48.0* A2	42.5* A3

Number with a * next to them were found to be statistically significant when compared to *E.coli* O157:H7 alone with $p<0.05$.
A different letter within each column is significantly different from the same antagonist in the same media with $p<0.05$.
A different number within each row is significantly different from other values in the same row with $p<0.05$.
Initial incoulum density of 1×10^4.
NG designates that no growth on the plate was observed after 72 hours of incubation.

Table 6: Plate counts of *E.coli* O157:H7 when co-cultured with select antagonists *in vitro*

Bacteria	12 hours		24 hours		36 Hours		48 Hours	
	TSA	M9 Minimal Media w/ 20% Cellobiose	TSA	M9 Minimal Media w/ 20% Cellobiose	TSA	M9 Minimal Media w/ 20% Cellobiose	TSA	M9 Minimal Media w/ 20% Cellobiose
E.coli O157:H7	4.31 ^{A1}	0 ^{B2}	7.72 ^{A3}	0 ^{B2}	9.46 ^{A5}	1.62 ^{B5}	10.0 ^{A5}	4.11 ^{B1}
Antagonist bacteria in the co-culture with *E.coli* O157:H7								
Bac. sp.	3.93 ^{A*}	0 ^{B2}	5.54 ^{A3*}	0 ^{B2}	6.57 ^{A3*}	0 ^{B2*}	8.79 ^{A4*}	2.39 ^{B1*}
B. pumilus	4.24 ^{A1}	0 ^{B2}	4.43 ^{A1*}	0 ^{B2}	7.1 ^{A3*}	0 ^{B1*}	8.89 ^{A3*}	0 ^{B2*}
Ent. sp.	4.46 ^{A1}	0 ^{B2}	6.95 ^{A3*}	0 ^{B2}	7.53 ^{A3*}	1.47 ^{B4}	9.13 ^{A5*}	3.05 ^{B1*}
Erw. persicina	4.38 ^{A1}	1.48 ^{B2*}	5.31 ^{A1*}	1.96 ^{B2*}	7.23 ^{A3*}	3.65 ^{B1*}	8.46 ^{A3*}	4.8 ^{B1}
Paen. polymyxa	2.33 ^{A*}	0 ^{B2}	3.46 ^{A1*}	0 ^{B2}	7.1 ^{A3*}	0 ^{B2*}	7.27 ^{A3*}	0 ^{B2*}
Pan. ananatis	3.77 ^{A*}	1.79 ^{B2*}	6.13 ^{A3*}	2.09 ^{B2*}	7.06 ^{A4*}	3.22 ^{B1*}	8.46 ^{A4*}	4.62 ^{B1}
Ps. sp. KF2C3	2.78 ^{A*}	0 ^{B2}	5.83 ^{A3*}	0 ^{B2}	6.94 ^{A3*}	1.47 ^{B1}	8.94 ^{A4*}	3.74 ^{B1}
Ps. espejiana	0 ^{A1*}	0 ^{B1}	3.73 ^{A2*}	0 ^{B1}	7.04 ^{A3*}	0 ^{B1*}	7.59 ^{A3*}	0 ^{B1*}

Number with a * next to them were found to be statistically significant when compared to *E.coli* O157:H7 with $p<0.05$.
A different number within each row is significantly different with $p<0.05$.
Numbers with a different letter next to them were found to significantly different from the same antagonist/time count that was made in the other media.
Initial incoulum density of 1×10^4
Cultures were incubated in corresponding broth

Table 7: Co-culture plate counts of *E.coli* O157:H7 and the isolated antagonists incubated on spinach leaves dipped in either water or 20% cellobiose for 24 hours and plated on ½ TSA and incubated at 25°C for 24 hours

	Isolate	Spinach Dipped into Sterile Water (*E.coli* O157:H7 log CFU/ml)	Log Reduction†	Spinach Dipped into 20% Cellobiose Solution (*E.coli* O157:H7 log CFU/ml)	Log Reduction†
Individual	*E.coli* O157:H7	7.26		7.28	
Co-culture with *E.coli* O157:H7	*Bac. sp.*	7.25	0.01	7.36	-0.08
	B. pumilus	6.62*	0.64*	6.68*	0.6*
	Ent. sp.	6.49*	0.77*	6.40*	0.88*
	Erw. persicina	5.97*	1.29*	5.22*	2.06*
	Paen. polymyxa	7.00	0.26	7.22	0.06
	Pan. ananatis	6.72*	0.54*	6.58*	0.7*
	Ps. espejiana	7.19	0.07	7.25	0.03
	Ps. sp. KF2C3	6.42*	0.84*	6.66*	0.62*

Number with a * next to them were found to be statistically significant when compared to *E.coli* O157:H7 alone with $p<0.05$.

Initial incoulum density of 1×10^6

†Log Reduction refers to decrease in log CFU/ml compared to *E.coli* O157:H7 when grown alone and as a co-culture.

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Chapter 6

Conclusions and Future Directions

The overall purpose of this study was to identify bacteria present on the surface of spinach leaves that are antagonistic towards *E. coli* O157:H7, determine the mechanism through which the antagonism is displayed and establish if the addition of a carbon source not utilized by *E. coli* O157:H7 will increased the antagonistic potential of the isolated bacteria.

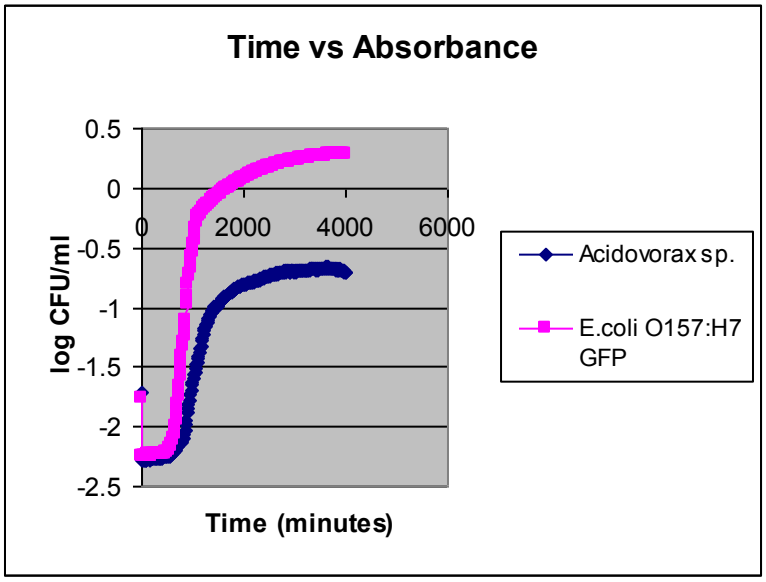
The presence of epiphytic bacteria on spinach leaves was shown to be impacted by the spinach cultivar, with the cultivars with the highest surface area having the greatest number of epiphytic bacteria. The cultivar could also impact the diversity of the epiphytic microbiota, but this was not shown conclusively in this study. Also, only conventionally grown spinach was compared in this study. Studying the impact of cultivar on diversity of epiphytic bacteria should be examined, along with the impact that organic growing conditions could have on the number and diversity of epiphytic bacteria on the spinach.

Following isolation and screening for antagonism towards *E.coli* O157:H7, the mechanism of antagonism for the isolated bacteria was studied. Acid production during antagonism was studied, with 14 of the isolated antagonists producing either moderate or heavy acid, indicating that acid production could play a role in the antagonism of these isolates. For two of the bacteria *Paen. polymyxa* and *Ps. espejiana* it was determined that a non-proteinaceous secreted molecule was partially responsible for the antagonism towards *E. coli* O157:H7. Growth curves that were performed also determined that 9 of the strains had curves that closely resembled the growth curve of *E. coli* O157:H7, suggesting that these bacteria could display

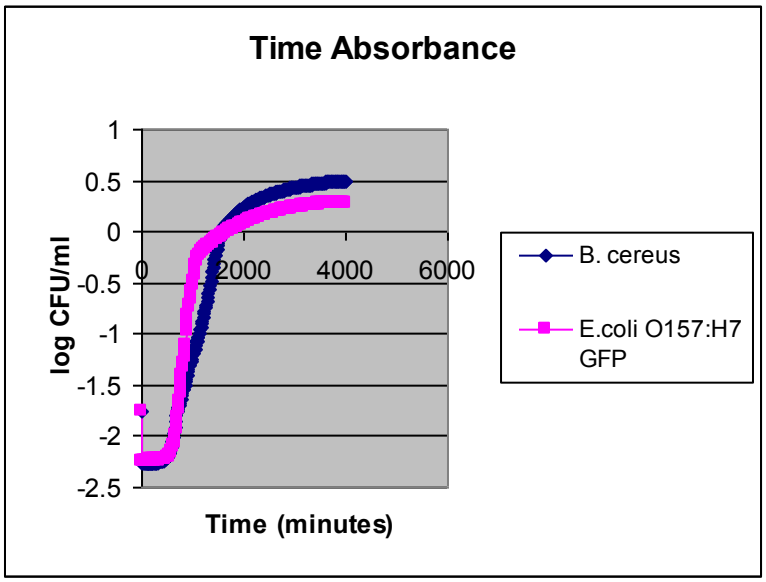
antagonism through nutrient or space competition. The secreted molecule produced by *Paen. polymyxa* and *Ps. espejiana* should be identified and future research could examine the impact of co-culturing the secreted molecule with *E. coli* O157:H7.

The isolated antagonists were co-cultured both *in vitro* and on spinach leaves with *E. coli* O157:H7 to determine the impact on the display of antagonism. The co-cultures were also performed in a minimal nutrient media with 20% cellobiose to determine the impact of having a carbon source not utilized by *E. coli* O157:H7 on the rate of antagonism. For the majority of the isolated antagonists, it was found that co-culturing them with *E. coli* O157:H7 resulted in a significant reduction in the population of *E. coli* O157:H7. On the spinach leaves, the majority of isolated antagonists still displayed antagonism towards *E. coli* O157:H7, though it was a reduced reduction from the study that was conducted *in vitro*. There was also no significant difference in the reduction of *E. coli* O157:H7 between the spinach leaves that were dipped in water and the ones dipped in cellobiose. This is possibly because although the antagonistic bacteria could utilize cellobiose, it was not a preferred carbon source, making their growth minimal. Future research could examine if the addition of another carbon source better utilized by the isolated antagonists and that is not broken down into a carbon source utilized by *E. coli* O157:H7 could improve upon the antagonism displayed towards *E. coli* O157:H7. This could be important in helping to determine if the alternate carbon source could increase the level of antagonism displayed towards *E. coli* O157:H7.

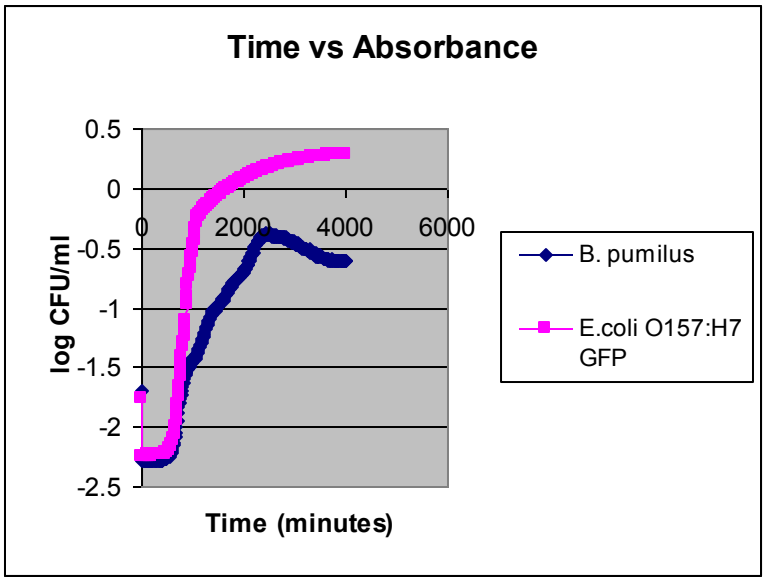
Appendix A: Growth Curves of Isolated Antagonists and *E.coli* O157:H7.



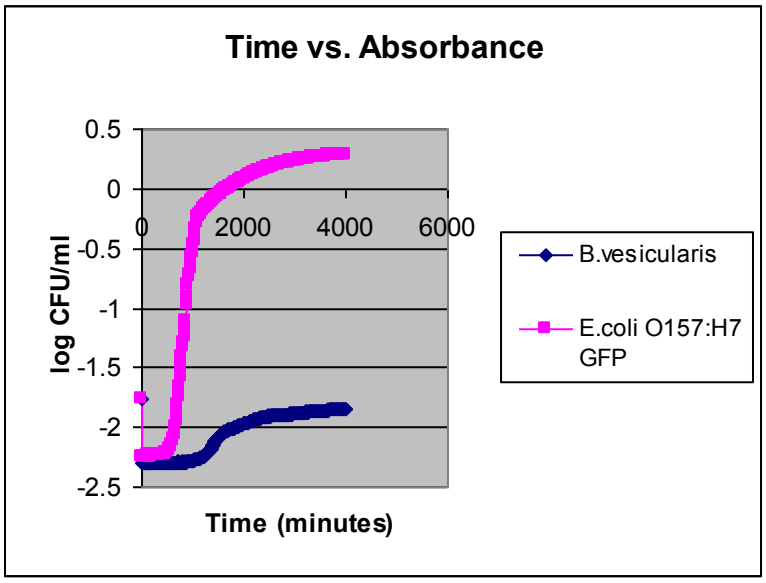
Growth curves of *Acidovorax sp.* and *E.coli* O157:H7.



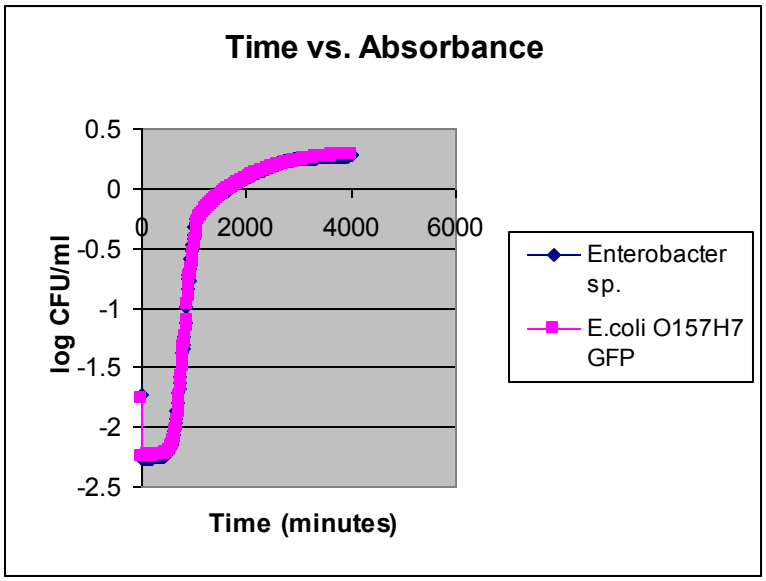
Growth curves of *B. cereus* and *E.coli* O157:H7.



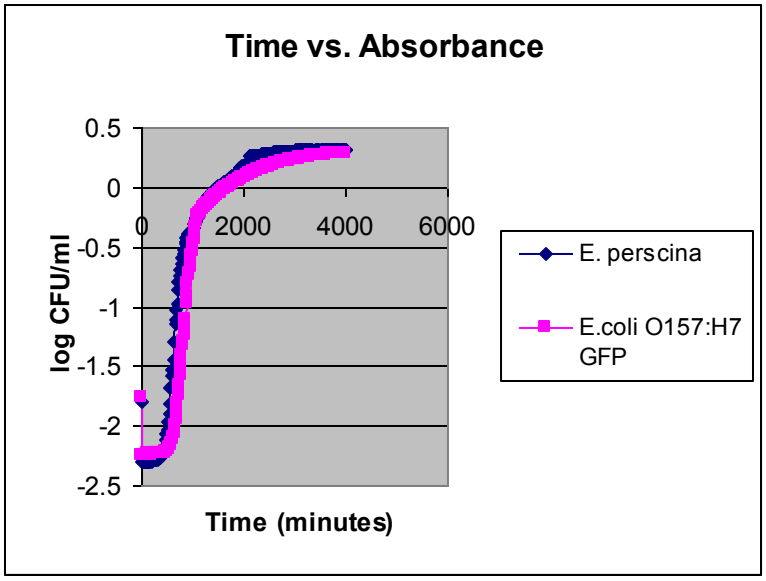
Growth curves of *B. pumilus* and *E.coli* O157:H7.



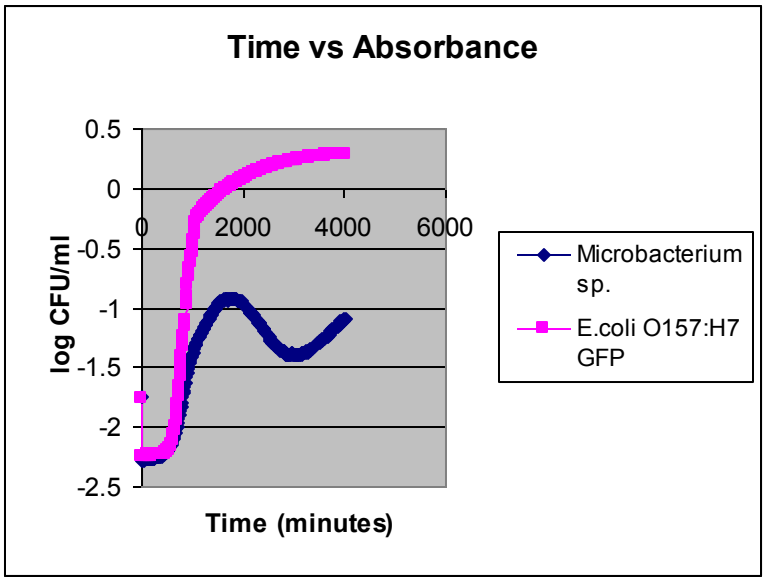
Growth curves of *Brev. vesicularis* and *E.coli* O157:H7.



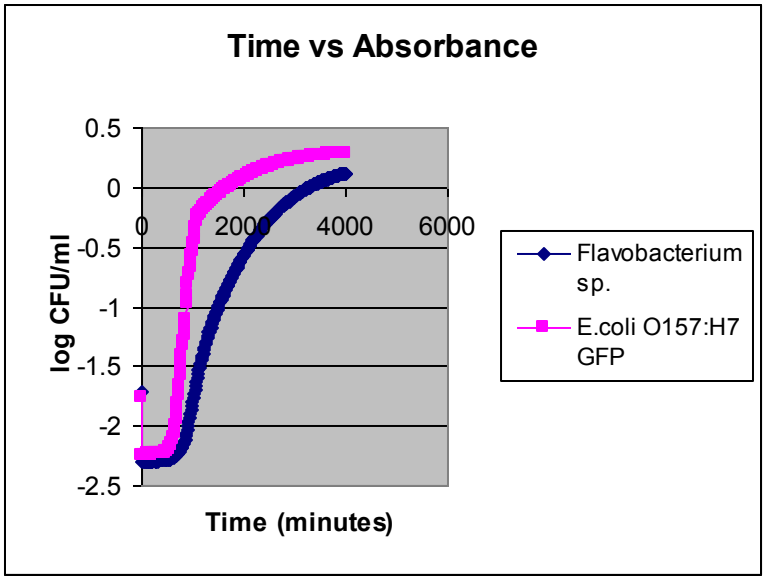
Growth curves of *Ent. sp.* and *E.coli* O157:H7.



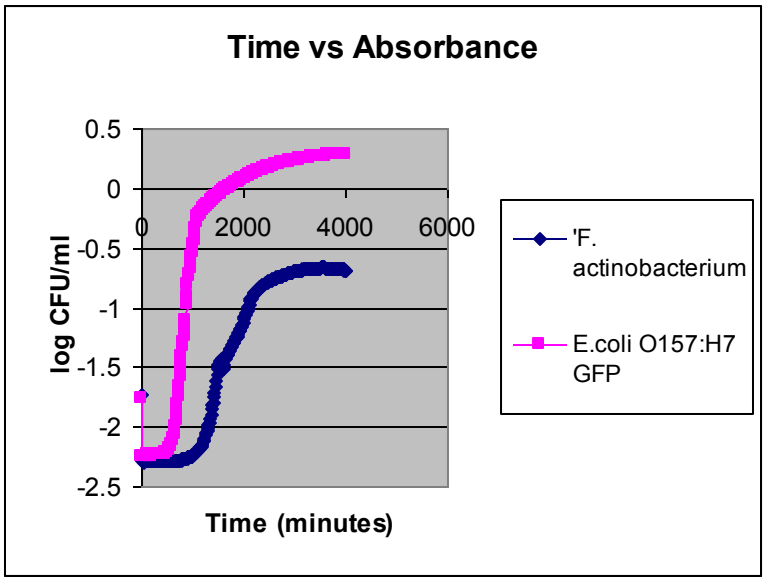
Growth curves of *Erw. persicina* and *E.coli* O157:H7.



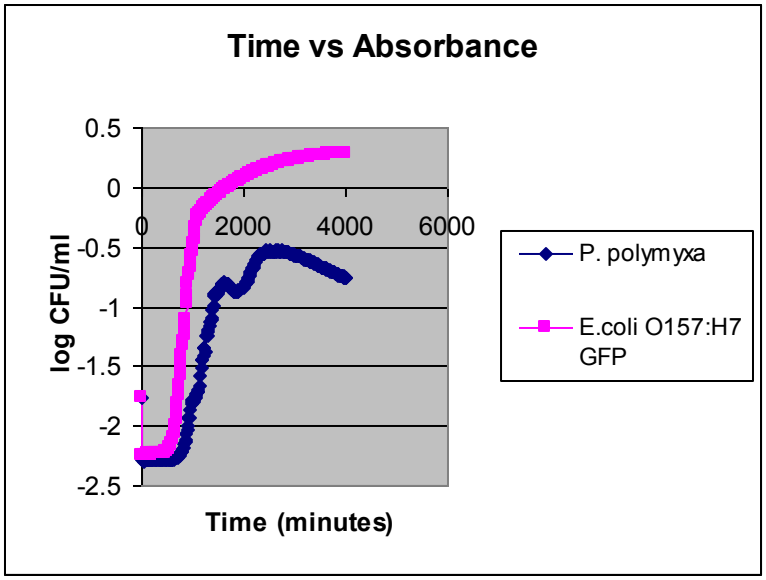
Growth curves of *Micro. sp.* and *E.coli* O157:H7.



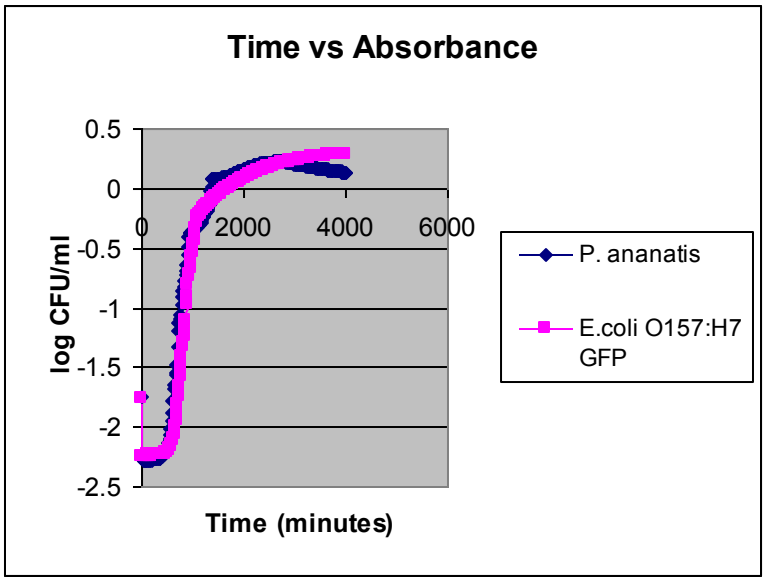
Growth curves of *Flav. sp.*. and *E.coli* O157:H7.



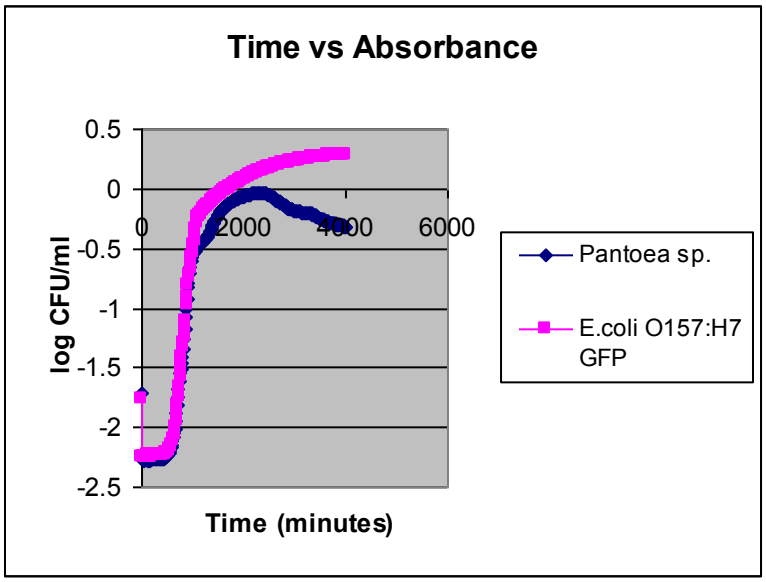
Growth curves of *Frigo. actinobacterium* and *E.coli* O157:H7.



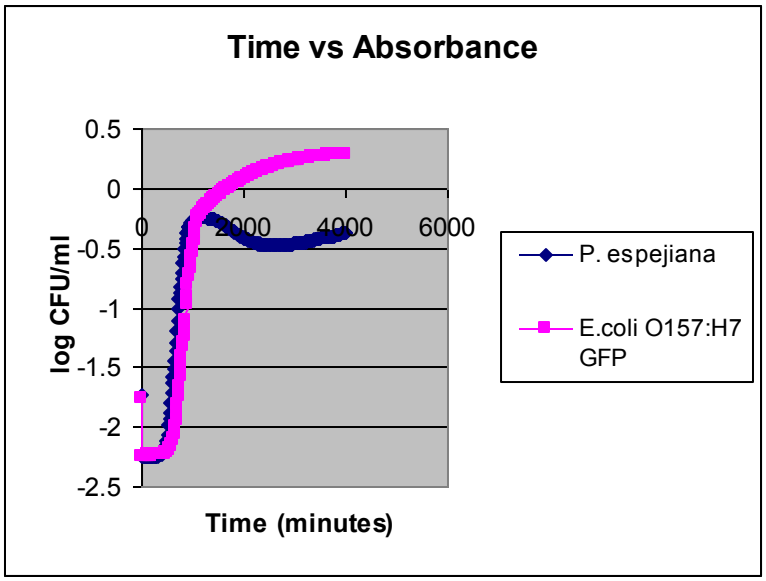
Growth curves of *Paen. polymyxa.* and *E.coli* O157:H7.



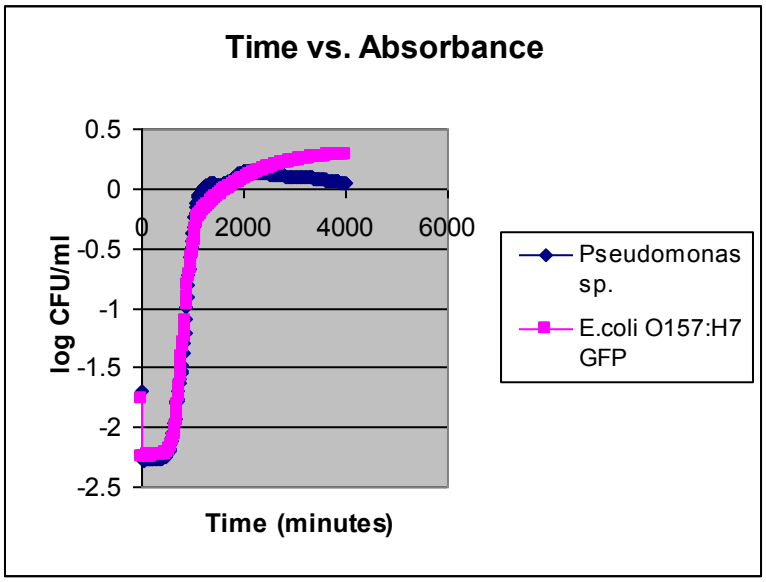
Growth curves of *Pan. ananatis* and *E.coli* O157:H7.



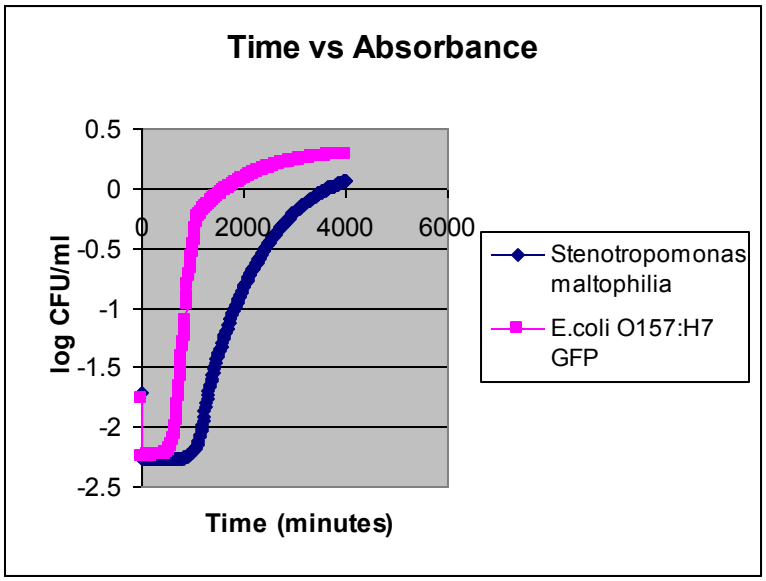
Growth curves of *Pan. sp.* and *E.coli* O157:H7.



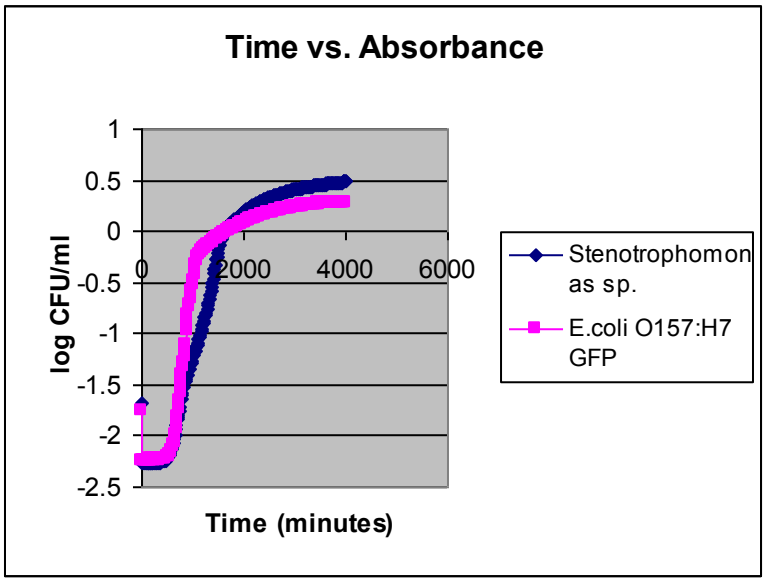
Growth curves of *Ps. espejiana* and *E.coli* O157:H7.



Growth curves of *Ps. sp.* and *E.coli* O157:H7.

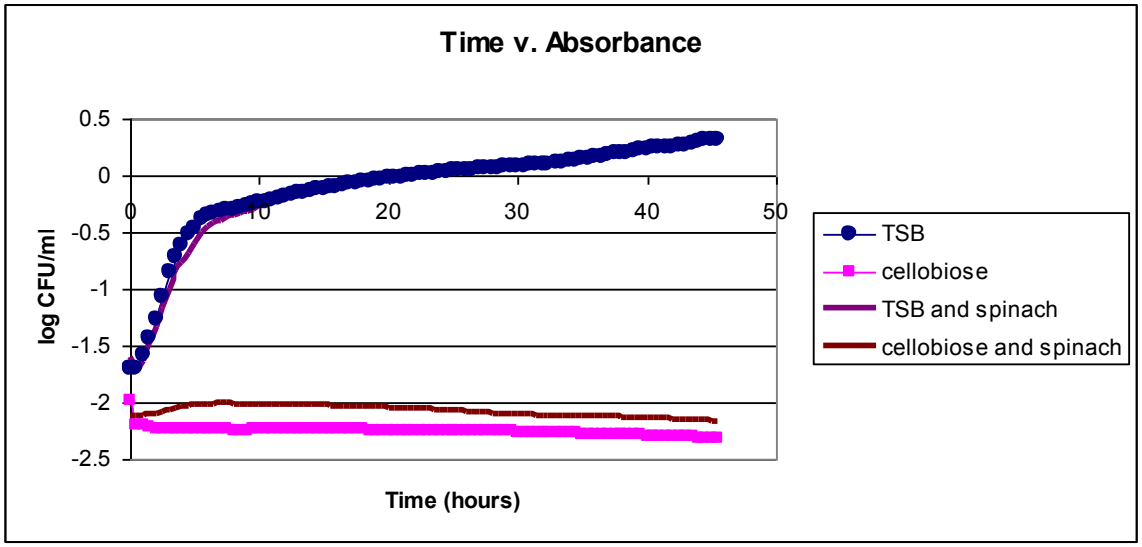


Growth curves *Sten. maltophilia* and *E.coli* O157:H7.

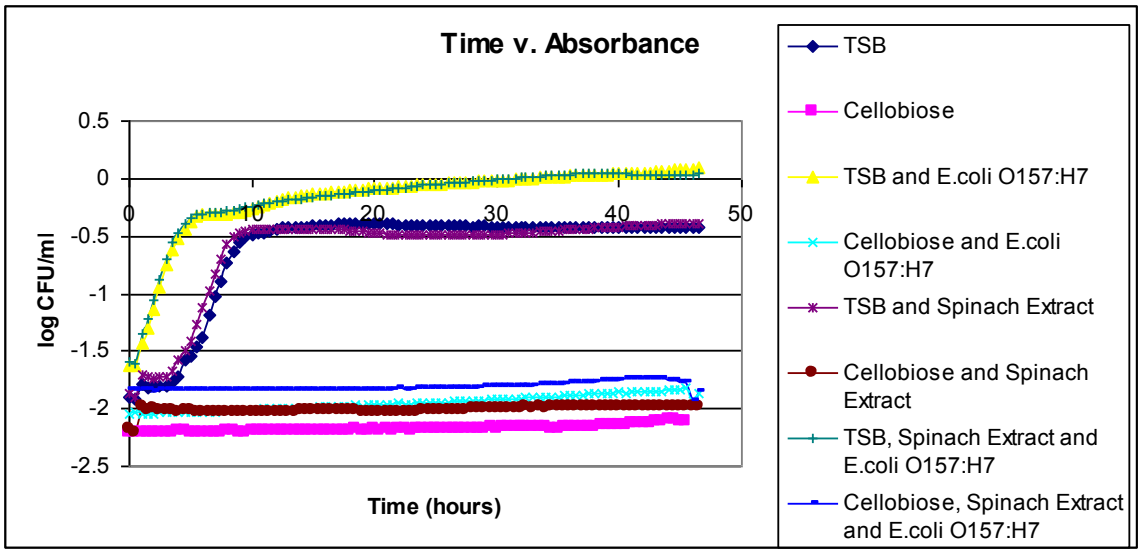


Growth curves of *Sten. sp.* and *E.coli* O157:H7.

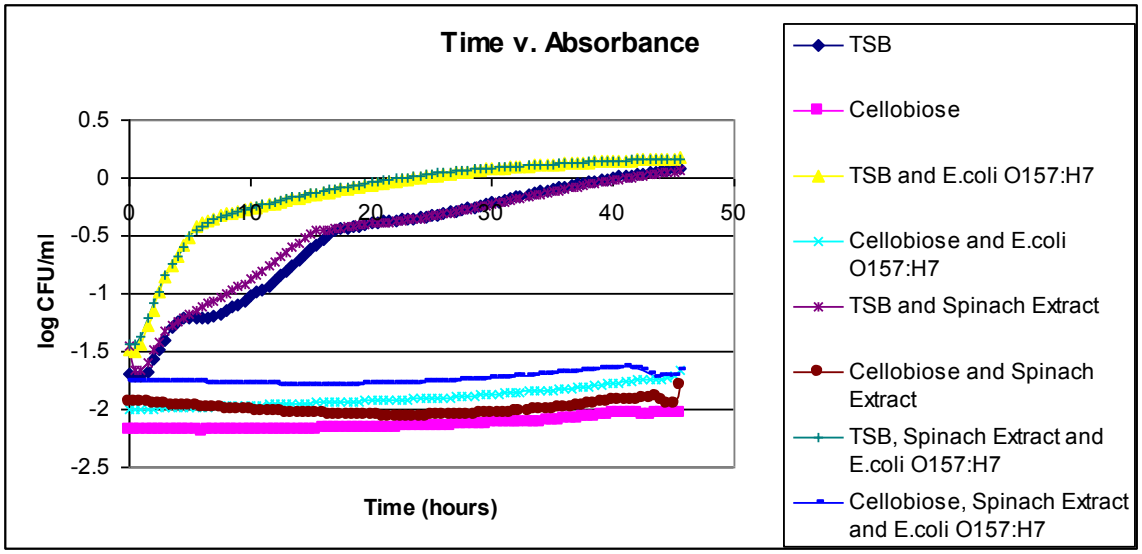
Appendix B: Growth Curves of Antagonists and *E.coli* O157:H7 grown in pure and Co-Culture in TSB and M9 Minimal Media with 20% Cellobiose and Spinach Extract.



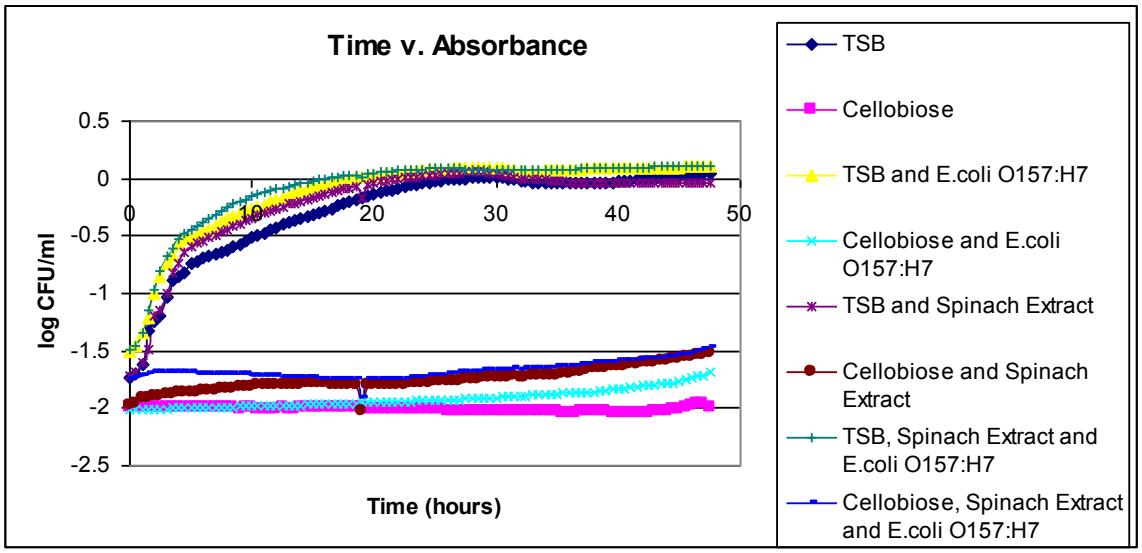
Growth curves of *E.coli* O157:H7.



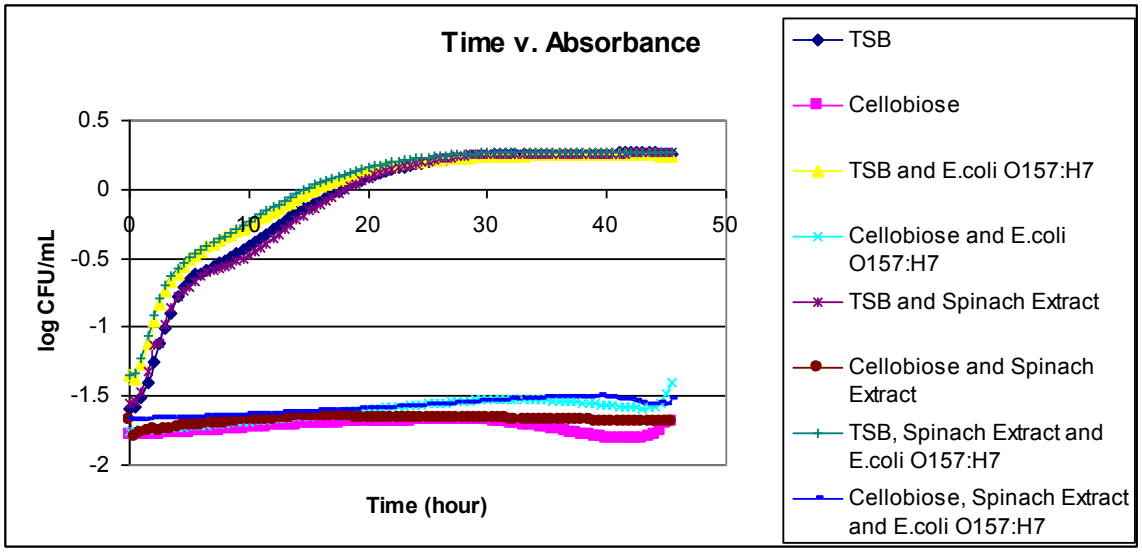
Growth curves of *Bac. sp.* and *E.coli* O157:H7



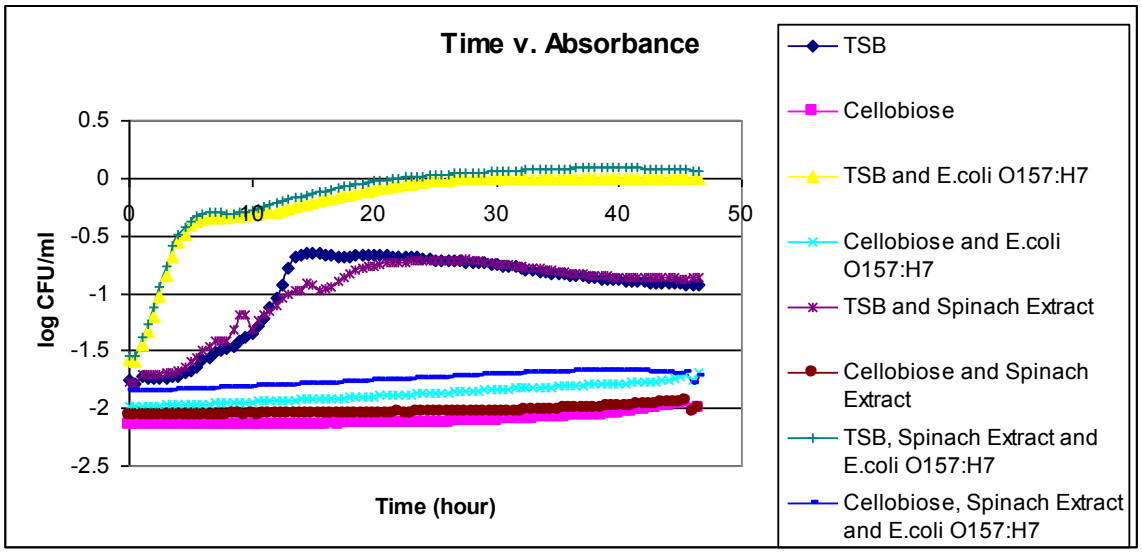
Growth curves of *B. pumilus* and *E.coli* O157:H7.



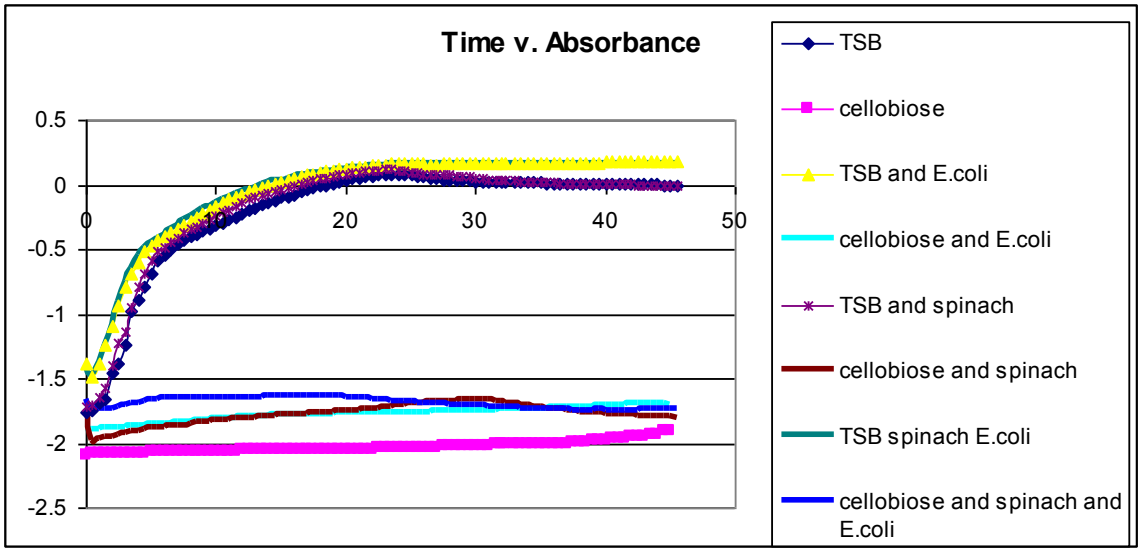
Growth curves of *Ent. sp.* and *E.coli* O157:H7.



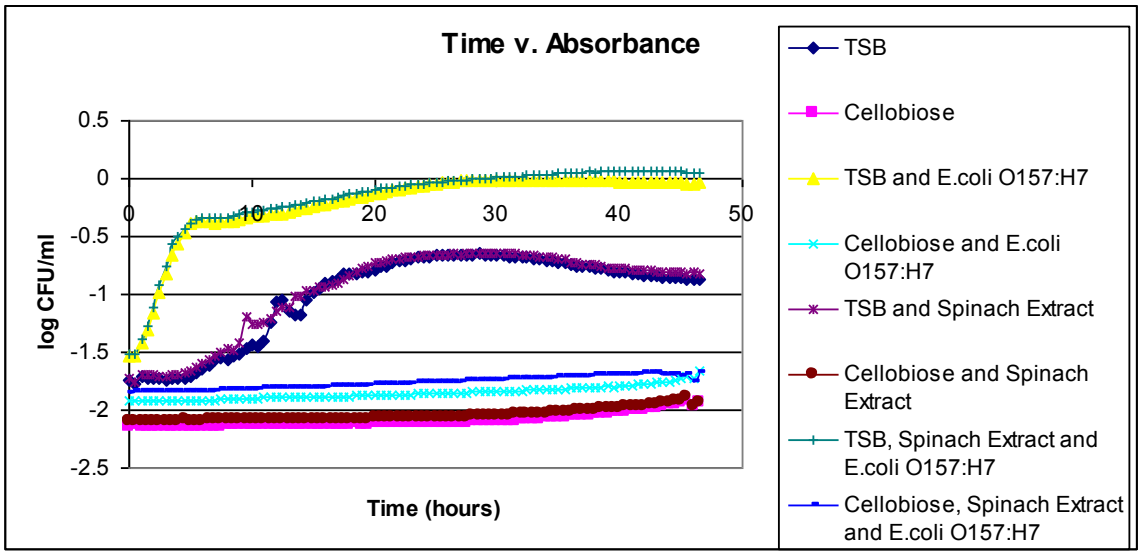
Growth curves of *Erw. persicina* and *E.coli* O157:H7.



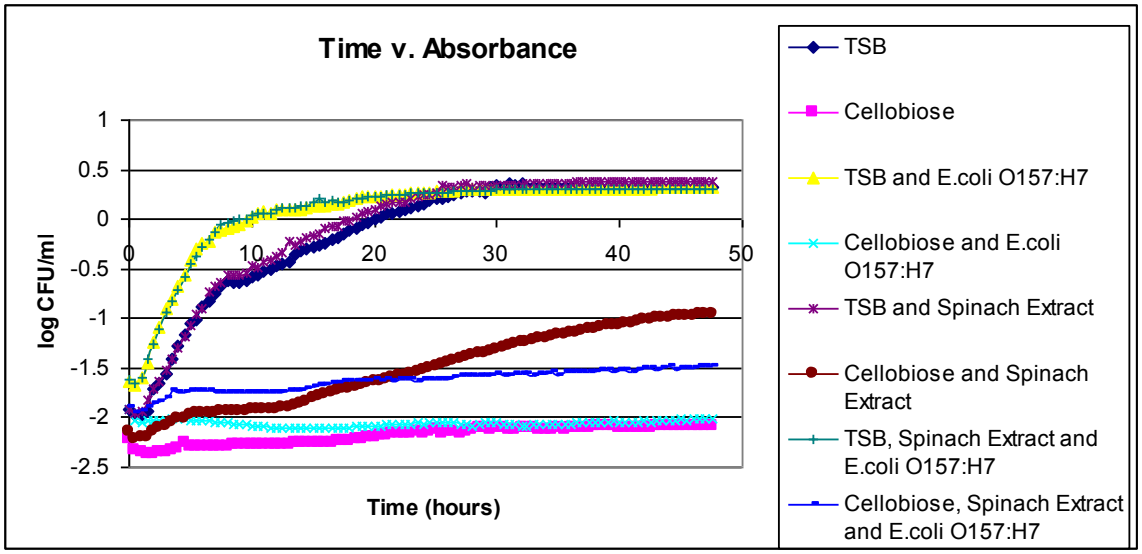
Growth curves of *Paen. polymyxa.* and *E.coli* O157:H7.



Growth curves of *Pan. ananatis* and *E.coli* O157:H7.



Growth curves of *Ps. espejiana* and *E.coli* O157:H7.



Growth curves of *Ps. sp.* and *E.coli* O157:H7.