ROLE OF EPIPHYTIC BACTERIA IN THE COLONIZATION OF FRUITS AND LEAFY GREENS BY FOODBORNE BACTERIAL PATHOGENS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2016

Major Subject: Food Science and Technology

ABSTRACT

The epiphytic bacteria content in fruits and leafy greens and their effect toward the colonization of foodborne bacterial pathogens was studied. Populations of mesophilic, lactic acid, coliform, and psychrotrophic bacteria were recovered from cantaloupe, tomato, pepper, spinach, endives, and parsley, and the effect of environmental and agricultural conditions toward epiphytic bacteria content was evaluated. The epiphytic bacteria content was variable by commodity, with cantaloupes and spinach being the most populated commodities. The environmental temperature and the irrigation method also affected the epiphytic bacteria content. To determine the inhibitory effect of epiphytic bacteria toward *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Saintpaul, 9,307 isolates were evaluated *in vitro*. In total, 2.6, 0.7 and 6.4% of the isolates were antagonistic toward *E. coli* O157:H7, *S.* Saintpaul, or both pathogens, respectively. Most antagonistic isolates were psychrotrophs and lactic acid bacteria. Overall, more antagonistic isolates from fruits were found in samples collected in the fall than the summer. Further biochemical identification revealed that most of the antagonistic psychrotrophs were *Alcaligenes faecalis* sbsp. *faecalis*. In fruits, most of the antagonistic isolates were *Leuconostoc*, *Enterococcus,* and *Streptococcus* species. Furthermore, the effect of epiphytic bacteria toward *S.* Saintpaul growth in fruits and toward *E. coli* O157:H7growth in leafy green leaves was studied in the plant surface. *Enterococcus kobei* and *Enterococcus casseliflavus* from cantaloupe, and of *Staphylococcus hominis* subsp. *hominis* from tomato inhibited *S.* Saintpaul on cantaloupe rind, and tomato skin, respectively. Similarly,

Enterococcus faecalis affected *S.* Saintpaul on peppers and *Gemella morbillorum*, *Enterococcus gallinarum*, and *Bacillus mycoides* affected *E. coli* O157:H7 growth on parsley. The effect of *Streptococcus alactolyticus*, *Bacillus licheniformis*, *Gemella bergeri*, *Staphylococcus sciuri*, and *Enterococcus gallinarum* toward *E. coli* O157:H7 growth and stomata invasion in endives was observed using confocal microscopy. After 24 h, *E. coli* O157:H7 growth was moderately inhibited by all epiphytic isolates tested. However, after three days, treated and control samples presented similar pathogen growth. The results from this study demonstrated that some epiphytic bacteria from fruits and leafy greens are potential biocontrol agents, able to reduce the proliferation of *E. coli* O157:H7 and *S. enterica* in fruits and vegetables.

DEDICATION

To my husband and best friend, Jorge,

I am forever thankful for your love, patience, and support throughout these years.

To Jacobo, my son, and my motivation, full of smiles.

For him, I hope curiosity will lead his path, open his mind,

brighten his spirit, and amuse him with every discovery he finds.

To my mom, Belia, and my dad, César, who taught me to reach for better things,

to work hard, to give everything I have, and to never give up.

To Marcela and Paulina, my sisters and best friends,

and to my handsome nephews, whose hearts are close and

endlessly connected to mine despite the long distance.

ACKNOWLEDGEMENTS

I want to extent my gratitude to my advisor, and committee chair, Dr. Alejandro Castillo, for the opportunity to pursue a doctoral degree and for his advice and support during my graduate studies, and to my committee members, Dr. Elsa Murano, Dr. Gary Acuff, and Dr. Leon Russell, for their willingness to help me throughout my research.

I also want to thank to Dr. Elisa Cabrera, and Mrs. Lisa Lucia for their support and expertise advice, but most importantly for their friendship.

I also want to thank to Ana M., Alexandra, Keila, Mary Pía, Grihalakshmi, and Verónica, for always being there, during uncountable hours in the laboratory, and for all the moments shared.

I want to express my gratitude to all my friends, especially to Alana, Amie Marie, Andrea, Andrés, Ayoola, Fanny, Jennifer, Jessie, Katie, Kathy Jo, Marco, Matt, Megha, Noo, Noushin, Sara, Sofía, Tanita, Tamra, Wendy, and Zahra, for their support, solidarity, and technical assistance.

I also want to thank to Dr. Juan Anciso and Ashley Gregory for the samples, to Dr. Joseph Sturino for the LAB control strains, to Dr. Roula Barhoumi for her assistance with the confocal microscope, to Ana Mercado for the *S.* Saintpaul growth curve data, and to Veronica Arias for the growth curve analyses.

Finally, I want to thank to the National Council in Science and Technology (CONACyT) of Mexico for the academic financial support and to the National Institute of Food and Agriculture, NIFA-USDA, for funding the present research.

NOMENCLATURE

AMP	Ampicillin
APT	All-purpose media containing Tween 80
CL	Coliform
GFP	Green fluorescent protein
IA	Inhibition area(s)
IPTG	Isopropyl β-D-1 thyogalactoside
*iv*AEB	*in-vitro* antagonistic, epiphytic bacteria
LAB	Lactic Acid Bacteria
MRS	De Man, Rogosa, and Sharpe medium
MS	Mesophile
PBS	Phosphate buffer solution
PW	Peptone water
PY	Psychrotroph
RIF	Rifampicin
STEC	Shiga-toxin producing *Escherichia coli*
TSA	Tryptic soy agar medium
TSB	Tryptic soy broth medium
VRBA	Violet red bile agar medium.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

INTRODUCTION

The consumption of fresh fruits and vegetables has steadily increased in the last three decades as a consequence of a better awareness of the health benefits provided by these commodities, especially after campaigns such as the five-a-day and nine-a-day programs led by the federal governments in the U.S. and U.K. *(52, 135)*. This increased demand has led producers to intensify their production, extend their distribution, and open new commercial channels. Unfortunately, this growth of the fresh produce industry has led to undesirable consequences, including a surge in the quantity and magnitude of foodborne disease outbreaks associated with the consumption of contaminated fresh produce *(255)*. As a result, consumption of fresh fruits and vegetables is now classified as a high-risk factor for gastrointestinal illness contagion *(135)*.

The contamination of fresh fruits and vegetables with foodborne pathogens has been linked to their production. Bacterial pathogens contaminating produce at pre-harvest are frequently related to the irrigation water, the cultivating soil, the use of soil amendments, human handling, and carrier animals *(23, 50, 131)*. The produce industry relies mostly on chemical contamination to reduce or eliminate pathogens in raw produce since other treatments, including pasteurization, blanching, and cooking would affect their desired freshness quality *(98, 163)*. Nonetheless, chemical antimicrobials are only effective when they can directly contact the pathogen, and this is often obstructed since pathogenic bacteria can reside in crevices and spaces where chemicals cannot reach, or be protected within biofilms *(4, 214)*. Food irradiation has been effective in reducing internalized

pathogens but unfavorable quality changes in particular commodities and partial consumer disapproval have limited its use in fresh produce *(42, 121, 210, 237)*.

Preventing food contamination is the ultimate goal in any food safety plan. For this, producers have followed numerous recommendations, rules, and guidelines to prevent the initial contamination with relative success. However, despite all efforts, foodborne disease outbreaks continue to occur *(49, 98, 110, 131, 135, 255)*.

The presence of pathogens after harvesting reveal their ability to survive in the fruit or vegetable surfaces despite the austere nutritional and environmental restrictions imposed by growers, the FDA and other government agencies to keep produce healthy. Nevertheless, enteric pathogens such as *E. coli* O157:H7 and *S. enterica* have overcome these challenging conditions and persist in fruit and vegetable surfaces by mechanisms not fully understood *(4, 35, 135)*. Some strategies demonstrated by these pathogens include their ability to migrate to the stomata and to the stem and bloom scars, and to localize spaces with high nutrient availability such as wounds, cuts, or bruises. Other pathogen attributes include their ability to form biofilms and provide protection against adverse environmental conditions *(19, 155, 214, 251, 258, 266)*.

The survival of bacterial pathogens in the produce surface is also influenced by the presence of epiphytic microorganisms *(19, 173)*. These microorganisms comprise the general microbial population of the plants and are well-established from early stages in plant development *(195)*. These indigenous microorganisms form a complex system in constant adaptation to their changing environment *(173)*. The ability of epiphytic bacteria to easily adapt to the fluctuating environment is noticeable since wholesome fruits and

vegetables can frequently contain a substantial number and variety of microorganisms at the moment of consumption *(137, 144)*. The continuous coexistence of epiphytic bacteria have evolved into community interrelations to ensure their subsistence *(218)*. Some of their community survival mechanisms include beneficial (agonistic) or adverse (antagonistic) interactions. The presence of enteric pathogens on produce surfaces have triggered a response from the epiphytic bacteria, including agonistic and antagonistic actions, at least under controlled experiments *(43, 47, 90, 144, 172, 218)*.

There is a current need of alternative antimicrobial agents for its use in fresh produce *(144)*. The use of epiphytic bacteria to reduce or eliminate pathogens would constitute an important progression in the antimicrobial treatments used in fresh produce, especially since these can be present before any other commonly used antimicrobial treatment can be applied. However, it is crucial to conduct exhaustive studies involving the analysis of the pathogen and epiphytic bacteria interactions and their responses at proximity *(99, 137, 166)*.

The objectives of the present study were: (1) to evaluate the microbiological content of fruits and leafy greens, (2) to identify antagonistic bacteria from their epiphytic bacterial community, and (3) to determine the interactions between epiphytic bacteria and enteric pathogens on the surface of fruits and leafy greens.

REVIEW OF LITERATURE

Several processing steps involved in the production of fruits and vegetables have been documented as risk factors for their contamination with foodborne pathogens *(32)*. To determine how some agricultural practices are involved in this contamination, it is important to know what those practices are, to further identify those that might increase the risk during the pre- and post-harvest processing. To illustrate this, three fruits and three leafy green commodities, previously involved in foodborne outbreaks, and presenting comparable and particular production practices were selected and further investigated.

Leafy green production

According to the Food and Agriculture Organization of the United Nations (FAO) *(110)* the term leafy greens includes "all vegetables and herbs of a leafy nature and of which the leaf (and core) is intended to be consumed raw." This comprises all varieties of lettuce, spinach, cabbages, chicory, and leafy herbs (including cilantro, basil, and parsley). Leafy vegetables are considered highly nutritious due to their significant content of dietary fiber and minerals, including Ca, P, Fe, K, and Na, and vitamins, including pro-vitamin-A, B_1, B_2, and C *(168)*. Their flavor, textures, aromas, nutritional content, and versatility have motivated their consumption around the world. The following is a summary of the production characteristics of three of the most commonly consumed leafy greens.

Spinach (*Spinacia oleracea*)

Spinach (*S. oleracea*) is an annual plant species belonging to the *Chenopodiaceae* family. Different varieties are produced worldwide including smooth and crinkled

varieties, based on their leaf shape and roughness *(212)*. Nowadays, the production of spinach is one of the largest in the leafy green industry. China is the largest producer, harvesting 20 million tons per year. The U.S. follows as the second worldwide producer with approximately 400,000 tons. Other countries, including Turkey, Japan, and Indonesia, are also significant spinach producers *(111)*. In the U.S., California produces the highest volume of spinach per state, cultivating 73% of the total spinach produced in the country. Texas and Arizona have also contribute significantly to spinach production, and16 other U.S. states are considered small producers *(278)*. In 2012, a total of 1,109 farms reported spinach as their main crop produced *(278, 279)*. About 94% of the spinach consumed in the U.S. is nationally produced. Imported spinach comes primarily from Mexico. Canada is one of the main exporting countries of U.S. spinach *(279)*.

Fresh spinach consumption is rising in several developed countries including the U.S., Canada, and several countries from the European Union. In the U.S. only, the averaged consumption reached 2.2 pounds per capita in 2006, where 75% of all marketed spinach is consumed raw. The commercialization of triple-washed, cello-bagged spinach has contributed to the rise in raw spinach consumption. Triple-washed and baby spinach are two of the fastest growing segments of the packaged salad industry, accounting for 10% of the groceries sales in a $2 billion fresh-cut salad industry *(278)*. Their popularity is enlarged mainly due to its convenient presentation, considered a ready-to-eat fresh product *(279)*.

Spinach is a cool-season crop of rapid growth, able to withstand frosts. The optimal temperatures for spinach cultivation are between 15 and 18 ºC, but it can also grow at

temperatures ranging between 5 to 30 ºC *(157)*. There are three types of spinach in the U.S. market: Savoy (wrinkled), semi-savoy, and smooth (or flat), according to their leaf flatness *(157, 278)*. Although the spinach produced in the U.S. was traditionally cultivated during the colder months of the year; nowadays, it is cultivated all year long, mainly in the coastal areas of the country. Its production at suboptimal environmental conditions extends its cultivation length, since cold weather delay its growth to the desirable harvesting size *(135, 157)*. In regions where cold winter and frosts affect crops, high-tunnels and greenhouses are employed.

Spinach seeds are commonly planted directly into the soil, at about 1-2 cm of depth *(157)*. The planting beds vary in width depending on the intensification of their production, and the harvesting size required. For example, baby spinach is planted in wider beds with more seeds per area since the leaves do not require much space to reach the required size. The production of bulked spinach takes approximately 30 to 60 d, whereas baby spinach can be harvested in 20 to 40 d. Some older spinach, commonly used in the freezing and processing industries are harvested between 50 and 90 d after planting *(157)*. Spinach irrigation include sprinklers, drip tape hoses and furrow irrigation, or a combination of these. Spinach intended to be sold as fresh-cut salad mixes is triple-washed and bagged. The baby spinach is harvested mechanically. Automatic equipment is used to cut spinach by the stems and to deliver the leaves into truck beds for transportation to processing areas. Post-harvest processing might include some soil and debris removal, washing, and disinfection, including chlorinated water baths, and one or two rinses in potable water baths *(122)*. After washing, dripping water is removed using centrifugation

and aeration to prepare the spinach for the packaging process. Washed spinach can get mixed with other leafy greens for mixed salad processing, before it is sealed in cello bags. Spinach is also commercialized unwashed after being hand-harvested and tied in bunches, or only clipped and packed in bulk. Refrigerating spinach is a common practice following washing and during transportation since the harvested leaves would spoil at warm temperatures *(122)*.

Curly endive (*Cichorium endivia*)

Endive (*C. endivia*) is a popular leafy green in Europe, although, with the internationalization of food commodities, its production and consumption has increased in the U.S. in recent years. Endive is part of the family *Asteraceae* and of the genus *Cichorium* that comprises two species: *C. endivia* and *C. intybus (280)*. The first is mainly a cultivated leafy vegetable, while *C. intybus*, known as chicory, can be found in natural environments or cultivated primarily for their roots, which are used to extract inulin and as a coffee substitute *(174)*. Leafy endive varieties include escarole and curly endive. Both varieties present leafy configurations although escarole leaves are broader. It is mainly consumed mixed in salads, and combined with other leafy greens due to their slightly bitter flavor. In the U.S., it is mostly produced in California, whereas Oregon, Washington, Texas, Michigan, Pennsylvania, Connecticut, and Maine, produce endives at a smaller scale *(280)*. Some important nutritive characteristics are attributed to endives, including high levels of antioxidants, minerals, pro-vitamin A and vitamins (B1, B2, and C) *(247, 286)*.

Endive cultivation is comparable to lettuce, although endives are more tolerant to heat and cold weather changes. Seed planting occurs in the early spring, or early fall, and greenhouse cultivation and transplantation can be used to produce endives earlier in the season *(181)*. Endives, as other leafy greens, require high levels of nitrogen in the soil. Therefore, chemical fertilization is a common agricultural practice *(3)*. Tender and light green colored endive leaves are preferred, while older, dark green colored leaves are rough and bitterer. The plant is commonly harvested by hand, cutting the whole head and removing some outer leaves. The head can be tightened and packed in 9- or 12-count waxed carton boxes. The boxes are transported and stored under refrigeration since the plant is highly sensitive to hot temperatures, and damaged leaves would turn an unattractive brown color if exposed to warm temperatures *(181)*.

Parsley (*Petroselinum crispum*)

For culinary purposes, parsley (*P. crispum*) is classified among the group of leafy spices known as herbs, which comprises "any vegetable products or mixtures, free from extraneous matter, used for flavoring, seasoning or imparting aroma in foods"*(109)*. Parsley is considered an aromatic herb, often used to garnish food, and it is the most consumed fresh herb in the U.S. *(109)*. The growth in the demand of parsley within the herb market is evident as it is no longer a seasonal herb as in the past, but parsley is currently sold all year long *(252)*. Botanically, it is considered a biennial plant belonging to the *Apiaceae* family. There are three varieties of parsley cultivated in the U.S. The common parsley (var. *crispum*), with curled leaves, commonly used as garnish; the flat leaved parsley, also known as Italian parsley (var. *neapolitanum* Danert), used in sauces,

soups and stews; and the less known turnip-rooted parsley, from which roots are sold in specialized markets *(253)*. Parsley can be harvested gradually or all at once. Long petioles are preferred for bunching since most of the crops are harvested by hand. Parsley is also considered a good source of vitamins, and as an antioxidant, such as apigenin, which is a flavonoid with antioxidant and anti-inflammatory properties studied for its potential anti-cancer activity *(133, 268)*.

Fruit production

The definition and classification of fruits and vegetables is dissimilar among areas of study. In botany, based on their anatomical features, fruits are the mature ovary including carpel tissues in part or whole. Many fleshy fruits, important in human consumption, also develop mature fruit tissues with edible or non-edible pericarp, known as peel, skin or rind. Other structures, such as non-fleshy and dry cereals are also considered fruits, although their classification exclude them from the general classification of a fruit *(119)*. However, for commercialization and classification purposes, fruits that are not sweet in flavor are commonly included in the vegetable section, for example, peppers, and tomatoes *(106)*. According to the Food and Agriculture Organization of the United Nations (FAO), fruit crop classification includes only those fruits and berries from permanent crops, harvested from trees, bushes, and shrubs as well as vines and palms. Although botanically and due to its sweet flavor, melons should be considered fruits, the FAO categorize them as vegetables since these are temporary crops *(106)*. For purposes of this review, the botanical definition is considered to describe fruits, including cantaloupes, peppers, and tomatoes.

Fruits are nutritious plant-origin products that are delicate to transport and handle, and have a short shelf life. Fruits are considered highly perishable once harvested. The industry uses different protective procedures to preserve the quality and extend the shelf life of fruits including but not limited to unripe harvesting, mechanical harvesting, chemical application, specialty packaging, and temperature and humidity control. Ripening is desirable for quality purposes, improving the aroma, flavor, and nutrient content. Nonetheless, during ripening, the susceptibility of the fruits to pathogen growth and spoilage increases along with some unfavorable characteristics, such as softening *(119)*. The water content in fruits ranges from 70 to 90% of their weight and contain variable degrees of minerals, vitamins, and sugars. The content of fat and protein is insignificant with the exemption of avocados *(106)*. Differences in the pH of the fruit flesh are related, to some extent, to their sugar profile and organic acid production *(128)*. The following is a general summary of three common fruit products, produced and consumed worldwide. All have been implicated in recent foodborne outbreaks.

Cantaloupe (*Cucumis melo* var. *cantaloupensis*)

Cantaloupe (*C. melo cantalupensis*) is one of the several existing varieties of melons, easily differentiated by its characteristic netted rind. The "melon" term includes watermelons, honeydew melons, casaba, Persian melons, muskmelons and cantaloupes. China is the largest producer of melons, producing nearly 15 million tons every year. Turkey, Iran, Egypt and India also produce significant amounts of melons, and the U.S. produces about 1 million tons annually *(111)*. Cantaloupe melons belong to the *curcubitaceae* family, which also includes cucumbers, pumpkins, squash, gourds, and

other melon plants. Cantaloupe fruits are varied in shape, rind, and color, and hybrid varieties have been developed using cross-pollination *(256)*. They are an important source of vitamins A, and C, and minerals, including potassium, copper, and magnesium. The flesh and seeds are edible and contain nutritive oils. The cantaloupe plant is temporary, meaning that replanting is necessary after each production season *(107)*. Cantaloupe plants grow as trailing vines, and develop better at higher temperatures; thus, they are cultivated in the warmer months of the year *(107)*. Irrigation practices used in cantaloupe production include furrow and drip, and the bed should be built sufficiently high to avoid irrigation water reaching the developing fruit, to help prevent rotting and ground spots *(132)*. The harvested fruits have a shelf life of approx. 15 days. Refrigeration is recommended to preserve their quality characteristics of sweetness and texture, after full maturity is reached. Ripening is associated with stronger aroma and softness of the rind, mostly around the stem scar *(239)*. Cantaloupes are manually harvested, and workers will enter the fields within 10–15 days to select and pull the cantaloupes with desired ripeness and similar size. The fruits are transported in bulk or packed in carton or wood crates. Post-harvesting processes intended to remove field heat and extend shelf life include cold forced air, cold water baths, and ice cooling. Hot water dips might also be applied to control fungal rotting *(132, 152, 184)*.

Tomato (*Solanum lycopersicum*)

Tomatoes (*S. lycopersicum*, syn. *Lycopersicon esculentum* Mill.) are part of the family *Solanaceae*, native to Central and South America *(259)*. It is considered one of the most commonly cultivated crop in the world *(93)*. Tomato popularity is related to its

characteristic flavor, nutritive qualities as source of vitamin A and C, and its intense red color *(241)*. It is estimated that approximately 113 million tons are produced worldwide every year *(93)*. There are two agronomic types of cultivated tomatoes: determinate and indeterminate depending on their growth behavior. Determinate tomato plants grow in bushes of determined sizes, flower around the same time within the cultivar, and fruits develop within a relatively similar time. Indeterminate varieties grow as vines and would need several harvesting times since their fruit develops at different times, while the plant keeps growing to a variable size during its cultivation *(241)*. There is a large number of varieties for fresh production, varying in flavor (sweet to sour), color (yellow, orange, red, and purple) and size (from dwarf tomatoes, such as cherry varieties, to large-size varieties, such as the beefsteak variety). Based on their morphological characteristics, there are five main types of cultivated tomatoes: Classic round, cherry and other dwarfs, plum and baby plum, beefsteak, and vine tomatoes *(91)*.

The cultivated tomato is a perennial crop, and commonly grows in temperate climates, since is highly susceptible to temperatures below 12 ºC, and requires 90–120 frost-free days for the full development of its fruit *(91)*. Tomato production is divided into processing tomatoes, used for canning and commercial pastes, and freshly-marketed tomatoes used for salads and as recipe additives *(91)*. There are numerous varieties of processing and fresh-market tomatoes with diverse growth habits. Plants of processing tomatoes are grown in open fields, directly planted, and do not require staking. Fresh-market varieties are grown both in greenhouses and open fields. Those grown in greenhouses are generally indeterminate varieties and require trellising, while varieties

grown in open fields are determinate, and could require staking to keep the fruit away from the ground. Both start by transplanting seedlings. Tomatoes are grown in a wide range of soils and production systems. Covering rows with plastic or similar material is a common practice for small young plants to protect them from drastic environmental changes *(93)*. Drip and furrow irrigation are the two most common irrigation systems used for this crop. Overhead sprinkling is also used to provide water and to reduce heat damage in the plant during hot days *(93)*. Harvesting is done by hand in fresh-market tomatoes, while processing tomatoes are harvested mechanically *(91)*. Tomatoes are climacteric fruits that can be harvested immature and then ripened under controlled environmental conditions of temperature and humidity. Although maturation in the plant improves their overall color and flavor qualities, tomatoes are harvested at some degree of greenness to facilitate harvesting and reduce rotting during transportation *(241)*. During the harvesting process, the fruits might be dipped in large tanks filled up with water to avoid injury. Postharvest practices can also include washes in tap or chlorinated water, sorting, grading, and packaging in cardboard boxes. Refrigeration conditions during storage (13–15 ºC), and 90–95% relative humidity (RH), can extend their shelf life from 4–7 weeks. If necessary, the fruits can be treated with ethylene to accelerate and homogenize ripeness *(93)*.

Pepper (*Capsicum annuum*)

Peppers are grown in most countries for spice and vegetable uses. Worldwide, China is the largest producer of pungent and non-pungent peppers producing approximately 13 million metric tons, followed by Mexico, producing 2 million metric tons. Turkey,

Indonesia, and the U.S are also important pepper producers *(33)*. Peppers vary in size, shape, color, flavor, and pungency or spiciness. Their nutritional content also varies by type, color, size, and ripeness, with green peppers being higher in carotenoids, and mature and dry peppers higher in pro-Vitamin A content. Fresh peppers are an important source of vitamin C. Besides being used as a condiment, pungent peppers have been used as a natural remedy for inflammation, arthritis, and itching since the capsaicin that produces their pungency, might interfere with the pain sensation by depleting certain neurotransmitter compounds *(33)*. The capsaicin compound is produced by the plant as a deterrent mechanism against mammal predation. Birds are not sensitive to this substance; hence, they eat the fruit and disperse the seeds contained in their droppings *(270)*. Pepper heat, pungency, or spiciness, is expressed in Scoville heat units, a system based on trained panel sensory tests *(33)*. Some varieties, including bell peppers, produce none to very low levels of capsaicin. Human selection, genetic mutations, and breeding, have influenced the preservation of their sweet flavor and absence of spiciness *(27)*. Peppers are categorized as wild, domesticated, and cultivated depending on the degree of human attention necessary for their growth. Within the cultivated peppers, popular pungent varieties include jalapeño, and serrano peppers. Jalapeño, also spelled jalapeno, are conical shaped, light to dark green, and red when matured. The skin might show some netting, called corkiness, and are usually produced for canning, pickling and, on a smaller scale, for fresh consumption as a spice and condiment *(33)*. Jalapeño peppers are cultivated in open fields and greenhouses. Seeds are germinated in soil trays in greenhouses, and irrigated using sprinklers or hand-held spray tanks. They are

transplanted after the first development of leaves to furrows *(240)*. The pepper plants are transplanted in previously prepared rows at a shallow depth. Furrow and drip are the most common irrigation techniques used, with drip technology preferable due to its efficiency in the water use *(146)*. Soil fertilization using chemicals or composted manure and application of pest control chemicals are common agricultural practices to improve yield and control insect invasion, respectively *(240, 285)*. The pepper plants are commonly staked to avoid contact of fruits with the cultivating soil and ground water *(240)*. The fruits are harvested progressively, continuing for several weeks until the fruit reaches the desired size, and color *(28)*. It takes approximately 50–60 days for the first fruits to reach the harvesting qualities of color and size, and this time varies by cultivar and environmental conditions *(28)*. During the last harvesting days, the whole plant is commonly plucked and any fruit remaining is harvested *(113)*. Peppers are harvested by cutting or breaking the stem close to the fruit, since pulling risks damaging the plant branches *(178)*. Washing treatments are unadvised since they would promote spoilage, unless the fruit is kept below 10 ºC until sold *(28)*.

Contributing factors during the contamination with foodborne pathogens in fruits and vegetables

The possible sources and practices identified as contributing factors for foodborne pathogen contamination in fruit and vegetables before, during, and after harvesting are further reviewed.

Pre-harvest contamination

Produce in the growing fields can become contaminated with foodborne pathogens through several routes related to one or more reservoirs. Water, soil, animals, human

handling, feces, and air are some of the principal contamination sources during the harvest period *(30, 32)*.

Water has been described, along with soil, as one of the principal vehicles of pathogenic microorganisms that contaminate fruits and vegetables *(14, 50, 206, 258)*. The irrigation water and cultivating soil can act as a niche and vehicle of bacterial pathogens, viruses, and parasites *(118, 287)*. Irrigation water, as an important cause of pre-harvest pathogen contamination, was evident during the largest *Salmonella* outbreak related to produce in the U.S. *(57)*. Environmental or agricultural events causing flooding such as heavy raining, can distribute pathogens to the fields and ultimately to the crops *(171)*. Contaminated water from sewage, wildlife living spaces, animal production plants, and water bodies (rivers and lakes) can reach the cultivating soils during these events *(22, 50, 51)*. Fruits growing in trailing vines, such as melons, might be at higher risk of contamination by runoff water or by direct contact with contaminated soil transferring pathogens to the fruit surface *(31)*. Although other fruits, not developed in the ground might not contact ground water directly, they can become contaminated with water splashes or aerosols formed during and after raining episodes *(83, 84)*. The cultivating soil, contaminated through flooding, can hold bacterial pathogens for extended periods of time, with the moisture level and the desiccation rate given as important determinants for extended pathogen survival *(51, 171)*. For example, artificially contaminated soil supported *Salmonella* presence for at least 45 days in moistened soil *(126)*. Other authors have determine survival of pathogens for several months *(216)*.

One more source of pathogens transferred to the cultivating soil are soil amendments. The utilization of biosolids, such as composted manure, is a common practice in agriculture. Manure improves the cultivating soil quality by providing supplemental nutrients including minerals, phosphates, and nitrogen *(285)*. For its use in agriculture, manure is processed by different means to eliminate pathogens potentially present in the raw manure, which include heat and solar drying as well as composting *(206)*. Nonetheless, the use of incorrectly composted or raw manure as fertilizer can lead to microbial contamination of the crops *(110, 285, 293)*.

Animal presence in the cultivating fields has also been described as a risk factor in the contamination of produce during pre-harvest. Grazing animals, birds, and other wildlife have been reported as carriers of human pathogens *(32, 44)*. Wild and domestic animals entering the fields and nearby animal production farms can serve as a source and vehicle of pathogens. These can directly introduce the pathogens into the fields through fecal depositions, which can be transported by air or water *(192)*. Animal production farms close to agricultural fields are a constant threat of foodborne contamination since some of these animals serve as reservoirs of human enteric pathogens. For example, beef cattle are recognized as an important source of *E. coli* O157:H7 and other pathogenic *E. coli* shed in feces, principally during the warmest months of the year *(161, 243)*. Sheep, goats, water buffalo, and deer can also carry this pathogen *(44, 293)*. Although swine is not a common source, feral swine have also been tested positive to *E. coli* O157:H7 and suspected of pathogen contamination on leafy greens *(44, 192)*. Other pathogens, such as *S. enterica* have multiple animal sources *(281)*.

Insects have also been reported as possible carriers of enteric pathogens. However, the direct association of insects with produce contamination in the fields is still under investigation *(267)*. Filth flies obtained from beef feedlots have been tested positive for *E. coli* O157:H7 *(250, 267)*. A study using house flies demonstrated the transmission of *E. coli* O157:H7 to spinach leaves under laboratory settings. Nevertheless, more research is necessary to demonstrate the direct transportation of enteric pathogens by insects into the fields and most importantly, to the edible portions of the plants *(267)*.

There are certain environmental conditions that have also increased the risk of pathogen contamination in the cultivating fields. Weather changes, increased environmental humidity, wind speed, dust, rain, and environmental catastrophes (e.g., hurricanes) have been associated with the dispersal of bacteria, viruses and pathogenic fungi to plants, animals, and humans *(51)*. Air samples taken from cattle feedlots in close proximity to cultivating fields have resulted positive to *E. coli* O157:H7, verifying that air contamination is an important risk factor for the transmission of enteric pathogens to crops. However, the entire air-plant contamination route and the impact extent on fruit and vegetable contamination have not been fully proven *(29)*.

One more source of possible contamination in fruits and vegetables are the employees, equipment and utensils, that can transport and deposit pathogenic bacteria directly to the produce. Workers involved in the cultivation, harvesting, and packing of fresh fruits and vegetables might act as carriers of pathogens if personal hygiene rules and good agricultural practices are not strictly followed. Similarly, unsanitary utensils and

equipment used in different cultivating practices can transport pathogenic bacteria into the fields or directly to the produce *(131)*.

Post-harvest contamination

Pathogen contamination in fruits and vegetables might initiate in the growing fields and expand during collecting and handling of fruits and vegetables, or it can start during the post-harvest processes, which include cooling, packing, storing, shipping, distributing, retail marketing, and preparation of foods in commercial and home kitchens *(31, 144)*. Not only can pathogens be dispersed but other detrimental bacteria might increase during post-harvest procedures *(144, 245)*. Although the processing steps in the farm-to-table distribution chain for different fruits and vegetables are considerably different, there are similar sources of possible contamination with human pathogens. These include the use of contaminated water, unsanitary handling, and fecal contamination by in-line workers, introduction of pests, wild, and domestic animals to the processing areas, dust, and insects as well as contaminated utensils, processing equipment, trucks, and facility structures *(32, 293)*. Cross-contamination of the fruits and vegetables increases under deficient sanitation practices or without proper sanitation programs established. Improper packaging, high humidity, and temperature abuse can also support the proliferation of pathogens during processing, and storage *(32)*.

The use of water for post-harvest processes is highly variable depending on the commodity, customary practices, and intensification of the process. For example, processing practices of some commodities do not involve water; this is the case with Californian cantaloupes, which are directly packed in the fields without the direct use of

water. On the other hand, other products can require triple-washing (spinach) and involve several steps that require high volumes of water *(265)*. Some processing steps where water is essential include baths or rinses to reduce heat from the field (hydrocooling) or to remove soil and debris. Other processing steps require receiving containers containing water to reduce bruising; water solutions to apply antifungals or other quality enhancers in sprays, mists or in baths. Ice and iced-water is often used during storage and transportation to preserve product freshness *(209)*. Washing utensils, equipment, storage containers, truck beds, conveyor belts, crates, and processing facilities also include the use of water. The large amount of water required for some processes have forced producers to reutilize water. To avoid the propagation of pathogens and rotting microorganisms in recirculating water, chlorination is commonly used. This treatment is regularly used to treat postharvest cooling water, and water used for washing or rinsing baths *(264)*. Chlorine, as sodium or calcium hypochlorite is inexpensive and convenient, and when included as part of the sanitation program, it is an effective chemical that controls bacteria loads in large or small processing plants. Nonetheless, the use of chlorine has some disadvantages including its inactivation in the presence of organic matter and the possible formation of undesirable substances, such as trihalomethanes and chloramines *(249, 294)*. The use of improperly chlorinated water can lead to the transference of pathogens to fruits and vegetables *(136)*. Submerging fruits, such as cantaloupes and tomatoes for hydrocooling, into contaminated water baths, can lead to pathogen internalization. Water can become infiltrated in fruit due to the heat differential between the fruit and water, mainly through the stem scar *(136, 187, 308)*. This is one of the reasons why hydrocooling

is being replaced by other cooling methods such as pressurized cool air to remove heat and improve the produce shelf life *(31, 131, 132, 265)*.

Workers are another important source of contamination during the processing chain *(30)*. Some commodities require manual handling during harvesting, packaging, sorting and cutting. The contamination of produce through manual handling is related to the dismissive attention of personal hygiene and improper sanitization of hands, gloves, and garments. Hand washing, proper and hygienic restroom facilities, management programs to control infected workers from entering the plant, and other similar measurements should be rigorously applied to avoid employees cross-contaminating the produce during handling *(32)*.

Facilities in general can also act as sources of contamination if they are not properly cleaned and sanitized. The floor and walls can harbor pathogenic microorganisms if these are not properly sanitized. Equipment can also act as a source and niche for pathogenic bacteria, especially those prone to water retention and harboring of other nutrients used by pathogenic bacteria, including rubber or foam-covered conveyor belts made of absorbent material. Storage facilities where moisture and temperature are suitable for bacterial growth are particularly hazardous. High moisture, moderately high temperature, and atmospheric characteristics inside packaging, can contribute to the survival and proliferation of pathogens during storage and transportation *(95)*.

After arriving at the retail market, faulty practices can also lead to cross-contamination of produce, especially when raw products such as meat, and poultry are in close proximity to the fruits and vegetables during meal preparation *(32)*. Unhygienic

practices in restaurants can also lead to the contamination of pre-cut fruit, through transference of pathogens from the food handlers. Furthermore, improper holding temperatures of pre-cut fruits in salad bars, and in commercial and home kitchens, can lead to the proliferation of pathogens *(165)*. Improper washing and sanitizing of utensils and other surfaces when preparing fresh cut produce can also lead to pathogen cross-contamination *(225)*. Waxes applied in the surface during processing for preventing mold might have a detrimental effect for pathogen removal, since this can interfere the cleaning and sanitizing action of commercial soaps before meal preparation. All these conditions during acquisition, meal preparation, and serving have been identified as causative factors leading to several foodborne outbreaks *(56, 203, 288)*. Any sanitizing activities can contribute to safety in commercial and home kitchens with varied effect *(13, 153)*.

Prevention of produce contamination

The prevention of any possible contamination, the elimination of hazards, and the reduction of contamination risk should be primary goals of the fresh produce industry along with their overall mission to supply wholesome products to consumers. Several recommendations, rules, and good practices guidelines have been developed to help producers prevent contamination *(49, 98, 110)*. Good agricultural practices (GAP) following the hazard analysis and critical control points (HACCP) approach, supply-chain controls, good manufacturing practices (GMP), and risk analyses have helped prevent and control fruit and vegetable contamination with relative success *(135)*. From planting seedlings to harvesting, and even between each growing season, all practices must follow specific strategies to avoid possible contamination of the products. Some GAPs are

specifically adapted to each production system and product and are intended to minimize risks of contamination. GAPs include: Selecting fields with minimal risk of contamination for fruit and vegetable production, water source control and testing, manure composting and testing, pest control, and restrictions on entrance of humans and animals. Other GAPs might also involve surveying workers' health status, personal hygiene practices, and behavioral training. Also included in the GAPs is the location and maintenance of sanitary facilities for employee use. Other practices incorporated in GAPs are the sanitization of equipment, utensils, tractors, and any other machinery in contact with the products. GAPs can also include product-specific harvesting practices that prevent or control the potential pathogen contamination *(85, 108)*. During, and after harvesting, these rules and recommendations follow the same goal of preventing contamination and minimizing the risks during the entire food chain. Packing houses and processors also use Sanitary Operating Procedures (SOPs) and GMPs, including facilities sanitary design, pest control, sanitation plans, workers hygiene and health, and temperature control. Some production plants have also developed a system to trace and control distribution for possible recall of products *(108, 185)*. Nevertheless, after the development and implementation of commodity-specific guidelines and after creating food safety systems for the entire produce operation to prevent the contamination of fruits and vegetables, foodborne outbreaks related to produce contamination continue to occur *(135)*. Some important outbreaks and pathogens related to produce are further reviewed.

Worldwide impact of fruit and vegetable foodborne pathogen contamination

In 2010, The World Health Organization estimated a total of 600 million foodborne illnesses which included 420,000 deaths worldwide. In total, 31 agents causing different diseases were part of this evaluation, and included 11 diarrheal diseases, seven invasive infectious diseases, 10 helminths, and three chemicals *(297)*. Foodborne diarrheal diseases caused 55% of the reported deaths, particularly non-typhoid *S. enterica* infections. The most common foodborne causing agents were diarrheal agents, mainly norovirus and *Campylobacter* spp. Forty percent of the foodborne disease burden involved five-year and younger children, mainly in low-income regions *(297)*. On a global scale, some of the largest outbreaks have been related to contaminated produce, causing extensive damage to public health. For example, during 1996, Japan suffered of one of the largest foodborne outbreaks in the human history. White radish sprouts contaminated with *E. coli* O157:H7 sickened more than 9,000 children and resulted in about 400 hospitalizations *(193)*. On a smaller scale, yet highly significant, in 2008 a foodborne outbreak related to contaminated peppers reached 1,442 cases of salmonellosis in 44 U.S. states *(57)*. In 2011, a multinational outbreak related to cucumbers contaminated with *E. coli* 104:H4 originated in Germany, causing 798 cases of hemolytic uremic syndrome (HUS) and sickened another 2,294 people from 15 countries *(295)*. Recently, in 2015, a foodborne outbreak related to cucumbers contaminated with *S.* Poona imported to the U.S. resulted in 888 salmonellosis cases distributed in 29 U.S. states *(81)*

Since not all diseased people have access to medical assistance or seek medical help, nor all patients with foodborne illness symptoms get a diagnosis of the causative agent,

and not all diagnosed cases are reported to surveillance agencies; therefore, the foodborne outbreaks reports underestimate the real impact of the fruit and vegetable contamination to the public health *(297)*.

The Centers for Disease Control and Prevention (CDC) estimate the occurrence of 48 million foodborne illnesses every year in the U.S. *(61)*. Almost 50% of the total foodborne outbreaks in the U.S. have been attributed to produce *(221)*. Table 1 summarizes the final burden of multistate foodborne outbreaks linked to fruits and vegetables, resulting in confirmed cases, hospitalized, and deceased persons, documented by the CDC in the U.S. during the last decade. The data is not inclusive; nonetheless, it illustrates the extent of some produce-borne outbreaks and the magnitude of the problem.

TABLE 1. U.S. multistate foodborne disease outbreaks related to contaminated fruits and vegetables from 2006 to 2016

Year	Pathogen	Commodity	U.S. States	Confirmed cases	Hospitalizations	Deaths	Reference
2006	*S.* Typhimurium	Tomatoes	21	183	22	0	*(54)*
2006	*E. coli* O157:H7	Spinach	29	199	31	3	*(53)*
2008	*S.* Litchfield	Cantaloupe	16	51	16	0	*(56)*
2008	*S.* Saintpaul	Raw produce (peppers, tomatoes)	44	1442	286	2	*(57)*
2010	*S.* Typhi	Mamey fruit (frozen)	2	9	7	0	*(59)*
2010	*E. coli* O145	Romaine lettuce (shredded)	5	26	12	0	*(58)*
2011	*S.* Panama	Cantaloupe	10	20	3	0	*(63)*
2011	*S.* Agona	Papaya	25	106	10	0	*(62)*
2011	*E. coli* O157:H7	Romaine lettuce	9	49	33	0	*(65)*
2011	*L. monocytogenes*	Cantaloupe	28	147	143	33	*(203)*
2012	*S.* Newport and *S.* Typhimurium	Cantaloupes	24	261	94	3	*(68)*
2012	*S.* Braenderup	Mangoes	15	127	33	0	*(67)*
2012	*E. coli* O157:H7	Spinach, spring mix blend	5	33	13	0	*(69)*
2013	*C. cayetanensis*	Salad mix	25	631	49	0	*(70)*
2013	*S.* Saintpaul	Cucumbers	18	84	17	0	*(71)*
2013	*E. coli* O157:H7	Ready-to-eat salads	4	33	7	0	*(72)*
2014	*C. cayetanensis*	Cilantro	19	304	7	0	*(77)*
2014	*S.* Newport	Cucumbers	30	275	48	1	*(204)*
2015	*S.* Poona	Cucumbers	39	888	191	6	*(81)*
2016	*C. cayetanensis*	Cilantro (suspected)	31	546	21	0	*(76)*
2016	*L. monocytogenes*	Salad mix	9	19	19	1	*(80)*
2016	*L. monocytogenes*	Frozen vegetables	3	8	8	2	*(79)*

Current surge of foodborne disease outbreaks related to fruits and vegetables

The human diet is determined by food availability and affordability. Other factors such as cultural background and education, also play a role in the selection of nutrients *(150, 189)*. Fruit and vegetable consumption have experienced a constant growth in the last decades due to public demand. People are looking for an abundant supply of fruits and vegetables as part of a healthier diet at a reasonable price with good variety and accessibility all year long *(226)*. This demand has led the produce industry to improve and adapt its production, amplifying distribution channels, and opening the market to new international supply chains *(224)*. Consequently, internationalization of fresh produce has also experienced a growing explosion in the last three decades. For example, in 1970, produce consumed in the U.S was principally supplied by national producers with the exception of bananas. Nowadays, the national consumption includes several imported commodities to guarantee year-round supply of tropical and exotic fresh fruits and vegetables *(120)*. Not only has the importation of fruits and vegetables increased, but national production has experienced an expansion in production and consumption. These market changes have forced all industries involved in the production chain to be in constant transformation *(120, 229)*. Production changes include intensive farming, with new and more drastic use of pesticides, the expansion of acreage, the use of newly introduced or genetically modified crops, improved agricultural practices, such as new irrigation methods, and harvesting technologies, faster transit from the fields to the packing houses, shorter processing, including direct packaging in the fields, and improved cold chains, extending shelf life, and allowing farther distribution with the use of improved

cold chain technologies. All these factors have amplified the availability of fresh produce, but its growth has come with some undesirable consequences. One of them is the increased amount and extent of foodborne outbreaks related to contaminated produce in the last decades *(255)*. For example, in the U.S., from the 48 million foodborne illnesses calculated to occur every year, nearly 50% are attributed to the consumption of contaminated produce *(61, 221)*.

Important foodborne outbreaks related to leafy greens

Although leafy greens is an ample group, the majority of the outbreaks have been related to lettuce, spinach, and salad mixes, while curly and escarole endives, kale, arugula, and chard are rarely linked to foodborne illnesses *(183)*. In 2006, bagged spinach was related to a large, multi-state outbreak of *E. coli* O157:H7 causing 199 cases confirmed, 33 HUS cases, and three deaths, in 29 U.S. states *(53)*. In 2010, contaminated romaine lettuce was the cause of 26 confirmed, and six possibly related cases of *E. coli* O145 infections in five U.S. states *(58)*. In 2012, an outbreak related to the consumption of organic spinach and spring mix blend caused 33 cases of *E. coli* O157:H7 infections. In the same year, romaine lettuce was linked to an *E. coli* O157:H7 outbreak where 58 cases were confirmed in nine U.S. states *(65)*. During the 1998-2008 decade, ten outbreaks of salmonellosis were linked to leafy greens caused by different serotypes including *S.* Enteritidis, *S.* Typhimurium, *S.* Newport, *S.* Javiana, S. Thompson, and *S.* Seftenberg *(140)*. Other pathogens related to leafy green-originated outbreaks include *Shigella sonnei* in parsley, *Cyclospora cayetanensis* in cilantro and salad mixes, and viruses including norovirus in lettuce and salads *(70, 77, 78, 198)*.

Important foodborne outbreaks related to fruits

Although other fruits have been also involved in several foodborne outbreaks, including strawberries, apples, and cucumbers, this review only includes cantaloupes, tomatoes, and peppers, important commodities recently involved in bacterial foodborne outbreaks.

Cantaloupes have been linked to several foodborne outbreaks. In the U.S., from 1973 to 2011, 19 outbreaks linked to cantaloupe were reported, causing 1,012 illnesses, 215 hospitalizations and 37 deaths *(288)*. In 1990, cantaloupes contaminated with *Salmonella* Chester caused 245 confirmed cases in 30 U.S. states *(197)*. In 1991, *Salmonella* Poona was the causative agent of an outbreak in the U.S. that extended to Canada, involving more than 400 confirmed cases of salmonellosis *(197)*. In 1997, *Salmonella* Saphra was the causative agent of an outbreak with 25 cases confirmed in the U.S. *(100)*. In 2000, 46 cases of salmonellosis were related to consumption of cantaloupe contaminated with *S.* Poona. The same year, three outbreaks including 47, 50, and 58 cases, involved the consumption of contaminated cantaloupes in the U.S. *(199)*. In 2004, an outbreak of *E. coli* O157:H7 was reported in the U.S. state of Montana, with several cases of HUS and TTP. In 2008, *S.* Litchfield was related to 51 cases of salmonellosis involving 16 U.S. states *(56)*. In 2011, another outbreak of *Salmonella* was reported, identifying *S.* Panama as the causative serotype of the 20 cases reported *(63, 68)*. In 2012, cantaloupes consumed in the U.S. were again involved in a large outbreak reporting 261 cases and three deaths; *S.* Newport, and *S.* Typhimurium were identified as the causative agents. Other than *S. enterica* and *E. coli* O157:H7 pathogens have been related to

outbreaks caused by cantaloupes, including *Campylobacter jejuni*, *Listeria monocytogenes,* and noroviruses *(34, 66, 288)*. One particular outbreak linked to cantaloupes contaminated with *L. monocytogenes* was of high concern in the U.S. in 2011, since it caused 147 listeriosis cases, and 33 deaths, being classified as the deadliest foodborne outbreak occurred in the country in more than 90 years *(203)*.

In the case of tomatoes, the U.S., has experienced several outbreaks, principally related to *Salmonella* contamination. In 1990, there were 176 salmonellosis cases confirmed, the causative agent was *Salmonella* Javiana *(201)*. In 1993, *Salmonella* Montevideo was related to a multistate outbreak with 100 salmonellosis cases confirmed *(201)*. In 1998, 86 cases were related to consumption of contaminated tomatoes in eight U.S. states. The causative serotype was *Salmonella* Baildon *(94)*. In 2002, *S.* Javiana contaminated diced tomatoes caused 141 salmonellosis cases. The contaminated tomatoes were consumed at a theme park in Florida, and most of the patients were children *(200)*. In the same year, *Salmonella* Newport caused 512 related cases after the consumption of round tomatoes. In 2004, *S.* Javiana in sliced Roma tomatoes caused 429 cases of salmonellosis *(201)*. During the outbreak investigations, other *Salmonella* serotypes were also related, including Anatum, Typhimurium, Thompson, Muenchen, and Group D untypable *(201)*. The same year, an outbreak of salmonellosis involving 125 cases was identified. The causative agent, and source were *Salmonella* Braenderup, and Roma tomatoes *(201)*. In 2005, tomatoes contaminated with *S.* Newport caused 72 confirmed cases of salmonellosis *(202)*. The same year, *S.* Braenderup in diced tomatoes caused 76 confirmed cases of salmonellosis in three U.S. states *(202)*. In 2006, two outbreaks related

to consumption of contaminated tomatoes were reported, the first one involved *S.* Newport, causing 115 confirmed cases, and was related to the previous outbreak from 2005 *(202)*. The second outbreak, resulted from the consumption of tomatoes contaminated with *S.* Typhimurium and caused 183 cases of salmonellosis in 21 U.S. states *(54)*.

One of the largest foodborne outbreaks reported in the U.S. was caused by peppers contaminated with *S.* Saintpaul. This outbreak was firstly attributed to tomatoes, and later to jalapeño and serrano peppers *(205)*. To date, the most numerous foodborne outbreak linked to *Salmonella* infections recorded 1,440 cases in 2008 including almost 300 hospitalizations and two deaths associated *(57)*. Although no more foodborne outbreaks have been related to hot peppers, several recalls have been reported due to contamination with *Salmonella* spp. found during routine monitoring *(213)*.

Pathogens linked to foodborne outbreaks in fruits and vegetables

Several etiological agents have been involved in produce contamination including parasites, such as *Cyclospora cayetanensis*, contaminating berries, lettuce and basil. *Cyclospora parvum* has been found in unpasteurized apple juice and *Giardia lamblia* can be found in sliced vegetables. *Fasciola hepatica* can contaminate watercress *(135, 293)*. Viruses such as Hepatitis A contaminate leafy greens, tomatoes, and strawberries; rotavirus is found in lettuce, and a Norwalk-like virus can contaminate salads and celery. Pathogenic bacteria, including *Aeromonas* spp., *Bacillus cereus*, *Campylobacter* spp., *Clostridium botulinum*, *E. coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*,

Shigella spp., *Vibrio cholerae,* and numerous serotypes of *S. enterica* have also contaminated different fruits and vegetables in the past *(135, 293).*

In particular, *S. enterica* and *E. coli* O157:H7 have jeopardized the public health, and harmed the reputation of the fresh produce industry. These bacterial pathogens are considered two of the most important human pathogens contaminating fruits and vegetables, causing numerous outbreaks. In 2012 only, salmonellosis cases reached 7,842 cases. Another 533 cases were related to Shiga-toxin producing *E. coli*, and together, these pathogens caused 2,500 hospitalizations. The majority of the *Salmonella* outbreaks and almost half of the *E. coli* O157 outbreaks were of foodborne origin, principally by consumption of contaminated produce *(64).* From 1998 to 2008 in the United States, 18 to 22.5% STEC O157 foodborne outbreaks were related to fruits and nuts, 19–31.5% to leafy vegetables and 1.1 to 1.7% to sprouts. In the same years, 0.1 to 48.5% of foodborne outbreaks of salmonellosis were related to fruits and nuts, 0.2 to 25.2% to leafy vegetables, 1.8 to 7.0% to sprouts, and 1.7–40.8% to vine and stalk vegetables *(221)*.

***Escherichia coli* O157:H7**

E. coli O157:H7 was recognized in the U.S. as an important foodborne pathogen after two foodborne outbreaks related to the consumption of contaminated beef hamburger patties in 1982 *(291).* Afterwards, *E. coli* O157:H7 presence in foods became a high public health concern since the disease can lead to life-threating conditions such as hemorrhagic colitis, HUS, and thrombotic thrombocytopenic purpura (TTP) and might cause the death of immunocompromised individuals as well as the elderly and children *(103, 104, 297).*

Generalities

The genus *Escherichia* belongs to the *Enterobacteriaceae* family which includes six species: *E. albertii, E. blattae, E. coli, E. hermannii, E. fergusonii,* and *E. vulneris (5). E. coli* was firstly isolated from human feces by Theodor Escherich in 1885 *(5)*. The majority of the E. coli serotypes are harmless, inhabiting the intestinal tract of human and warm blooded animals. However, certain serotypes can act as opportunistic pathogens, including the serotype O157:H7.

E. coli is a nonsporulating, facultative anaerobic, mesophilic microorganism *(284)*. The cell presents a rod shape with attached flagella, in a peritrichous arrangement *(142)*. Its optimal growth temperature is 37 ºC, but it can grow from 7 to 50 ºC. Some of the biochemical characteristics used for its identification include its inability to liquefy gelatin and to utilize citrate as a sole carbon source. *E coli* is also known for its catalase production and glucose and lactose fermentation with gas and acid production *(5, 142)*.

Classification and serology

The classification of Kauffman *(149)*, included the identification of the somatic (O) and flagellar (H) antigens. Diarrheagenic *E. coli* share particular virulence factors including plasmids and toxin production. They also have a similar preference for certain enterocytes and fall in similar O:H classification. Disease-related *E. coli* have also been classified based on clinical lesions as Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC), and diffusely adherent *E. coli* (DAEC) *(142)*. *E. coli* O157:H7 is the most important serotype included in the enterohemorrhagic group (EHEC)

(236). This classification is merely practical, since this subdivision of *E. coli* strains into specific groups and fails to address fundamental intrapathotype variation and interpathotype similarities *(89)*. Since the serotype related to the first outbreaks of *E. coli* O157:H7 did not present the toxigenic or invasive mechanisms seen in ETEC and EIEC, nor fever, which indicates an EIEC infection, and had caused profuse bloody diarrhea resembling gastrointestinal bleeding uncommon in EIEC and EPEC infections, the serotype could not be classified within those groups *(236)*. Later studies reported that strains in the O157 serotype included the expression of toxins similar to those found in *Shigella dysenteriae* (Shiga-toxin) named Stx1 and Stx2 with its variants a, b, and c *(138, 162)*. Thus, the EHEC group was first recognized and *E. coli* O157:H7 and was considered the most important serotype of the group. *E. coli* O157:H7 toxins were found toxic to vero-cultured cells (African green monkey kidney cells) and lethal to mice, thus the serotype was identified as vero-toxigenic *E. coli* (VTEC) and later was called Shiga-toxin producing *E. coli* (STEC). Other STEC serovars had been reported linked to foodborne outbreaks, and are considered important pathogens associated with food contamination *(175)*. Further differentiation by clonal analysis of serotypes positive to Stx genes have separated STEC into four groups: STEC 1, STEC 2, EHEC 1, and EHEC 2. *E. coli* O157:H7 and its non-motile related strains are enlisted in the EHEC 1 group, along with the serovar O55:H7, alleged antecessor of the O157 serovar and is recognized as the most common cause of STEC-associated human illness *(103, 292)*. The EHEC 2 group includes serotype O111 and O26. STEC group 1 includes several O types that do not carry the

intimin gene and the pathogenicity island for the enterocyte effacement (LEE); for example, O113, OX3, and O91. The STEC 2 group includes the serotypes O103 and O45.

Virulence factors

All STEC contain genes encoding the cytotoxins Stx1 and Stx2 while some other virulence factors are only present in a few serotypes *(138).* The pathogenicity of EHEC is related to the Stx toxins, endotoxins, and host-derivated cytokines such as the tumor necrosis factor alpha (TNF-a) and interleukin-1 β. Shiga toxin 1 (Stx1) and (Stx2) inhibit protein synthesis in endothelial cells. The host receptor for these toxins is globotriasylceramide (Gb3). Cells in the human kidneys contain large amounts of Gb3. Therefore, this tissue is highly sensitive to the Stx toxins producing the hemolytic-uremic syndrome (HUS) symptoms such as hemolytic anemia, thrombocytopenia and acute renal failure *(217). E. coli* O157:H7 also contains distinctive virulence factors including the *eae* (*E. coli* attachment effacement) gene encoding the intimin protein that is essential for attachment/effacement (A/E) and microvillus effacement, and the gene ehxA encoding for enterohemolysins *(89, 102).*

Epidemiology

The clinical manifestation of the *E. coli* O157:H7 infection varies by host, entry way and pathogen dose. Five-year-old and younger children are the highest risk group while elderly and immunocompromised people are also considered highly susceptible to infection. *E. coli* O157:H7 infections commonly occur by one of three main routes: through consumption of contaminated foods (in about 50% of cases), person-to-person (in about 14% of cases), and directly from animals (3% of cases, from farm animals, domestic

pets, deer, sheep, dogs, and wild birds) *(123, 142, 234)*. Other contracting routes include laboratory settings, and water, while approximately 20% of the cases have an unknown origin *(234)*. Beef cattle is considered the primary reservoir for *E. coli* O157:H7, although it has been found in other ruminants *(86)*. Weaned calves are important shedders of EHEC strains due to their immature biota *(103, 142)*. Shedding of the pathogen through cattle feces increases during the summer months, although occurrence of foodborne outbreaks is not considered related to this peak in cattle shedders *(20)*. The most common food vehicles of *E. coli* O157:H7 are ground beef and produce, which account for 40 and 20% of total *E. coli* O157:H7 foodborne outbreaks, respectively *(234)*. Some produce commodities related to foodborne outbreaks of *E. coli* O157:H7 include apples, cabbage, celery, cilantro, coriander, cucumber, lettuce, spinach, and sprouts *(135, 293)*. Produce-associated outbreaks peak in the summer and fall months. This is related, to some extent, to the seasonal production of some commodities including lettuce, apples, salads, coleslaw, melons, sprouts and grapes. In approximately 53% of the outbreaks, the contamination of produce has been related to sources other than the kitchen-level cross-contamination. This suggests that the contamination of produce with *E. coli* O157:H7 occurred during their production, transportation, retail marketing, or storage *(234)*. The possible routes of pathogen contamination during the fruit and vegetable production have been discussed in a previous section of this review. However, it is worth mentioning that the use of incorrectly composted manure and direct or indirect contact with potential carriers and their fecal depositions are considered particularly important contaminating sources of *E. coli* O157:H7 in the produce fields *(293)*.

Manifestation of illness

From the first two outbreaks reported in 1982, related to *E. coli* O157:H7, it was determined that the disease characteristic symptoms included severe abdominal pain, initial watery diarrhea followed by bloody diarrhea, and little to no fever *(236)*. According to the worldwide medical data from 2007-2015 related to STEC, and summarized by the World Health Organization, severe diarrhea occurs in 2% of cases, moderate diarrhea in 18% of cases and mild diarrhea in 80% of cases. STEC manifestation of the disease in other organs are related to renal infection including HUS in 0.8% of cases, and 3% of HUS cases lead to end-stage renal disease and death *(297)*. Diarrhea symptoms persist for five to 10 days, while HUS can extend 14–42 days. The fatality rate for HUS patients is 3.7%. Cases related to the serotype O157 of STEC are more commonly found in the U.S., Canada, Latin America, European Union and Australia (36%), whereas in Asian and African countries, such cases are uncommon *(297)*. End-stage renal disease leads to lifelong disability including regular dialysis and related deaths. In developed countries, including U.S., Canada, E.U countries and Australia, expedited medical service and overall patient care reduces the case fatality for end-stage renal failure patients to about 20%, although patients must undergo constant dialysis treatments. In other countries, the case of fatality increases significantly, and has been calculated as high as 100% *(297)*.

Salmonella enterica

The genus *Salmonella* was named after Dr. D.E. Salmon, by J. L. M. Lignières, and has been related to human illnesses for more than 125 years *(5, 60)*. *S. enterica* is one of the most widely distributed foodborne bacterial pathogen affecting public health with

significant economic losses *(5, 297).*This species is distributed in nature and all species are considered pathogenic with varied severity of the disease. It is estimated that tens of millions of salmonellosis cases and 100,000 related deaths occur worldwide every year *(296)*. In the U.S. only, nearly 1.2 million cases occur per year *(60)*. Even though salmonellosis has been investigated for decades, it is still considered an emerging pathogen due to the increased number of outbreaks reported and the more frequent antibiotic resistance found in the serotypes related to these outbreaks *(296)*.

Salmonella spp. are a non-spore forming, facultative anaerobic bacteria. Cells are rod shaped, generally motile with flagella in peritrichous arrangement *(142)*. Some biochemical reactions used to identify *Salmonella* spp. include its inability to ferment lactose and sucrose, the absence of cytochrome C oxidase complex (Oxidase negative), and inability to convert tryptophan into indole (indole negative) and to produce acetoin from glucose (Voges-proskauer negative). *Salmonella spp.* are positive to catalase production, and have a positive reaction to the Methyl red test since it ferments glucose with formation of acid and gas *(5)*. Due to its growth conditions requirements, it is considered a mesophilic microorganism, with an optimal growth temperature of 37 ºC *(142)*. However, *Salmonella* growth has been documented at temperatures of 5 to 40 ºC. Optimal pH for growth is between 6.6 and 8.2, but it is able to survive at a pH as low as 4.05 *(142)*. *Salmonella* spp. is able to grow at water activity (Aw) values of 0.93 and above, but it can also survive low Aw, which is a major concern in the dried and semidried food industry *(142)*.

Serology and classification

Salmonella spp. belong to the *Enterobacteriaceae* family. The genus contains only two species: *Salmonella bongori*, and *S. enterica*. The first is unimportant as a human pathogen with less than 1% (22) of *Salmonella* serotypes. The species *S. enterica* is subdivided into six subspecies: *S. enterica* subsp. *enterica* (I) with 1,531 recognized serotypes; *S. enterica* subsp. *salamae* (II) with 505 identified serotypes; *S. enterica* subsp. *arizonae* (IIIa) with 99 serotypes, *S. enterica* subsp. *diarizonae* (IIIb) including 336 serotypes; *S. enterica* subsp. *houtenae* (IV) with 73 serotypes; and *S. enterica* subsp. *indica* (VI) with 13 serotypes *(41, 124)*. Hosts of *S. enterica* sbsp. *enterica* are warm blooded animals while cold blooded animals and the environment are the common hosts for other subspecies *(41)*.

The classification of *Salmonella* spp. includes serology studies, initially led by F. Kauffman to determine the somatic (O) and flagellar (H) antigens *(124)*. Although new molecular methods have been utilized to classify *Salmonella* variants, the serology method is considered the "gold standard" technique for the classification of *Salmonella* below the subspecies level *(41, 164)*. The serotype list, based on the Kauffman-White scheme for *Salmonella* is maintained and updated every year by the World Health Organization (WHO) and the Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute in France *(41)*. Based on this list, there are 2,503 serotypes of *Salmonella (124)*. However, only about 200 have been reported as associated with illnesses affecting the public health *(293)*. The way that serotypes nomenclature are designated have changed with time. Some serotypes denote the causing syndrome, while other their initial place of

isolation, or their specific host. Only with the subspecies *enterica*, names are used to designate different serotypes. All other subspecies serotypes are assigned with a combination of letters and numbers to specify their antigenic differences. In the U.S, some of the most important serotypes related to foodborne diseases are: Enteritidis, Typhimurum, Newport, Javiana, 4,5,1 2 :i:-, Heidelberg, Saintpaul, Infantis, Muenchen, and Oranienburg *(75)*.

Virulence factors

The outcome of the infection with *S. enterica* alone and with other infectious agents, depends on the host and the bacteria. Age, genetics, and environmental factors determine the susceptibility of the host, while virulence factors determine the possible disease manifestation *(283)*. *Salmonella* is a facultative intracellular bacteria, capable of invading enterocytes, M cells, and dendritic cells in the gastrointestinal tract *(182)*. It can also disseminate through the bloodstream after entering macrophages in the intestinal submucosa. The bacteria can enter the host cells through two mechanisms involving invasion or phagocytosis. In addition, other mechanisms, independent of fimbrial adhesins have been studied, including the replication in intracellular *Salmonella*-containing vacuoles *(182)*. Survival into the host cell is dependent on multiple factors including nutrient availability and avoidance of host antibacterial activity *(139)*. Most of their gene encoding for virulence factors are located in clustered areas in the chromosome, known as pathogenicity islands. The distinctive functions of these pathogenicity islands include protein binding leading to uptake of the *Salmonella* cell by the enterocytes, secretion of proteins to control host response, and to aid in the survival of the *Salmonella*

cell inside the macrophages, toxin secretions, and host response control of inflammation and intestinal secretions *(179)*. Some serotypes also contain plasmids where virulence genes are contained, which have been related to the particular adaptation of host-specific *Salmonella* serotypes *(283)*. *Salmonella* also produces toxins, including the endotoxin lipid A, and exotoxins with unknown mechanisms of action *(283)*. *Salmonella* cells are motile due to the presence of flagella, and their motility has been related to the evasiveness of the pathogen to the host defense mechanisms. Motility is also useful to locate, adhere and invade the target cell *(283)*. The formation of fimbriae has also been connected to *Salmonella* colonization of target cells, although its role is not fully understood *(283)*.

Epidemiology

For their epidemiological study, *Salmonella* serotypes are classified in different groups. The first group includes those serotypes that only affect humans, such as *S*. Typhi, and *S*. Paratyphi A and C, causing typhoid disease. The second group include those that are host-adapted, such as *S.* Gallinarum in chicken, *S.* Choleraesuis in swine, *S.* Abortus-equi in horses, etc. The last group includes those serotypes that are not host-adapted, but are pathogenic to humans and animals. Members of this group are mainly those causing foodborne outbreaks, for example, *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport, *S*. Heidelberg, *S*. Muenchen, *S*. Montevideo, and *S*. Saintpaul *(142)*.

Salmonella is generally contracted through consumption of contaminated animal- or vegetable-origin foods, and water. Products commonly related to salmonellosis cases include meat, poultry, eggs, and milk, green vegetables and fruits. *Salmonella* has shown resistance to heat lethal treatments in high fat, and low water activity (Aw) foods, for

example, those involved in recent outbreaks including peanut butter, nut butter, and dried protein meal powder *(74, 82, 248)*. Nevertheless, unprocessed, fresh foods have also been contaminated with this pathogen, including fruits and vegetables. Among produce, some of the commodities related to *S. enterica* outbreaks include tomatoes, artichokes, sprouts, chili, parsley, cilantro, broccoli, cauliflower, lettuce, spinach, watercress, beets, celery, cabbage, eggplant, endives, fennel, potato, mustard cress, peppers, and unpasteurized apple and orange juices *(231, 293, 296)*. Children are at a higher risk of contracting salmonellosis, along with the elderly and immunocompromised population *(73)*. The contamination of fruits and vegetables with *S. enterica* has been related to unsanitary conditions in the growing fields and use of sewage water. Washing produce with contaminated water or by employees carrying the infection can also serve as vehicles of contamination *(293)*.

Manifestation of illness

Salmonellosis is the name commonly given to the contracted disease related to non-typhoidal *Salmonella* infection. Clinical outcomes include diarrhea with variable manifestation: severe in 2% of the cases, moderate diarrhea in 25% of the cases, and 73% mild diarrhea in the remaining cases. In children aged 5 or less, severe diarrhea persists for 4 to 8 d, and in older patients the average duration of diarrhea is 3 d *(297)*. Most people recover without treatment although antibiotic treatment is required when septicemia is developed *(296)*. Vomiting and mild fever may accompany the infection, and rehydration treatments might be necessary to patients with severe body fluids loss. If dehydration is not attended promptly, it can lead to death, especially in infants and elderly people. A

small number of patients develop reactive arthritis as a consequence of the infection, and this can last from months to years *(296)*.

Factors involved in the pathogen colonization of produce

Plants are not considered natural hosts of enteric pathogens, although particular strains of the *Enterobacteriaceae* family have been related to plant tissues, including *Klebsiella* and *Serratia* species *(269)*. Factors inducing the survival and growth of microorganisms in produce surfaces include inherent characteristics of the bacteria, the state of the plant, the environmental conditions (e.g., pH, water activity, atmospheric composition) and the pre- and postharvest processing steps *(98)*. The presence of enteric pathogens in the plant ecosystem, known as the phyllosphere, is also dependent of these factors as further reviewed.

Electrostatic forces and hydrophobicity

Inherent conditions of the plant surface can influence bacteria attachment including the nutrient availability in the surface, the hydrophobicity of the waxy cuticle, and the electrostatic forces. The first contact of the bacteria cells with the plant surface is subjected to the external conditions of the surface *(303)*. During this initial contact, electrostatic forces and the hydrophobicity of the plant surface and bacterial cell can play an important factor determining the further stronger attachment of the bacteria, although its role in the attachment of enteric bacteria to plant surfaces is not well understood.

Plant surfaces present hydrophobic forces, due to their external waxy cuticle. This natural waxy surface is produced intentionally by the plant to counteract the invasion of plant pathogens and to repel water *(166)*. Some plant pathogens have adapted to the

hydrophobic charges of the plant surface. In lettuce, *Pseudomonas fluorescens* and *Pasteuria* spores have demonstrated a preference for attachment to intact surfaces presenting high hydrophobicity, whereas *E. coli* O157:H7 proliferated better in the cut edges, possibly due to higher nutrient content, or perhaps due to the interrupted hydrophobicity effect *(246)*. The contact angle (CA) measurement is most commonly used to determine the wettability of a surface *(156)*. The hydrophobicity of any surface can be measured by calculating the contact angle of a water droplet. The greater the hydrophobic force, the less attached the water droplet is, thus a larger contact angle is calculated *(187)*. Plants in general present high hydrophobicity, with contact angles (CA) around 150–160º *(211)*. Although bacterial cells are also hydrophobic, and this might influence in the initial bacterial adhesion, electrostatic forces might repel this contact further since the plant and the bacteria surfaces are negatively charged *(187)*. Pili structures might assist the initial adhesion of certain plant pathogens including *Pseudomonas syringae* pathovar *phaseolicola* adhering to non-hydrophobic substances such as carbohydrates in the plant surface *(263)*. Further synthesis of substances aiding the adhesion, such as curli fimbriae and cellulose, might also help bacteria to tightly attach despite the hydrophobic and electrical repelling forces.

Surface roughness can also play an important role in the survival of pathogens in fruits and vegetables. For example, in the case of cantaloupes, their netted rind has been indicated as a possible cause for persistence of pathogens. The rind netting, being a rough surface, forms numerous crevices where the pathogens can be protected from externally applied antimicrobial treatments *(223, 282)*. The stems attached to peppers have been

reported as a possible source of *Salmonella*, since their wrinkled surface might serve as a protective environment for pathogens *(45)*.

The survival, and other growth abilities of bacteria, including biofilm formation, is influenced by the nutrient availability in the plant surface *(251)*. Nutrients in healthy leaves are scarcely available for bacteria in most of the leaf surfaces. Carbon sources including glucose, sucrose and fructose found in the leaves, are not only limited but heterogeneously distributed *(166)*. In fruits, spoilage bacteria, and fungi, are able to attach to the outer spaces of the fruit and damage the cuticle. Surface damage including bruising, fissures, and wounds will further assist in the nutrient provision to bacteria that comes with spoilage where pathogen microorganisms will attach, and possibly penetrate past the skin, cuticle or rind into the fruit. *(21, 127)*.

Plant-associated microbiota

One important factor in all plant surfaces that might affect the initial attachment and further survival of enteric pathogens on their surface is the presence of naturally-occurring epiphytic and endophytic bacteria *(170, 274)*. According to Hirano and Upper *(137)*, the term *epiphytic* in regards to plant-related bacteria, consist of all bacteria, from any structure of the plant above the ground, which are able to be removed through washing. Fresh produce present a natural epiphytic microbial load, and generally these microorganisms are not harmful to the plant, or humans. This microbial mass might be the result of intentional or accidental input to the growing field environment by water, soil, wildlife, livestock, farm equipment and farm workers *(110)*. In the produce surfaces, the native microbial community forms a complex system where different microorganisms

adapt, survive, and grow regarding the fluctuating conditions of their surrounding environment *(173)*. This continuous coexistence leads microorganisms to develop interaction mechanisms to benefit their survival *(218)*. Some microorganisms are highly adapted to the plants that populate them during the early development stages *(195)*. This effective adaptation is also demonstrated by the bacterial diversity found in produce at the moment of consumption, even after disinfection treatments, and further produce processing *(137, 144)*. Adaptive mechanisms by epiphytic bacteria include beneficial agonistic and antagonistic interactions. Microorganisms capable of inhibiting the colonizing bacteria are known as antagonistic *(274)*. The plant-associated microbiota might influence the colonization of other microorganisms such as plant and human pathogens *(137)*. The presence of enteric pathogens on produce have triggered this community response *(145, 170, 172)*. In previous studies, epiphytic bacteria had shown agonistic and antagonistic activities toward enteric pathogens *(90, 144, 218)*. The response mechanisms of the epiphytic bacteria to the presence of enteric pathogens on produce in addition to the different mechanisms used by the pathogens to limit their effect are still being investigated.

Environmental factors

Environmental factors influencing the colonization of pathogens in the plants include solar UV irradiation, atmospheric temperature, and relative humidity. Variations in the environment influence the content and diversity of microorganisms in the plant *(137)*. Although the environmental conditions might affect the survival of pathogens in fruits and vegetables, some studies have demonstrated the presence and survival of enteric

pathogens such as *E. coli* O157:H7 and *Salmonella* under stressful conditions including desiccation. Brandl and Mandrell *(35)* reported the survival of *Salmonella* Thompson in cilantro, under dry conditions and its growth in humid environment. In this study, two epiphytic bacteria, *Pantoea agglomerans* and *Pseudomonas chlororaphis*, demonstrated better adaptation to the moisture changes on cilantro leaves than the pathogen. One study describing the variation of epiphytic bacteria in different Mediterranean plants found water as one of the first descriptors for the variation of epiphytic bacteria load, accounting for approx. 55% of the variance changes *(302)*. Medina-Martinez et al. *(186)* described the rapid change in the proliferation of epiphytic coliforms in baby lettuce toward the end of the winter that was not observed during the first days of the same season. Marine et al. *(180)* also found significant differences between sampling dates while studying epiphytic bacteria on spinach, lettuce and other leafy greens samples and related these results to differences in the humidity and temperature of the harvesting days. It is evident that changes in the environmental humidity and in the leaves surface can modify and influence the survival and possible proliferation of human enteric pathogens. However, since these pathogens are foreign to the fruit and vegetable surfaces, their proliferation or presence is relatively poor compared to epiphytic bacteria, although inherent characteristics of the pathogenic bacteria might aid in their survival on the plant surfaces.

Pathogen-inherent factors

Enteric bacterial pathogens have developed survival mechanisms to subsist in the gastrointestinal tract of their hosts *(148)*. Essentially, these mechanisms are used by the bacteria to protect them from the host defense mechanisms, to resist and/or control their

surrounding environment, and to benefit their proliferation. Enteric pathogens including *E. coli* O157:H7 and *Salmonella* spp. might use similar strategies to survive until further contact with a new host, or until a more suitable environment allows their proliferation *(303)*. Bacteria will locate at the initial contact area, which depends on the pathogen mode of transmission. Therefore, pathogenic bacteria may be located in the rhizosphere or in the phyllosphere *(303)*. This initial contact will influence the survival of the pathogens. Mechanisms used by bacteria to survive in the phyllosphere include the ability to attach to the plant surface, to locate high nutrient areas, to form biofilms, and to migrate to protective areas such as the stomata and the interior of the plant tissue, known as bacterial internalization.

Biofilms

Biofilms are the accumulation of different microorganisms protected by a complex matrix of extracellular polymeric substances and complex carbohydrates, tightly attached to any surface *(92)*. Some of the components studied in the extracellular matrix, include the curli fimbriae and cellulose, which was previously mentioned as being able to attach to surfaces, expedite biofilm formation, and stress tolerance of different bacteria including *S. enterica (17)*. The formation of biofilms has been observed in biotic and abiotic surfaces and has been located in almost any surface tested *(7, 303)*. Several pathogens including *Campylobacter*, *Shigella* spp., *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* have demonstrated the ability to form biofilms on animal and plant surfaces *(8, 87, 125, 129, 134, 246)*. Bacteria contained in biofilms are more resistant to acidic conditions, antimicrobial substances, and environmentally harsh conditions such as UV radiation,

osmotic stress, and desiccation *(4, 87, 214, 303)*. Enteric pathogens have also demonstrated this improved resistance to stress conditions and to antimicrobial treatments in produce. *S. enterica* embedded in biofilms on lettuce leaf surfaces demonstrated higher resistance to acidic conditions than planktonic cells *(160)*. In several studies, pathogenic *E. coli* embedded in biofilms demonstrated higher resistance to decontamination treatments including chlorine, hydrogen peroxide, and ozone, in fresh commodities such as lettuce, spinach, and cantaloupes *(214, 251, 258, 266)*.

Internalization

Internalization of pathogens into the fruits and vegetables is highly concerning since pathogens allocated inside crevices and spaces are not reached by antimicrobial treatments and disinfectants *(4, 214)*. The possible internalization of enteric pathogens into the plant through the roots and aerial parts has been studied in edible leaves and fruits. The internalization through the root system to the plant xylem has been better explained than their counterpart aerial surfaces. Klerks et al. *(155)* demonstrated the ability of *Salmonella* Dublin to internalize lettuce through the root of the plant, and suggested the activation of molecular markers in the plant as seen in the invasion of certain plant pathogens, but could not simulate the internalization of the pathogen on the leaves. Other studies have concentrated in elucidating the leaf internalization with inconclusive results *(258)*. Nonetheless, some studies reveal important possible routes for this invasion. Kroupitsli et al. *(159)* reported the migration and internalization of *S. enterica* on iceberg lettuce. In this study, the pathogen migrated to the stomata where it was stimulated to produce photosynthetic nutrients, using light activation, possibly using motility and chemotaxis

abilities attracted by high nutrient content of newly synthetized substances by the plant stomata; the pathogen was microscopically observed within these plant structures. Furthermore, the possible adaptation of enteric pathogens to the interior of the leaves has been observed. Mitra et al. *(196)* studied the colonization of *E. coli* O157:H7 in spinach stems simulated by puncture-inoculating the stems. The pathogen was able to persist as an endophyte for 2 weeks, showing that once the pathogen reaches internal areas, the likelihood of survival is augmented.

Biocontrol in produce

Biocontrol refers to the use of one or more organisms to inhibit the proliferation of undesirable organisms in the environment or in particular products *(142)*. This inhibition might be related directly to the organisms, such as phages, or indirectly through the production of inhibitory agents or actions. Microorganisms as biocontrol agents include those able to produce inhibitory substances including antibiotics, volatile organic compounds, and antimicrobial peptides including bacteriocins *(2, 16, 191)*. Biocontrol agents also include bacteriophage viruses, capable of infecting bacteria *(2)*. Bacteria commonly act as nonspecific biocontrol agents interfering with the propagation of harmful or undesirable microorganisms. Although some fermenting microorganisms produce metabolites with strong inhibitory activity toward enteric pathogens, these are mostly used in foods intended to be modified by these, including milk transformed by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* into yogurt, and fruit. Cereal extracts from *Saccharomyces cerevisiae* are used to make alcoholic beverages *(25, 142, 228)*. Nonetheless, other lactic acid bacteria, that do not substantially modify the products,

are nonspecific inhibitors exploited in various industries. The production of several inhibitory substances have been identified in lactic acid bacteria (LAB) including bacteriocins, diacetyl, H_2O_2, and antibiotics in addition to their common transformation of sugars into organic acids, including lactic and acetic acids.

Biocontrol of specific targeted pathogens are conducted principally by bacteriophages. Bacteriophages, also known as phages, are viruses capable of infecting bacteria. These can either lysate or intracellularly grow and burst the invaded bacterial cell *(117)*. While bacteriophages were discovered in the last century, their application in patient treatment for bacterial infection was disregarded with the discovery and application of antibiotic treatments. Nowadays, the increased resistance to antibiotics shown by several pathogenic bacteria have forced the scientific community to investigate bacteriophages as an alternative therapy for those multidrug resistant bacteria *(141)*. The use of phages in the food industry is still an uncommon alternative. A current concern in their application is the possible transference of genetic material to already virulent pathogens and the lack of technology for its application and dispersal *(117)*. Bacteria acquiring the phages and transporting them to niches were pathogenic bacteria and have been studied as adequate vehicles for its dispersal. However, this requires further identification of ideal carrier bacteria *(117)*. Furthermore, the use of bacteriophages is subjected to government regulation and requires approval prior to its application on specific food products, a process that can delay the expansion of this technology *(40)*.

The potential use of antagonistic bacteria, epiphytic to fruits and vegetables, as biocontrol agents against enteric pathogens has been studied in *in vitro* and *in situ*

experiments with promising results *(43, 47, 170, 172)*. However, exhaustive studies involving the analysis of pathogen and epiphytic bacteria interactions and their responses at proximity must be evaluated *(99, 137, 166)*. Thus, the identification of epiphytic bacteria, with antagonistic activity toward enteric pathogens, and the study of their role during the pathogen colonization of fruits and vegetables is necessary to recognize biocontrol agents and their potential use during the production of fruits and vegetables.

MATERIALS AND METHODS

Preparation of media

Trypticase soy agar (TSA, Difco™, BD, Sparks, MD), de Man, Rogosa and Sharpe agar (Difco™ MRS Agar), violet red bile agar, (Difco™ VRBA), MacConkey Sorbitol Agar (Difco™), xylose lysine tergitol 4 agar (XLT4), and all-purpose Tween agar (Difco™ APT Agar) were prepared following manufacturer instructions. To prepare agar plates for spread plating and isolation streaks, approximately 15 ml of sterile molten media (50 ± 2 ºC) was transferred aseptically into individual 100 x 15 mm sterile, disposable petri plates. After pouring, the plates were allowed to solidify at 25 ± 2 ºC for 24 h before storing at 4 ºC. Lactobacilli MRS Broth, tryptic soy broth (Difco™ TSB), APT broth (Difco™), 0.1% peptone water solution (Difco™ PW), and phosphate buffer solution (PBS, Calibrochem, EMD Biosciences Inc., La Jolla, CA) were dispensed into 8 x 150 mm test tubes and sterilized in an autoclave for 15 min at 121 ºC. To prepare MRS deep agar tubes for lactic acid bacteria (LAB) stabs, 10 ml of MRS agar were dispensed into test tubes before sterilization. MRS deep agar tubes were allowed solidify before storing at 4 ºC. Similarly, TSA was poured into test tubes to prepare TSA slants and then sterilized. After sterilization, the tube racks were placed at an inclined position to allow slant formation during solidification.

Media for overlay including MRS agar and VRBA agar were prepared and sterilized following manufacturer instructions and held for up to 1 h in a water bath (50 ºC) until needed. Semisolid TSA for *in vitro* antagonistic effect experiments was prepared by

adding 5 g of granulated agar to 1 L of TSB and heated to boil for 1 min. The medium was dispensed in 9 ml aliquots into test tubes, and sterilized in an autoclave for 15 min at 121 ºC. After sterilization, the semisolid TSA tubes were held at 50 ºC in a water bath until needed, within 2 h after sterilization.

To prepare tryptic soy agar (TSA) supplemented with 100 mg/L of rifampicin (TSA-RIF), 0.1 g of rifampicin, (RIF, Sigma-Aldrich, St. Louis, MO) was dissolved in 5 ml of methanol. This solution was added to 1 L of sterile molten TSA and immediately poured into petri plates. To prepare TSA supplemented with 100 mg/L of ampicillin (TSA-AMP), 1 g of ampicillin (AMP, Sigma-Aldrich), was dissolved in 10 ml of sterile distilled water and 1 ml of this solution was added to 1 L of sterile molten TSA and immediately poured into petri plates. For TSA-IPTG preparation, 480 mg/L of isopropyl β-D-1 thyogalactopyranoside (IPTG, Novagen EBM Biosciences, Inc., Madison, WI) were suspended in 5 ml of sterile distilled water and 1 ml of this solution was added to sterile molten TSA and immediately poured into petri plates. TSA-RIF, TSA-AMP and TSA-IPTG plates were stored at 4 ºC and utilized within 7 days. Other surface agar plates, PBS and PW solutions were stored for up to 4 weeks at 4 ºC. Before use during experiments, media were allowed to reach 25±2 ºC on benchtop.

For the discriminatory tests, when differentiation between two or more bacterial species was needed, the following were used. Sugar-fermentation tests were carried out by adding a filter-sterilized suspension of the corresponding sugar to a sterile phenol red broth base (Difco™). Litmus milk (Difco™) and motility media (Difco™) were prepared following manufacturer instructions.

For the determination of biofilm formation using Red Congo Agar, four different media were prepared: TSA supplemented with 5% sucrose (Difco™) and red Congo (0.08%) (TSA-Suc-RCA), TSA supplemented with red Congo dye (TSA-RCA), brain heart infusion agar (BHI, Difco™) supplement with sucrose (5%) and red Congo dye (0.08%) (BHI-Suc-RCA), and BHI supplemented with red Congo dye (0.08%) (BHI-RCA). BHI and TSA were prepared, supplemented with 5 g of sucrose, and sterilized following manufacturer instructions. After sterilization, media was supplemented with 8 g of red Congo, previously suspended in 10 ml of sterile distilled water, and mixed. This solution was filter-sterilized and added to 1 L of sterile molten agar and immediately poured into petri plates.

Procurement of pathogenic bacteria

For those experiments involving the use of pathogenic enteric bacteria, strains of *S. enterica* serovar Saintpaul, and of *E. coli* O157:H7 were selected. One strain of *S.* Saintpaul strain, identified as *S.* Saintpaul FDA/CFSAN 476398, and isolated from a U.S. raw produce-related outbreak in 2008, and one strain of *Escherichia coli* O157:H7, identified as K3999, related to a U.S. spinach-related outbreak in 2006, was selected for the *in vitro* antagonistic effect test and preliminary experiments *(55, 57)*. Both pathogens were obtained from the Food Microbiology laboratory stock culture collection (Texas A&M University, College Station, TX) and maintained at − 80 °C in CryoCare bead storage system vials (Key Scientific Products, Stamford, TX). Whenever propagation of these pathogens was required, incubation conditions were established as 35 ºC for 24 h for all experiments unless otherwise specified.

For the growth inhibition experiment on leafy greens and fruits skin/rind surfaces, a naturally occurring RIF-resistant derivative strain of *S.* Saintpaul was used. This RIF-resistant *S.* Saintpaul strain had been previously derived from *S.* Saintpaul 476398 and its similar growth behavior to parent strain has been documented in previous studies *(188)*. A naturally occurring RIF-resistant *E. coli* O157:H7 K3999 was derived following a modification to the method described by Kaspar and Tamplin *(147)*. A 24-h culture of *E. coli* O157:H7 K3999 in TSB was centrifuged at 1,623 x *g* in a Jouan B4i centrifuge (Thermo Electron Corp., Madison, WI) for 15 min, and resuspended in 1 ml of PBS to reach an approximate concentration of 10 log CFU/ml. The suspension was spread-plated onto TSA-RIF and incubated. From the few colonies that were able to grow, one colony was selected, streaked in TSA-RIF, and incubated. One colony from streaked culture was selected and transferred to CryoCare bead vials for storage at − 80 °C. Growth curves of the *E. coli O157:H7* parent and derivative strains were compared as further described in the preliminary experiments section. For experiments, TSA-RIF agar was used for enumeration of the inoculated pathogens in the fruit and leafy greens samples. For confocal microscopy, an *E. coli* O157:H7 strain previously inserted by electroporation, with a plasmid codifying for green fluorescent protein expression (GFP), and resistance to AMP was used. AMP resistance was confirmed using TSB cultures of *E. coli* O157:H7 GFP streaked in TSA-AMP and incubated. Expression of GFP was confirmed by observing the characteristic color in well-grown bacterial colonies under UV light (365 nm). To improve expression of the plasmid proteins, the *E. coli* O157:H7-GFP was streaked in TSA-IPTG with three consecutive transfers and incubations. With every

transference, a more intense fluorescent colony was selected. After three consecutive transferences, one colony was streaked in working slants to be used within 4 wks for the experiments. During this experiment, TSA-AMP was used for selection purposes to confirm and enumerate the pathogen.

All *E. coli* O157:H7 and *S.* Saintpaul strains were revived in order to prepare working slants. A CryoCare bead containing the pathogen was transferred to TSB and incubated for 24 h at 35 ºC. A loopful of the cultured pathogen was streaked for isolation on TSA or TSA-IPTG for the GFP *E. coli* strain and incubated for 24 h at 35 ºC. One colony was transferred to TSA slants and incubated. After incubation, the caps were covered with Parafilm (Bemis flexible packaging, Oshkosh, WI) to avoid dehydration and stored at 25 ± 2 ºC. Working slants were prepared every 4 wks for the duration of the experiments.

Preliminary experiments

Transformation of microbiological content by g to content by cm^2

In an effort to compare microbiological content of all the commodities studied, a transformation of counts expressed by g to be expressed by cm^2 was calculated. For this, spinach and endives were acquired from a local distributor and aseptically transported to the laboratory for immediate sample processing. After removing damaged leaves, 100 circles each of spinach and endive leaves were cored from the middle areas of 100 leaves and weighed. The area calculated was 10 cm^2 x 200 (100 leaf circles multiplied by 2, to consider adaxial and abaxial surface areas of the leaf). The corresponding area and weight calculated were: 2000 cm^2 of spinach = 30.375 g, and 2000 cm^2 = 48.147 g of endives;

thus, 1 g of spinach corresponded to 65.84 cm^2, and 1 g of endives corresponded to 45.54 cm^2.

Incubation time for *in vitro* experiments

To determine the incubation time and temperature required for the possible epiphytic bacteria to exhibit inhibitory activity *in vitro* against *E. coli* O157:H7 and *S.* Saintpaul, a preliminary experiment was carried out using a modification of the spot agar test by Fleming et al. *(114).* The incubation temperature of the antagonistic LAB and targeted bacteria to be inhibited reported by Fleming et al. *(114)* was modified to better fit the mesophilic growth characteristics of *S.* Saintpaul, *E. coli* O157:H7 and of the LAB used from 30 ºC to 35 ºC. In addition to this modification to the test, the incubation time was studied. Fleming et al. *(114)* allowed the possibly antagonistic LAB to grow for 24 h before the targeted bacteria was overlaid. Brashears et al. *(39)* allowed the probable antagonistic LAB to grow for 24 to 48 h before being overlaid with molten TSA inoculated with a cocktail of 4 strains of beef-isolated *E. coli* O157:H7. Neither study provided edification on the reasoning behind this pre-incubation of LAB before co-incubation with the targeted bacteria. It is rational to consider that a prolonged incubation of LAB before pathogen inoculation in an overlay would allow the LAB to adapt and grow with possibly further accumulation of antimicrobial substances. Hence, the lack of information about the use of pre-incubation of LAB led to the following experiment.

Two treatments involving the time allowed for antagonistic bacteria to grow were studied. Treatment 1 allowed 1 h attachment after spot inoculation with antagonistic LAB

and Treatment 2 included a 24 h incubation after spot inoculation. Both treatments were overlaid with pathogen suspended in overlay media as further described.

LAB cultures of *Lactobacillus amylovorus* NPC M-35, *Lactobacillus animalis* LA-51 and *Pediococcus acidilactici* D-3 were used due to their demonstrated inhibition activity against pathogens such as *E. coli* O157:H7 and *S. enterica (38, 114, 260, 290).* Frozen cultures of these LAB in MRS (20% glycerol) were donated by Dr. Joseph Sturino, from the Cater-Mattil Protein Research Center (Texas A&M University, College Station, TX). A loopful of each culture was suspended in MRS broth and incubated at 35 °C for 24 h. The cultures were streaked for isolation in MRS agar, and incubated for 24 h at 35 °C. One colony of each strain was selected from streaked cultures and transferred to CryoCare bead vials and stored at − 80 °C. MRS stabs were prepared 1 wk before the experiment. One bead of each LAB from the CryoCare beads vials was transferred to MRS broth and incubated for 24 h at 35 ºC. After incubation, each culture was streaked in MRS agar for isolation and incubated for 24 h at 35 ºC. One colony was picked and stored in agar stabs using MRS agar deep tubes. The stabs were overlaid with 1 ml of sterile mineral oil (Avantor, Center Valley, PA) and incubated for 24 h at 35 ºC. After incubation, the stabs were stored at 4 ºC until needed.

For the experiment, individual cultures of *L. amilovorus, L. animalis,* and *P. acidilactici* were prepared by suspending a loopful of each strain from MRS storage stabs in 10 ml of MRS broth. Inoculated MRS tubes were incubated at 35 °C for 24 h. Concentrations of LAB were calculated by CFU enumeration after spread plating serial dilutions of each 24-h culture in MRS agar and incubation for 24 h at 35 ºC.

For the experiment, 1 µl of each 24-h LAB culture was spot inoculated in MRS agar using a sterile micropipette tip. Three different LAB spots were spot inoculated in one plate leaving a 2.5 cm space between spots and two plates were used per treatment and pathogen. The LAB spots were allowed to dry and attach for 1 h at 25 ± 2 ºC. After attachment, two plates were overlaid with molten semisolid TSA previously inoculated with *E. coli* O157:H7, and two more with *S.* Saintpaul. This procedure was carried out after 1 h attachment (Treatment 1) or after 24 h incubation at 35 ºC for 24 h (Treatment 2). For the inoculation of the molten semisolid TSA, cultures of *E. coli* O157:H7 and *S.* Saintpaul were prepared by suspending a loopful of each pathogen from storage slants into 9 ml of TSB. After incubation at 35 ºC for 24 h, the cultures were centrifuged at 1,623 x *g*, for 15 min, decanted and resuspended three times with 9 ml of PBS to remove waste material from incubation. After the final suspension, each culture was diluted in 0.1% PW to reach a concentration of 7 log CFU/ml. One ml of this dilution was transferred to individual tubes containing 9 ml of molten semisolid TSA at 50 ± 2 °C, and the entire content of each tube was slowly poured over the plate to completely cover the spots and the rest of the agar surface. Overlays solidified for 1 h at 25 ± 2 ºC and plates were upturned and incubated for 24 h at 35 ºC. After incubation, plates were examined for clear zones surrounding each isolate spot. These clear zones indicated growth inhibition of the pathogen contained in the overlay due to the presence of antagonistic LAB spot growth. Diameter size (in mm) of the inhibition zone (inhibition halo) and isolate spot growth were measured using a dial caliper (Scienceware Bel-Art, Pequannock, NJ). The area sizes of the inhibition halo and the spot were calculated using the formula to calculate the ellipse

area: Area = π x a x b; where a is the radius of the longest side (greatest width) and b is the radius of the shortest side (greatest height) of the ellipse. The total inhibition area (IA), in mm^2 was calculated by subtracting the distance of each spot area from the distance of each corresponding halo area.

Medium selection for lactic acid bacteria

Bacterial growth in laboratory settings is determined by the ability of the bacterial cells to adapt and to obtain nutrients from culturing media. This adaptation might also impact the expression of antagonistic activity against enteric pathogens since this antagonistic activity relates, to some extent, to metabolites production which requires different nutrient consumption. Since LAB are fastidious microorganisms requiring several nutrients for their laboratory culturing, several complex media have been developed *(97, 105)*.

A preliminary experiment was designed to determine the effect of the propagation media over the antagonistic activity of LAB. For this experiment, epiphytic bacteria isolated from spinach were selected. These isolates had been collected from cultured MRS plates incubated anaerobically at 35 ºC for 24 h and were considered presumptive LAB. The treatments consisted of 4 combinations of propagation broth and agar of MRS and APT media. These two media are commonly used for cultivation of hetero-fermentative lactobacilli, and homo-fermentative lactobacilli respectively *(97, 105)*. The treatment combinations of APT and MRS media during the agar spot test were: Treatment 1 was implemented by propagation of LAB isolates in MRS broth and spot inoculation in MRS agar (MRS-MRS); Treatment 2 used propagation in APT broth and spot inoculation in

MRS (APT-MRS), Treatment 3 involved propagation in MRS broth and spot inoculation in APT agar (MRS-APT), and Treatment 4 used propagation in APT broth and spot inoculation in APT agar (APT-APT). The antagonistic effects against *E. coli* O157:H7 and *S.* Saintpaul were measured for spinach-isolated LAB individually. The spinach isolates were grown on MRS broth (Treatment 1 and 3) or APT broth (Treatment 2 and 4) and incubated for 24 to 48 h at 35 ºC. After incubation, 1 µl of each broth was spot inoculated onto MRS agar (Treatment 1 and 2) or APT agar (Treatment 3 and 4). Spot inoculated plates were incubated for 24 h at 35 ºC. After incubation, spots were overlaid with molten semisolid TSA containing 6 log CFU/ml of *E. coli* O157:H7 or *S.* Saintpaul. Overlays were solidified at 25 ± 2 ºC for 1 h and the plates were upturned and incubated for 24 h at 35 ºC. After incubation, plates were examined for clear zones surrounding each isolate spot. These clear zones indicating pathogen growth inhibition were measured with a dial caliper and recorded. The area sizes of the inhibition halo and the spot area, and the total IA was calculated and total inhibition area (IA, in mm^2) was calculated as previously described.

***E. coli* O157:H7 lag phase**

The adaptation or lag phase time required by *E. coli* O157:H7 was studied. The determination of this time was important, in order to establish the maximum time that the samples would be incubated for the pathogen to recover before being overlaid with selective media (MacConkey agar). For this, a culture of *E. coli* O157:H7 was prepared by suspending a loopful from storage slant into 9 ml of TSB. The 24 h culture in TSB was centrifuged at 1,623 x *g*, for 15 min. using sterile tubes containing 9 ml of TSB; these were

then inoculated with 1 ml of a dilution in 0.1% PW of the bacteria with an approximate concentration of 2 log CFU/ml. The tubes were incubated for 3 h at 35 ºC. Every 20 min, three TSB tubes were removed from incubation, and the serial dilutions of each tube were spread plated onto TSA O157:H7 and incubated at 35 ºC for 24 h.

Differential media to isolate *E. coli* O157:H7 from leafy green samples

The medium Sorbitol MacConkey agar was tested to evaluate the differentiation and selection of *E. coli* O157:H7 from background bacteria previously inoculated on spinach leaf samples. For this, a culture of *E. coli* O157:H7 was prepared by suspending a loopful from storage slant into 9 ml of TSB. After incubation at 35 ºC for 24 h, the culture was centrifuged at 1,623 x *g*, for 15 min, decanted and resuspended three times with 9 ml of PBS to remove waste material from incubation. The culture was diluted in 0.1% PW to a concentration of approximately 4.0 log CFU/ml. Three circles of 10 cm^2 of previously washed and disinfected spinach leaves were cut using a sterile stainless steel borer. Spinach leaves were selected from recently purchased whole, bunched spinach. Leaves were selected for similar appearance and size, and visibly wounded or broken leaves were discarded. After sorting, leaves were washed with running tap water, rubbing the surface gently with gloved hands to remove soil and debris for 1 min. Excess water was removed by shaking gently and using a salad spinner (OXO, El Paso, TX). Individual leaves were disinfected with 70% ethanol sprayed to cover the surface of the adaxial and abaxial sides and were air-dried for 2 h at 25 ± 2 ºC. One sample consisted of 3-10 cm^2 cutlets. The corresponding pieces per sample were placed into a sterile petri plate containing VWR grade 415 filter paper (415, 7.5 mm, VWR) moistened with 2 ml of sterile distilled water

to keep them from drying during incubation as described by Khalil and Frank *(154)*. Each circle was inoculated with 10 drops of 1 µl of *E. coli* O157:H7 inoculum. After the plate lid was positioned, the specimens were incubated for up to 12 h at 20 ºC. For negative controls, one sample per each time point was processed equally to those inoculated samples with exception of the pathogen inoculation that was substituted with inoculation of 10 drops of 1 µl of sterile peptone water. For enumeration, three inoculated samples and one non-inoculated sample (negative control) were removed from incubation at 0,1,2,4,6,10 and 12 h. Specimens were suspended in 25 ml of TSB, pummeled in a stomacher for 2 min and incubated for 1.5 h at 35 ºC. This incubation time was previously determined as the lag phase for *E. coli* O157:H7 in TSB. This incubation was utilized to allow *E. coli* O157:H7 cells time to recover from possible stress during their incubation on the spinach leaves. An aliquot of 1 ml was obtained from samples, and serial dilutions of the sample in 0.1% PW were plated onto SMAC agar. Plates were incubated for 24 h at 35 ºC.

Growth curves

To determine the effect of antagonistic bacteria over the growth of *E. coli* O157:H7 on leafy greens, and of *S.* Saintpaul on fruit rind/skin, a derivative strain from the *E. coli* O157:H7 K3999 strain, showing resistance to RIF was obtained as previously described and growth curves were constructed to analyze the growth similarities between the parent and the corresponding RIF-resistant derivative. A loopful of parent and derivative RIF-resistant strains were transferred to 9 ml of TSB and incubated at 35 ºC for 24 h. After incubation, serial dilutions of each strain were prepared in 9 ml PW to obtain a

concentration of 4 log CFU/ml, and 0.1 ml of this suspension was transferred to test tubes containing 9.9 ml of TSB to reach an approximate concentration of 2 log CFU/ml. The tubes were incubated at 35 ºC. Every h for 3 h and every 2 h for additional 10 h, triplicate tubes were retrieved from incubation and serial dilutions in 0.1% PW were spread plated on TSA. Plates were incubated for 24 h at 35 ºC. After incubation, CFU were enumerated.

In the case of *S.* Saintpaul and its RIF-resistant derivative, the constructed growth curves were previously developed by Mrs. Ana Mercado and the information of each curve was kindly shared for statistical analysis *(188)*.

Effect of antagonistic bacteria toward parent and rifampicin-resistant pathogens

Since the epiphytic bacteria isolates that would exhibit an *in vitro* antagonistic effect (also referred to as *in vitro* antagonistic epiphytic bacteria, or *iv*AEB), toward *E. coli* O157:H7 and/or *S.* Saintpaul, during the *in vitro* spot agar test were going to be further studied on nonsterile fruit and leafy green surfaces, derivative strains showing resistance to RIF, for selective purposes, were used. Thus, samples could be spread plated onto RIF-TSA agar where the background microbiota would be inhibited. These RIF-resistant derivatives of the *E. coli* O157:H7 and *S.* Saintpaul were studied to determine if their susceptibility to antagonistic bacteria was similar to their corresponding parent strain. Additionally, this experiment helped confirm the inhibitory activity of the previously identified epiphytic isolates before their use in further experiments. Also, the results from this experiment allowed the statistical comparison of the inhibitory effect of each ivAEB. An *in vitro* antagonistic effect test was carried out using produce-isolated *iv*AEB. Only leafy green-isolated *iv*AEB toward *E. coli* O157:H7 and fruit-isolated *iv*AEB toward *S.*

Saintpaul were used since only those *iv*AEB treated specimens were to be used in the growth inhibition experiments on leafy greens and fruit surfaces, respectively.

For the experiment, 24-h cultures of *iv*AEB were prepared. LAB, CL, and MS *iv*AEB were incubated for 24 h at 35 ºC on TSA (CL, and MS) or MRS (LAB), and PY *iv*AEB were propagated on TSA and incubated for 48 h at 25 ± 2 ºC. One µl of the culture was spot inoculated in MRS agar or TSA agar and incubated for 24 h at 35 ºC (MS, LAB, CL) or 48 h at 25 ± 2 ºC (PY). One isolate was spot inoculated 4 times per plate using 4 plates. After 1-h attachment, one plate was overlaid with 10 ml of semisolid TSA containing *E. coli* O157:H7; a second plate was overlaid with *S.* Saintpaul; a third plate was overlaid with RIF-resistant *E. coli* O157:H7, and a fourth plate was overlaid with RIF-resistant *S.* Saintpaul. The pathogen concentration of the overlay media was 5.8–6.2 log CFU/ml. The overlays solidified for 1 h at 25 ± 2 ºC and the plates were upturned and incubated at 35 ºC (LAB, CL, and MS) or 25 ± 2 ºC (PY) for 24 h. After incubation, plates were examined for inhibition areas, and the total IA was calculated as previously described. The experiment was repeated two times (n = 8).

Determination of biofilm using the crystal violet method

In an attempt to estimate the inhibitory effect of *iv*AEB toward the biofilm formation by *E. coli* O157:H7 and *S.* Saintpaul, two methods were preliminarily evaluated. Biofilm formation can be evaluated by staining biofilm formed on sterile surfaces with crystal violet, during *in vitro* experiments. However, the evaluation of the inhibition of biofilm formation caused by other bacteria is challenging. The rationale was that even if the target bacteria did not express biofilm formation due to the presence of the antagonistic bacteria,

these antagonistic bacteria could form biofilm which would be non-specifically stained by the crystal violet. To confirm this, an experiment was carried out including 70 ivAEB and both pathogens, *S*. Saintpaul and *E. coli* O157:H7. For this, the protocol described by Head and Hongwei *(134)* was followed. One loopful of *E. coli* O157:H7 and *S*. Saintpaul from stock cultures were separately transferred to TSB and incubated for 24 h at 35 ºC. One loopful of each ivAEB isolates was separately transferred to TSB or MRS and incubated at 35 ºC (MS, LAB, and CL) or 25 ºC (PY) for 24 h or 48 h. The 24 or 48 h cultures were diluted 1:100 in TSB, and 125 µL were dispensed onto three wells of two 96-well microtiter plates (Microtest™, Becton Dickinson and Co.). One microtiter plate was processed immediately, and another one was incubated at 35 ºC or 25 ºC (PY) for 24 h. The staining process consisted of dispensing 100 µL of crystal violet into each well. After 30 min at 25 ºC, the contents were discarded by turning the plate and vigorously shaking using a vertical movement, to expel the content of the wells. The plate was rinsed three times by submerging slantways into sterile distilled water, and dried by shaking and tapping the microtiter plate on paper towels. Suspensions of the biofilm-attached crystal violet was achieved by adding 200 µL of 95% ethanol to each well. The absorbance of each well was read using an EL800 absorbance microplate reader (BioTek® Instruments, Inc., Winooski, VT) with an OD set to 570 nm. The same procedure was repeated using the incubated plate after 24 h. In all readings, blank wells, and wells inoculated with sterile TSB, were included as controls

Determination of biofilm formation using red Congo agar

Since the formation of biofilm using crystal violet was unspecific to the biofilm forming bacteria (pathogenic or antagonistic), one more biofilm detection alternative was studied *(307)*. Red Congo agar is commonly used to detect the formation of amyloids. Amyloids are the principal proteins included in the curli formation by gram negative bacteria *(307)*. Since curli is linked to biofilm formation, the estimation of the amyloid presence is an indirect indication of biofilm formation. Thus, the detection of amyloid production as an indicator of biofilm formation would detect biofilm produced by enteric pathogens, even when other bacteria is present in the same environment. To study possible application of the red Congo agar technique in biofilm formation, *S.* Saintpaul and *E. coli* O157:H7 were tested on different variations of the red Congo agar.

A loopful of a 24 h culture of *S.* Saintpaul and *E. coli* O157:H7 were separately streaked in TSA-Suc-RCA, TSA-RCA, BHI-Suc-RCA, and BHI-RCA. Also, 5 ivAEB isolate cultures were streaked onto these agar media, and all isolates were incubated for 24 h at 35 ºC.

Evaluation of the microbiological content in fruits and leafy greens

To determine the content and nature of the epiphytic bacteria found on the surfaces of different fruits and leafy greens and the environmental impact over the bacterial content, the following procedures were observed.

Produce samples

The leafy green commodities utilized in this study were: Spinach (*Spinacia oleracea*), curly endives (*Cichorium endivia* var. *crispum*), and curly parsley

(*Petroselinum crispum* var. *crispum*). The fruit commodities included jalapeno peppers (*Capsicum annuum*), Roma tomatoes (*Solanum lycopersicum*), and cantaloupe melons (*Cucumis melo* var. *cantalupensis*). Samples of each commodity were obtained from fields located in Weslaco, TX, U.S. For fruit sample collection, four fields were selected. Two separate fields of each fruit commodity were harvested during the fall (October-December) and two more during the summer (May-June) harvesting seasons. Leafy greens were collected only during the winter (February-March) season in two different fields.

For sample collections, the field was divided into five sections (center, left front, right front, left back, right back), to a total of 25 sampling sites per field (Fig. 1). A sample of approximately 100 g of each leafy green commodity, including parts of the stems, leaves and petioles of the same plant were collected at each sampling site. For fruits, one piece was collected at each sampling site. All samples were aseptically collected, using scissors and disposable gloves previously disinfected with 70% ethanol, and placed in individual zip-lock bags. During collection, tomatoes and cantaloupes were separated from the stems while peppers preserved 1–2 cm of the stem attached. Samples were not washed or decontaminated at any point during or after collection. After collection, samples were stored in insulated containers with frozen coolant packs and shipped to the Food Microbiology Laboratory at Texas A&M University (College Station, TX) to be analyzed within 24 h from collection.

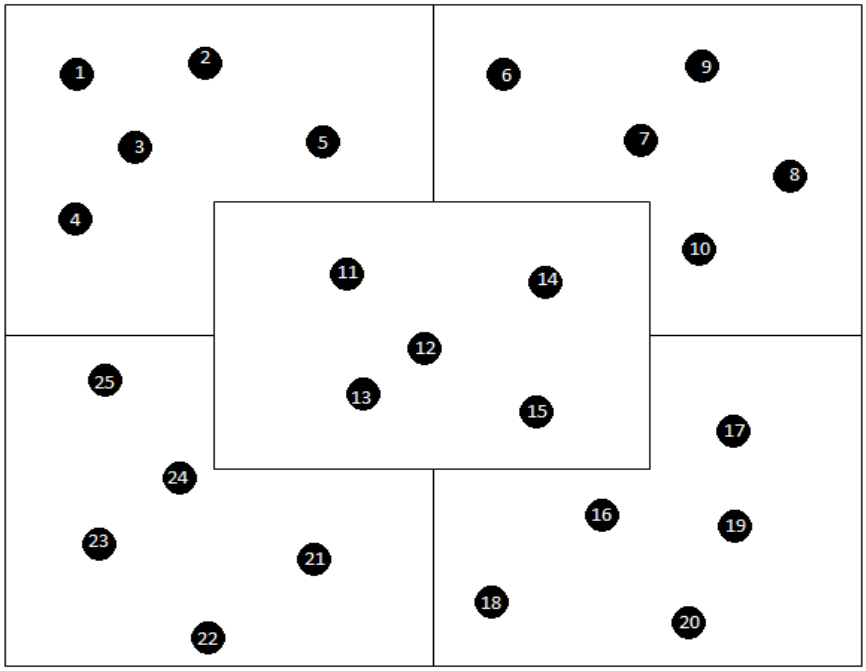

FIGURE 1. Sampling pattern for fruits and leafy greens collection within fields. Numbers in circles indicate the sample identification number. Five areas are represented by this simple schematic diagram showing that five samples were collected per area. Diagram is not to scale.

Weather conditions, including temperature (ºC), and relative humidity (%) were obtained from the Weslaco weather station for each collection day at all fields and during the seasons when the sampling took place. Irrigation methods and water sources were recorded.

Upon sample arrival, the cantaloupes, tomatoes, and peppers were subsampled by excising three circles of 10 cm^2 each from produce surface (rind or skin), whereas the endives, spinach, and parsley were subsampled by weighing 25 g including petioles, leaves and/or stems. All subsamples were aseptically transferred to sterile plastic bags.

Fruit skin samples were mixed with 99 ml of PW. Leafy green samples were mixed with 225 ml PW. Samples were pummeled in a stomacher blender (A.J. Seward, London, UK) for 1 min at 300 RPM to dislodge bacteria from the surface. Serial dilutions of the sample suspensions were spread plated onto TSA to determine PY and MS count, then spread plated onto MRS agar and overlaid with MRS agar to determine LAB count, and finally pour-plated and overlaid using VRBA for CL count determination. Incubation conditions included: PY in aerobic conditions at 7 °C for 5−7 d, MS at 37 °C for 24 h, and CL at 35 °C for 48 h. For selective purposes pertaining to LAB isolation, MRS plates were anaerobically incubated in jars (BBL GasPack system, BD, Sparks, MD) using gas packs (BBL GasPack system, BD) without a catalyst at 35 °C for 48 h. Colonies were counted after incubation and the colony counts were calculated as CFU/cm^2 for fruits or CFU/g for leafy greens. Using these methods, the minimum detection level of bacterial content for fruits and leafy greens was of 0.52 log CFU/cm^2, and 1 log CFU/g, respectively.

Selection of epiphytic bacteria isolates

The produce epiphytic bacteria used in this study were recovered from leafy greens and fruit samples collected from growing fields in Weslaco, TX. The isolates were obtained during the fruit and leafy greens microbiological content experiment and the detailed procurement of produce samples is explained in the methods section of that experiment. Individual bacterial colonies grown in TSA, VRBA, and MRS, and utilized for the respective enumeration of mesophilic (MS), and psychrotrophic bacteria (PY), coliforms (CL), and LAB were selected. Selection of isolates was adapted from Johnston, et al. *(145)*. For each sample and media type, one plate was selected. The particular plate

had to contain less than 250 CFU/plate. When the selected plate contained more than 10 CFU/plate, the colony selection was randomized using a Harrison disk *(130)*. A cutout of the disk was placed under the plate and the colonies were selected randomly. For plates containing less than 10 CFU/plate, all colonies were selected. Colonies were aseptically collected using a sterile needle, from the selected petri plates by commodity sample and bacterial group (MS, CL, LAB, and PY).

Storage of epiphytic bacteria isolates

Selected isolates were streaked in TSA slants for PY, MS, and CL or stabbed in MRS agar deep tubes for LAB. Inoculated MRS stabs were covered with 1 ml of sterile heavy mineral oil. TSA slants and MRS stabs were incubated for 24 h at 35 °C for MS, CL, and LAB isolates or at 25±2 ºC for PY isolates. After incubation, tube caps were covered with parafilm. TSA slants were stored at 25 ± 2 ºC and MRS stabs were stored at 4 °C for up to 4 wks. For extended storage an alternative method was used. A loopful of each bacterial isolate from MRS stabs or TSA slants was propagated in 5 ml of MRS broth or 5 ml of TSA correspondingly, and incubated for 24–48 h at 35 °C for MS, CL, and LAB isolates or at 25 ± 2 ºC for PY isolates. After incubation, 1 ml of the culture was mixed with 1 ml of sterile TSB containing 30% glycerol v/v (Avantor, Center Valley, PA) (for MS, PY, CL) or MRS broth containing 30% glycerol v/v (for LAB) in sterile microtubes (VWR, Radnor, PA) to obtain 2 ml of medium with 15% (v/v) glycerol as cryoprotectant. Microtubes containing the isolate suspensions were frozen at − 80 °C for 24 h and transferred to a commercial freezer at − 20 °C for extended storage. When needed, TSA slants or MRS stabs were prepared from frozen vials for experiments. Microtubes

containing epiphytic isolates were thawed at 25 ± 2 ºC for 5 min and a loopful of CL, MS, and PY isolates was suspended in TSB while LAB isolates were suspended in MRS broth. These were incubated at 35 °C for CL, MS, and LAB isolates, or at 25 ± 2 ºC for 48 h for PY isolates. After incubation, each isolate was streaked in a TSA or MRS agar and incubated at 35 °C for 24 h for MS, CL and LAB or at 25 °C for 48 h for PY. After incubation, a single colony of each CL, PY, and MS isolate was streaked in TSA slants, and one colony of each LAB isolate was stabbed in MRS agar deep tubes. The MRS stabs were overlaid with 1 ml of sterile mineral oil. Slants and stabs were incubated at 35° C for 24 h, except for PY slants, which were incubated at 25 ± 2 ºC. After incubation, the caps were covered with parafilm to avoid dehydration. TSA slants were stored at 25 ± 2 ºC and MRS stabs were stored at 4° C. All working slants and stabs were utilized within four weeks. One or two days before the experiments, the CL, MS, and PY strains were propagated in TSB. LAB isolates were propagated in MRS broth. MS, CL, and LAB isolates were incubated for 24 h at 35 °C, and PY for 48 h at 25±2 ºC.

Epiphytic bacteria recovery

In total, 15,742 isolates were recovered from the various media, from all cultivars in one season (for leafy greens) or two seasons (for fruits) and stored at room temperature on TSA slants. MRS deep agar tubes were kept under refrigeration, and TSB or MRS supplemented with 15% glycerol were frozen at – 20 ºC. The isolates recovered and stored in TSA slants and MRS tubes were propagated in TSB or MRS broth supplemented with 15% glycerol after 4 wks of the initial isolation. After the first attempt to propagate these isolates for further testing, several isolates were unable to grow on culturing broths (TSB,

for PY, CL, and MS, or MRS broth for LAB), neither at 25 ºC nor at incubation temperature (35 ºC for MS, LAB, and CL). Some other isolates were not able to grow in culturing broths after frozen storage. A total of 9,307 isolates (60% of the originally isolated specimens) were able to propagate and their inhibitory effect *in vitro* against *E. coli* O157:H7 and *S.* Saintpaul was studied.

In vitro antagonistic effect of epiphytic bacteria toward enteric pathogens

The *in vitro* inhibitory effect of epiphytic bacteria from leafy greens and fruit commodities toward *S.* Saintpaul and *E. coli* O157:H7 was studied following the further described modification of the spot agar test described by Fleming et al. *(114).*

Pathogen inoculum and overlay preparation

E. coli O157:H7 and *S.* Saintpaul cultures were prepared by suspending a loopful from storage slant in TSB and incubating at 35 ºC for 24 h. After incubation, the cultures were centrifuged at 1,623 x *g* for 15 min, decanted and resuspended three times with 10 ml of PBS to remove waste material from incubation. After the last suspension, each culture was diluted in 0.1% PW to reach a concentration of 7 log CFU/ml. The final concentration of the inoculum was calculated by enumeration of CFU after spread plating serial dilutions of the inoculum in TSA and inoculation for 24 h at 35 ºC. One ml of this dilution was transferred to individual tubes containing 9 ml of molten semisolid TSA (at 50 ± 2 °C), and the content was immediately poured to overlay epiphytic cultures as further described.

Epiphytic bacteria spot inoculum

Epiphytic isolate cultures were prepared as follows. A loopful of each epiphytic bacteria from TSA slants or MRS tubes was suspended in 10 ml of TSB for CL, MS, and

PY isolates or in 10 ml of MRS broth for LAB isolates, and incubated at 35 °C for 24 h (CL, MS, and LAB) or 25 ± 2 ºC for 48 h (PY). One µL of each culture was used as the inoculum to spot inoculate onto MRS or TSA agar as further described.

Spot agar test

One µl of each 24 or 48 h epiphytic bacterial culture was spot inoculated on two separate plates containing TSA agar (CL, MS, and PY isolates) or MRS agar (LAB isolates), using a sterile micropipette tip. Four isolates were spot inoculated on each plate allowing enough space between each spot. The spots were allowed to dry for 15 min, and incubated at 35 °C for 24 h (CL, MS, LAB) or at 25 ± 2 ºC for 48 h (PY). After incubation, previously inoculated semisolid TSA with either *E. coli* O157:H7 or *S*. Saintpaul were poured to cover each plate containing the well-grown bacterial spots. After pouring, the overlays solidified at 25 ± 2 ºC for 1 h and were incubated for 24 h at 35 ºC for CL, MS, and LAB isolates or at 25 ± 2 ºC for PY isolates. After incubation, plates were examined for inhibition zones surrounding each spot.

Estimation of the *in vitro* antagonistic effect

Plates containing each isolate with an incubated pathogen overlay were examined for clear zones surrounding each isolate spot. These clear zones indicated growth inhibition of the pathogen contained in the overlay due to the presence of antagonistic isolate spots. Diameters of the inhibition zone (halo) and isolate spot were measured with a dial caliper and recorded. The area sizes of the inhibition halo and the spot were calculated as previously described. Epiphytic isolates were considered positive to *in vitro* antagonistic effect when the calculated IA was > 1 mm^2. Further experiments including

these positive antagonistic strains are referred as *iv*AEB (*in vitro* antagonistic epiphytic bacteria).

Biochemical identification of *in vitro* antagonistic epiphytic bacteria

Epiphytic bacteria that presented the *in vitro* antagonistic effect (*iv*AEB) against *E. coli* O157:H7 and *S.* Saintpaul were identified based on its biochemical properties using a VITEK-2 system for microbiological identification (BioMérieux, Durham, NC) as further described.

Isolate selection for biochemical identification

To avoid repeated identification of identical isolates that originated from the same sample, a selection criteria was established. This included a pool of the total number of *iv*AEB toward *E. coli* O157:H7 from leafy greens samples, or *iv*AEB toward *S.* Saintpaul from fruits samples defined as *n*. If *n* was equal or less than 6, all isolates were selected for further biochemical identification. Conversely, if *n* was more than 6, the formula: *n*/2+1 was applied to determine the number of isolates to be identified. This conditions allowed the identification of < 50 % of the total antagonistic isolates from each sample.

Initial biochemical tests for card selection

The VITEK-2 system is an integrated automatic system for microbial identification of bacteria and yeasts using algorithms based on fluorescence and colorimetry. It also provides information about antimicrobial susceptibility testing based on kinetics analysis of bacterial growth *(115, 207)*. To prepare the sample according to manufacturer instructions, a loopful of TSA slants or MRS tubes containing the *iv*AEB isolate was suspended in 10 ml of TSB (CL, MS, and PY) or MRS broth (LAB) and incubated at 25

± 2 ºC for 48 h (PY) or at 35 °C for 24 h (CL, LAB, and MS). The incubated culture was streaked for isolation in TSA or MRS agar using a sterile needle and incubated for 18–24 h. After incubation, Gram staining, catalase test and oxidase tests were performed.

VITEK-2 card preparation

One to three CFU were selected from TSA (CL, MS, and PY) or MRS (LAB) plates and collected using a sterile cotton swab and suspended in 3 ml of saline solution (0.45% sodium chloride) aseptically dispensed in polystyrene tubes. The suspension was adjusted with saline solution using a turbidity meter, to the required McFarland standard specified by the manufacturer for each card type. The card for VITEK identification was selected according to Gram stain (positive, variable or negative), cell shape (bacilli or cocci), and catalase results (positive or negative). The respective VITEK-2 test cards were filled with cell suspension according to the manufacturer's instruction. A GP card was used to identify Gram positive bacteria; GN card was used to identify fermentative, and a non-fermentative Gram-negative, bacilli CBL card was used to identify spore-forming bacilli and CBC for Gram-positive, catalase negative bacilli. Each test tube containing an isolate suspension was attached to the selected card and placed in a loading cart, which was loaded into the VITEK-2 system following manufacturer procedures.

Discriminatory biochemical tests

When the results given by the VITEK system was nondiscriminatory for two or more species, the recommended further analyses specified by each VITEK-2 result were carried out to discriminate the species. These tests included one or more of the following: Antibiotic susceptibility test to vancomycin and clindamycin; fermentation of sorbitol,

galactose, lactose, dextrose, raffinose, and dulcitol; production of catalase and oxidase, production of urease; nitrate reduction, growth at 6.5% NaCl, hydrolyzation of tryptophan (indole test), litmus milk test, and motility *(176)*.

Effect of antagonistic epiphytic bacteria over the growth of *E. coli* O157:H7 on leafy greens and of *S.* Saintpaul on fruits

To evaluate the ability of previously identified *in vitro* antagonistic epiphytic bacteria (*iv*AEB) from fruits, to inhibit *S.* Saintpaul growth on rind/skin, and of *iv*AEB from leafy greens, to inhibit *E. coli* O157:H7 growth on leaves, the following procedures were applied.

Procurement of produce samples

Produce samples for this experiment were obtained from a local produce distributor. Sample selection criteria excluded waxed, bagged, mixed, chopped, prewashed, or organic produce. Due to the unavailability of unwaxed Roma tomato, the experiments including this commodity were conducted using unwaxed vine tomatoes var. salad. Samples were transported in individual plastic bags to the laboratory listed above and placed in plastic containers, covered with aluminum foil, and stored at 4°C for up to 24 h until further sampling was carried out, as will be further explained.

Preparation of leafy green samples

For leafy green samples, leaves were selected based on similarity in appearance and size. Visibly wounded or broken outer and core leaves were discarded. After sorting, leaves were washed with running tap water, rubbing the surface gently with gloved hands to remove soil and debris for 1 min. Excess water was removed by shaking gently and

using a salad spinner (OXO, El Paso, TX). Individual leaves were disinfected with 70% ethanol sprayed to cover the surface of both, adaxial and abaxial sides and air dried for 2 h at 25 ± 2 ºC. For spinach and endives, three circles of 10 cm^2 each were aseptically excised from one leaf using a borer. One sample consisted of 3-10 cm^2 cutlets from the same leaf. For parsley, since the leaf is a compound of leaflets, three leaflets from one leaf were separated and considered as one sample. The corresponding pieces per sample were placed into a sterile petri plate containing VWR grade 415 filter paper (415, 7.5 mm, VWR) moistened with 2 ml of sterile distilled water to keep them from drying during incubation as described by Khalil and Frank *(154)*.

Preparation of fruit samples

For fruit samples, wounded or damaged pieces were eliminated. Each fruit was rinsed in tap water and rubbed with gloved hands to eliminate soil and debris from surface. After rinsing, the fruits were air-dried on paper towels for 1-8 h at 25 ± 2 ºC. The skin or rind of each fruit was disinfected by spraying 70% ethanol to cover the surface and air-dried for 1 h at 25 ± 2 ºC. For tomatoes and cantaloupes, three circles of 10 cm^2 per fruit were identified on the skin/rind and the three circles were considered as one sample. For peppers, three peppers were marked with 1 area of 10 cm^2, and the three areas were considered as one sample. Areas of discoloration or wounds in the skin/rind were not used neither the stem nor the bloom scar. The cantaloupes were placed inside sterile beakers containing approximately 500 ml of sterile distilled water and a smaller beaker inside to hold the cantaloupe in place. Tomatoes and peppers were held on weighting boats with filter paper (415, 9 mm, VWR) moistened with 2 ml of sterile distilled water. Crinkled

sterile aluminum foil was placed between the filter paper and the fruit to separate the sample from direct contact with water. The weighting boats containing the samples were individually placed inside zip-lock bags and closed before incubation.

Pathogen inoculum

For the experiment, RIF-resistant *E. coli* O157:H7 and *S.* Saintpaul were individually cultured in 10 ml of TSB for 24 h at 35 °C. The culture was centrifuged at 1,623 x *g* for 15 min, decanted, and resuspended three times using 10 ml of PBS to remove waste material from incubation. The final concentration of the inoculum was calculated by enumeration of CFU after spread plating serial dilutions of the inoculum in TSA-RIF and inoculation for 24 h at 35 ºC. Two serial dilutions were made from each pathogen, using 9 ml PW 0.1% to achieve a concentration of approximately 6 log CFU/ml per pathogen.

***In vitro* epiphytic antagonistic bacteria (*iv*AEB) inoculum**

One or 2 d before the experiment, 1 culture of each *iv*AEB was prepared in TSB (MS, CL, and PY) or MRS (LAB) broth and incubated for 24 h at 35 ºC (MS, LAB, and CL) or 48 h at 25 ± 2 ºC (PY). After incubation, the cultures were centrifuged at 2191 x *g*, for 20 min (to maximize recovery), and decanted and resuspended in 1 ml of PBS to increase the concentration of each *iv*AEB in suspension to approx. 8−9 log CFU/ml, and to remove waste material from incubation.

Inoculation procedures

Fruit marked areas or leafy green cutlets were spot inoculated with 10 droplets of 1 µl of each commodity-corresponding *iv*AEB inoculum with a concentration of 8–9 log

CFU/ml and let attach for 2 h at 20 ºC (for leafy greens) or at 30 ºC (for fruits) to mimic growing field temperatures. The adaxial (upper) side of the leaf pieces was used for inoculation. After the initial 2 h attachment, the same inoculated sites were inoculated with 10 droplets of 1 µl of the pathogen inoculum containing 6 log CFU/ml of RIF-resistant *E. coli* O157:H7 (leafy greens) or RIF resistant *S.* Saintpaul (fruits) and incubated for up to 24 h at 20 ºC or 30 ºC, respectively.

Evaluation of the growth effect

Triplicate samples per *iv*AEB were retrieved from incubation at 0, 6, 12, and 24 h for all commodities except peppers which were retrieved at 0, 12, 18, and 24 h. Samples not inoculated with the pathogen or the *iv*AEB were included as negative control. Treatment control consisted of samples inoculated only with the pathogen. Leafy green samples from one petri plate each, were suspended in 25 ml of PW and hand massaged for 1 min to dislodge bacteria. For fruits, three marked skin/rind areas were aseptically excised using a flame-sterilized borer of 10 cm^2 diam., placed in a sterile bag and suspended in 99 ml of PW 0.1%. Fruit samples were pummeled in stomacher for 2 min at 300 RMP. Serial dilutions in 0.1% PW of each sample were spread plated onto TSA-RIF and incubated at 35 ºC for 24 h. After incubation, CFU were enumerated. The experiment was repeated three times per pathogen and *iv*AEB (n=3).

Evaluation of *E. coli* O157:H7 growth and stomata invasion on endives in the presence of antagonistic epiphytic bacteria

The effect of *iv*AEB on the *E. coli* O157:H7 ability to form biofilms and to migrate to stomata areas was studied on endive leaves and observed using confocal microscopy using the following procedure.

Sample selection and preparation

Endives were obtained from a local retail distributor and stored at 4 ºC for up to 24 h. Whole leaves were selected for similar appearance and size from the middle leaves of the plant. Visibly wounded or broken leaves were not selected nor were the outer and inner core leaves. Leaves were washed with running tap water for 1 min, rubbing gently with gloved hands to remove soil and debris. Dripping water was removed by shaking gently and by using a salad spinner. Endive leaves were disinfected with 70% ethanol sprayed to cover the surface of adaxial and abaxial sides, to reduce the epiphytic microbial load to approx. 4 log CFU/cm^2 (Aerobic plate count) and air-dried for 1–2 h at 25 ± 2 ºC. Leaf pieces of 1 cm^2 were cut using a flame-sterilized scalpel. Three leaf pieces were placed inside sterile petri plates containing filter paper moistened with 3 ml of sterile distilled water to keep them from drying during attachment and incubation.

Pathogen inoculum

For the experiment, a loopful from the TSA slant containing *E. coli* O157:H7 GFP was grown in 10 ml of TSB for 24 h at 35 °C. The culture was centrifuged at 1,623 x *g*, for 15 min, decanted, and resuspended in 10 ml of PBS. The final concentration of the

inoculum was calculated by enumeration of CFU after spread plating serial dilutions of the inoculum in TSA-AMP and inoculation for 24 h at 35 ºC.

Epiphytic bacteria inoculum

One isolate each of *Streptococcus alactolyticus, Bacillus licheniformis, Gemella bergeri, Staphylococcus sciuri*, and of *Enterococcus gallinarum* antagonistic toward *E. coli* O157:H7 on endive leaves were used for treatments. For the experiment, a loopful from each TSA slant or MRS stab was transferred to TSB or MRS using the same type of media during their isolation and incubated for 24 h at 35 ºC. After incubation, the isolate cultures were centrifuged at 2,191 x *g* for 20 min, decanted, and resuspended in 1 ml of PBS to remove waste material from incubation, and to increase the concentration to approx. 8–9 log CFU/ml. The final concentration of each bacterial strain was determined by serial dilutions and spread plating of each inoculum onto MRS or TSA agar and incubating at 35 ºC for 24 h.

Inoculation of *E. coli* O157:H7 and epiphytic bacteria on endive surfaces

The treatments consisted in inoculating 1-cm^2 endive pieces with one drop of 10 µl of epiphytic bacteria inoculum each and allowing these to attach for 2 h at 25 ± 2 ºC. After attachment, the leaves were inoculated with one drop of 10 µl of the *E. coli* O157:H7 GFP inoculum in the same area where the epiphytic bacteria inoculum was placed, and incubated at 20 ºC for up to 3 d. The final concentration of pathogen and isolates inoculum were ~ 6 log CFU/10 µl.

Determination of *E. coli* O157:H7 on endive samples

To complement the findings from the confocal microscopic analysis of samples for the presence/absence of *E. coli* O157:H7 GFP on the endive leaves, two samples per treatment (antagonistic bacteria isolate) and two negative (not inoculated with the pathogen or epiphytic bacteria) and two untreated (positive) controls were processed for bacterial enumeration. At 0 h, 12 h, and 60 h of incubation, two samples per treatment and controls consisting of three inoculated endive pieces were suspended in 99 ml PW, and pummeled in a stomacher for 1 min at 300 RPM to dislodge *E.coli* O157:H7 GFP from the surface. Serial dilutions in PW of the sample suspensions were spread plated onto TSA-AMP and incubated for 24 h at 35 ºC to enumerate the pathogen.

Preparation of samples for confocal microscopy

Five pieces per treatment were retrieved from incubation at 12 and 60 h for confocal microscopy analysis. The inoculated marked areas of each leave piece were aseptically excised with a sterile scalpel and surgical forceps to obtain a sample of approximately 0.5 cm diam. The sample was mounted between two 24 x 60 mm rectangular microscope glass slides, kept together using adhesive clear tape, and transported to the Imaging Analysis Laboratory at Texas A&M University (College Station, TX).

Evaluation of the growth and stomata invasion

Ten to 15 photomicrographs were captured using the confocal laser scanning microscope (CLSM, Zeiss LSM 780 NLO Multiphoton microscope, Carl Zeiss, Jena, GE) with lasers set at 488 nm (green) excitation wavelength and 600 nm (red) for contrasting background. The image size corresponded to 212.5 x 212.5 µm and was observed using

the 40x immersion oil objective and 1.4 of numerical aperture. Images were digitally captured and stored in a Tagged Image File Format (16 bits, TIFF) *(6)*. The images were observed using the MS Office Windows photo viewer program for Windows ver. 8.1. without any modifications or edits to the images *(194)*. The digital images had an approximate resolution of 2672 x 2672 pixels, and a printing resolution of 96 x 96 dpi. The presence of stomata, the growth of *E. coli* O157:H7, and the invasion of stomata by the pathogen were recorded. To qualitatively evaluate the pathogen presence in the leaf surface, a classification was used for the growth and stomata invasion. In the case of pathogen growth, images with no visible growth were graded as "No growth"; those images showing low growth (approx. 1 to 33% of the total image) were graded as "Low"; those images showing growth of 34-66% were recorded as "Moderate"; and those showing more than 67% of growth were graded as "High growth." The stomata invasion was graded similarly. Only open stomata were used for this evaluation even when closed stomata could present bacteria but the image would not allow their observation. Open stomata showing no growth of *E. coli* O157:H7 GFP in the lumen space or attached to the opening walls were classified as "no invasion" and assigned the number 0; if an open stomata showed invasion of 1–33% of this space, it was classified as "low invasion" and graded as 1; when stomata presented invasion of approx. 34 to 66%, it was classified as "moderate" and graded as 2, and stomata presenting an invasion of the pathogen in more than 67% of the lumen and walls was graded as 3, and classified as "high invasion." The frequency of each classification was compared using Z-test for two proportion comparisons.

Data analyses

All bacterial CFU counts were transformed to their corresponding logarithmic value and expressed as log CFU for statistical analyses. All statistical analyses were calculated using SAS 9.4 *(242)*. Statistical differences were considered significant when α-value was < 0.05.

For the preliminary experiment to determine the incubation time to be used in the *in vitro* inhibitory effect test, the T-TEST procedure was carried out using the overall mean inhibition area (IA) by treatment and pathogen tests.

For the preliminary experiment to select a media combination to be utilized during the *in vitro* spot agar test, the mean IA for each treatment by pathogen and overall mean IA were compared using the ANOVA procedure. When statistical differences were found ($P < 0.05$), Tukey's (HSD) test was used to separate the IA means per treatment. The proportion of detected antagonistic bacteria by treatment and pathogen tested were compared using a Z-test for comparison between two binomial proportions.

For the preliminary experiment to determine similarities in the growth of RIF-resistant and parent strain pathogens, the growth of *E. coli* O157:H7 and *S.* Saintpaul parent strains and RIF-resistant strains were compared by plotting log CFU/ml values as a function of time in h. Growth data were fitted to a Baranyi model equation for sigmoid curves, using DMFit Excel add-in ver. 2.1 (IFR, Colney, UK) *(18)*. For individual growth curves, the following growth parameters were estimated: initial population (N_0), maximum population (N_{max}), specific growth rate (μ_{max}), and doubling time (d-t). Due to the rapid growth of the bacterial strains in TSB, lag phase was not accurately predicted by

the software (Fit error > 0.20) and was not included in the analysis. Growth parameters of each pathogen were compared using the T-TEST procedure of SAS 9.4 *(242)*.

For the preliminary experiment to compare the susceptibility of parent and RIF-resistant *E. coli* O157:H7 and *S.* Saintpaul to the inhibitory effect of *iv*AEB, the mean IA by respective parent and RIF-derivative were compared using the T-Test procedure of SAS 9.4 software *(242)*. To compare the inhibitory effect of each isolate toward *E. coli* O157:H7 or *S.* Saintpaul, the mean IA for each treatment by pathogen was compared using the ANOVA procedure. When statistical differences were found ($P < 0.05$), Tukey's (HSD) test was used to separate the IA means per treatment.

For the preliminary experiment to determine biofilm formation by E. coli O157:H7, S. Saintpaul, and ivAEB isolates, using the crystal violet method, the absorbance of three replicates at 0 h and 24 h from the isolates were compared to each pathogen and to the negative control (sterile TSB) using the T-Test procedure of SAS 9.4 software *(242)*.

To analyze the microbial content on fruits and leafy greens, the mean bacterial counts (log CFU/g for leafy greens or log CFU/cm^2 for fruits) were calculated and compared by leafy green (spinach, endives, and parsley) or fruit (cantaloupe, tomatoes, and peppers) commodity, with ANOVA analysis for each bacterial group (MS, LAB, CL, and PY). When ANOVA indicated statistical differences ($P < 0.05$), a mean separation was carried out using Tukey's honestly significant difference (HSD) test. The effects of temperature, relative humidity, and season (only fruits) were analyzed using the ANOVA procedure separating each bacterial group. The effect of the irrigation method (drip or

flood) on tomatoes and peppers bacterial counts were analyzed using the T-Test procedure.

For the analysis of the antagonistic effect *in vitro* against *E. coli* O157:H7, *S.* Saintpaul or both pathogens by epiphytic bacteria, isolates presenting IA > 1.0 mm^2 were considered positive and included in the analysis. Mean IA of positive results by bacterial group and species were calculated to determine field, commodity, and season effect using ANOVA. To compare the content of epiphytic bacteria by commodity, field, and season, antagonistic to *E. coli* O157:H7, *S.* Saintpaul, or both pathogens, percentages of positives were compared using the Z-test for two binomial proportions. The mean IA for each bacterial group by inhibited pathogen was compared using ANOVA followed by mean separation using Tukey's HSD test *(242)*.

To analyze the effect of *iv*AEB over the growth of *E. coli* O157:H7 on leafy greens leaves and the effect of *S.* Saintpaul in fruit skin/rind, log CFU/ml values from sample processing were used to calculate the overall growth (OvGr), growth rate (μ_{max}), and doubling time (d-t) for each epiphytic isolate tested, and these values were compared to the untreated control using the SAS T-Test procedure *(242)*. The OvGr was calculated by subtracting the final count in log CFU/ml after 24 h of incubation, from the initial count at 0 h of incubation. Doubling time (d-t, in min) was calculated using the formula:

$$\text{d-t} = t / n$$

where n was the number of generations and t was the time interval in min. considered within the exponential growth. The number of generations was calculated using the formula:

$$n = \frac{\log N_1 - \log N_0}{\log_{10}(2)}$$

where N_1 is the CFU/ml count at the final time point, and N_0 is the CFU/ml count at the initial time point considering the time interval at the exponential growth. The growth rate was calculated using the formula:

$$\mu_{max} = \left(\frac{\log N_1}{\log N_0}\right)^{1/t} - 1$$

To analyze the effect of *iv*AEB toward the growth and stomata invasion of *E. coli* O157:H7 GFP using confocal images, total and open stomata were added by treatment and incubation time, and the proportion of open stomata were compared between treatment and control and between incubation times within treatments using Z-test comparison for two proportions. The grades obtained for growth and stomata invasion were averaged by incubation time, and the treatment and means were compared by incubation times between treated and untreated samples (*E coli* O157:H7 control) using the T-test procedure of SAS *(242).*

RESULTS AND DISCUSSION

Preliminary experiments

Transformation of microbiological content by g to content by cm^2

In order to compare the microbiological content of all commodities, a factor of conversion from g to cm^2 was attempted as previously described. However, this transformation was determined unreliable due to the variation of the weight given by the stems, since these were not considered in the transformation and were included in the original samples. Moreover, the areas calculated and the weight of the leaves would vary depending on the roughness of the surfaces; furthermore, the weight loss due to transpiration of the leaves during transportation could add another error factor to this conversion. Thus, it was decided to compare only those commodities that were studied using similar measuring units. Therefore, leafy greens were not compared to fruits.

Incubation time for *in vitro* experiments

The objective of this experiment was to determine the incubation time required by lactic acid bacteria to demonstrate inhibitory activity *in vitro* toward enteric pathogens. The spot agar test by Fleming et al. *(114)* included the incubation of the presumptive inhibitory LAB for 24 h at 30 ºC, before overlay with media containing the pathogen, followed by a second incubation for 24 h at 30 ºC. Treatment 2 methodology was similar to that reported by Brashears et al. *(39)*, who used a 24 h LAB well-grown spot in MRS, and overlaid with molten TSA, inoculated with *E. coli* O157:H7. The modification tested in this experiment included an attachment for 1 h (Treatment 1) and incubation for 24 h

(Treatment 2) prior to the pathogen overlay and further incubation of both for 24 h. treatment time. The temperature of incubation was set to 35 ºC for both treatments and pathogens tested. The initial concentration of each LAB was 5.8 log CFU/µl for *L. amylovorus*, 5.8 log CFU/µl for *L. animalis*, and 6.0 for *P. acidilactici* log CFU/µl per each spot inoculated. The overlay inoculated with *E. coli* O157:H7 contained an initial concentration of 6.6 log CFU/ml, and the *S.* Saintpaul overlay concentration was 6.8 log CFU/ml. The IA was averaged for the two replicates and three LAB isolates (n=6) since no significance difference was found between replicates and LAB strains within treatment. When the IA against *E. coli* O157:H7 was compared by treatment, mean IA in Treatment 1 was significantly smaller ($P < 0.05$), with a mean IA of 18 ± 7 mm^2 than that in Treatment 2 which presented a mean IA of 188 ± 69 mm^2 (Fig. E 2). A similar result was observed when *S.* Saintpaul IA means were compared. The mean IA for Treatment 1 resulted in 18 ± 5 mm^2 while Treatment 2 reached a mean IA of 171 ± 81 mm^2 ($P < 0.05$). *E. coli* O157:H7 and *S.* Saintpaul were similarly inhibited within treatments ($P > 0.05$) (Fig. E 2).

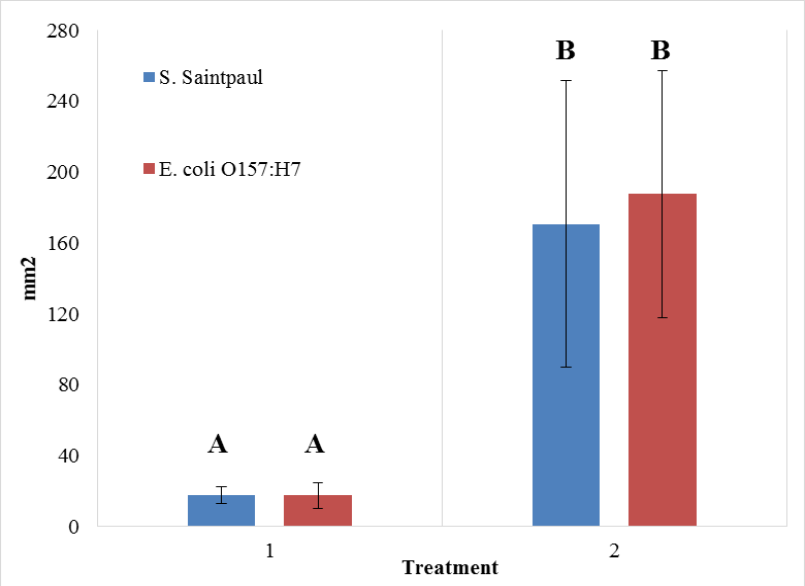

FIGURE 2. *In vitro* antagonistic effect against enteric pathogens using two incubation times for LAB during spot agar test. Graphed here with the mean inhibition area (bars) and standard deviation (vertical lines). Bars with same letter above are not significantly different (P > 0.05).

The LAB utilized in this study were expected to exhibit the inhibition activity toward *E. coli* O157:H7 and *S. enterica* since these have been extensively confirmed *in vitro* in previous studies *(12, 37, 38)*. Brashears et al. *(39)* reported inhibition halos with diameters no larger than 11.47 mm for the inhibitoriest strain (*P. acidilactici* in that study) toward a cocktail of 4 beef-isolated *E. coli* O157:H7 strains. In this study, the diameter of the inhibition halos, also called inhibition zones, were collected from the shortest and the longest diameter of the halo, since tracing only one line across the halo to measure its diameter would bias the results since most halos were not entirely circular. Using the averaged diameters, the results from the present study of the *E. coli* O157:H7 inhibition showed a slightly larger inhibition mean diameter of 16 mm ± 2.5 mm, when using Treatment 2, than the largest diameter reported by Brashears et al. of 11.47 mm. This

could be due to the different *E. coli* O157:H7 strains used or to different methods or measurements of this diameters. The halos obtained when using *Salmonella* cannot be compared since that study did not include *Salmonella* as a screening pathogen.

When Treatment 1 and 2 were compared, it was evident that the less LAB bacteria was incubated, the smaller the IA was. The differences found between these two methods can be related a larger number of LAB cells leading to a greater use of nutrients and an accumulation of inhibitory substances with an expanded diffusion of these in the inoculated media. In a study conducted by Parente et al *(222)*, the dose/response behavior of bacteriocins toward bacterial growth was demonstrated using partially extracted bacteriocins, expressing linear or sigmoidal dose/response curve behavior. Although bacteriocins activity in LAB as their main inhibitory characteristic is less common than the production of acids, and hydrogen peroxide, *P. acidilactici,* has demonstrated bacteriocin-like inhibitory activity toward enteric pathogens with the synthesis of pediocins *(12, 219, 260)*. Furthermore, these strains produce organic acids as metabolites of their fermentation of sugars. Diffusion of these antibacterial acids and a drop in pH might also be related to the higher inhibition areas after prolonged incubation. The LAB strains might have been producing organic acids during the entire incubation time, and they might have also continued growing since they are not highly sensitive to the low pH produced by its metabolites *(39)*.

Although less evident, the growth inhibition of *E. coli* O157:H7 and *S.* Saintpaul *in vitro* when using Treatment 1 denotes that some LAB could demonstrate their antagonistic activity at the early stages of their adaptation to a new environment, and within the first

24 h of coexistence with an enteric pathogen. However, since Treatment 1 was going to be designated for the screening of numerous produce-epiphytic LAB, Treatment 2, was deemed a better choice because it allows for easier differentiation of the antagonistic isolates *in vitro* due to the formation of larger inhibition areas which could possibly benefit from Treatment 2 and its subsequent improvement of inhibition expression from slow-growing bacteria. This method also appears to promise better facilitation in case the further tested epiphytic bacteria releases substances are not easily diffused in agar.

Medium selection for lactic acid bacteria

In total, 254 spinach-isolated presumptive LAB were tested using two different media (MRS and APT) for propagation and spot inoculation (four treatments in total) to identify the medium that would better allow the best expression of pathogen growth inhibition *in vitro*. In total, 151 (59%) showed an antagonistic effect toward either *E. coli* O157:H7 or *S.* Saintpaul or toward both pathogens, when tested using one or more treatments. Seventy-five isolates were antagonistic to both pathogens, while three isolates were antagonistic solely toward *E. coli* O175H7, and 73 isolates were antagonistic solely toward *S.* Saintpaul, using 1 or more treatments. The mean IA were compared considering all inhibitory results toward *S.* Saintpaul, or *E. coli* O157:H7 and toward both pathogens. Overall, the mean IA averaged for both pathogens was significantly larger ($P < 0.05$) when Treatment 1 and Treatment 2 were used obtaining a mean IA of 67 ± 62 and 66 ± 57 mm^2 respectively, than when Treatment 3 and 4 were applied resulting in 24 ± 71 and 19 ± 43 mm^2 mean IA, respectively. When the treatments were compared separating by pathogen inhibition, the mean IA for Treatments 1 and 2 were again consistently larger

(P<0.05) than those mean IA using Treatments 3, and 4 for *E. coli* O157:H7 and *S.* Saintpaul (Table 2). When the percentages of inhibitory isolates using each treatment were compared, the use of Treatment 1 and 2 resulted in more isolates identified as inhibitory to both pathogens (24.8 and 23.6 %, respectively) (P < 0.05), than when using treatments 3 and 4 (14.6, and 17.3 %), respectively (Table 2).

TABLE 2. Media combinations used during *in vitro* spot agar test to determine the antagonistic effect of spinach-isolated bacteria toward *E. coli* O157:H7 and *S.* Saintpaul[a]

Treatment [b]	μ ± SD[c]
Antagonistic against *E. coli* O157:H7	
1) MRS - MRS	53 ± 44 A[d], a[e]
2) APT - MRS	70 ± 51 A, a
3) MRS - APT	22 ± 83 B, a
4) APT - APT	21 ± 61 B, a
Antagonistic against *S.* Saintpaul	
1) MRS - MRS	53 ± 44 A, a
2) APT - MRS	52 ± 38 A, b
3) MRS - APT	18 ± 61 B, a
4) APT - APT	9 ± 14 B, a
Proportions of antagonistic isolates by treatment[f]	
1) MRS - MRS	63/254 (24.8 %) A
2) APT - MRS	60/254 (23.6 %) A
3) MRS - APT	37/254 (14.6 %) B
4) APT - APT	44/254 (17.3 %) B

[a] Spot agar test described by Fleming et al. (1975) *(114)*
[b] Treatments included incubation of spinach-isolated bacteria in MRS broth (1 and 3) or APT broth (Treatments 2 and 4), followed spot inoculation onto MRS agar (1 and 2) or APT agar (3 and 4).
[c] Mean ± standard deviation of *E. coli* O157:H7 or *S.* Saintpaul inhibition areas (mm^2)
[d] Within columns, and pathogen group, values followed by the same uppercase letter are not significantly different ($P > 0.05$).
[e] Within columns, and treatment, values followed by the same lowercase letter are not significantly different ($P > 0.05$).
[f] Number and percentage (%) of spinach-isolated bacteria antagonistic to one or both pathogens.

The mean IA were similar for *S.* Saintpaul and *E. coli* O157:H7 within treatments (P > 0.05), except when using Treatment 2 where *S.* Saintpaul was less susceptible,

presenting a smaller IA of 52 ± 38 mm^2, while the *E. coli* O157:H7 mean IA was 70 ± 51 mm^2 ($P < 0.05$).

The two media used in this study were developed for the propagation and growth of lactobacilli in the laboratory. The MRS medium developed by De Man, Rogosa and Sharpe *(97)*, included most of the recipe ingredients from the Evans and Niven *(105)* medium known as APT, although no thiamine was included, and our MRS medium incorporated a different sugar content and other chemicals *(24)*. The media ingredients used in this experiment are included in Table 3. The medium used to propagate LAB in the laboratory have affected the growth of different lactobacilli species and other fermentative bacteria in previous studies *(151)*.

Noticeably, the ability of LAB to express antagonism toward enteric pathogens *in vitro* was also affected by the medium used. A better adaptation and varied usage of the nutrients contained in MRS agar might have allowed the presumptive LAB to synthetize a higher volume or a more diversified quantity of antimicrobial compounds, including acids, bacteriocins or hydrogen peroxide *(158)*.

The protocol followed for further *in vitro* inhibitory effect tests included the use of MRS broth as a propagation medium, and for MRS agar to be used for LAB spots to test presumptive LAB antagonistic activity *in vitro*.

TABLE 3. Commercial formulation of MRS and APT media for proliferation of lactobacilli[a]

Purpose	Ingredients	
	MRS medium	APT medium
Peptides, amino acids, and nitrogen source	Proteose Peptone No. 3, 10 g Beef Extract, 10 g	Pancreatic digest of casein, 12.5 g
B-complex vitamins	Yeast Extract, 5 g	Yeast Extract, 7.5 g
Carbon source	Dextrose, 20 g	Dextrose, 10 g
Fatty acids source, surfactant	Polysorbate 80, 1 g	Polysorbate 80, 0.2 g
Buffering agent	Dipotassium Phosphate, 2 g	Dipotassium Phosphate, 5 g
Ions source	Manganese Sulfate, 0.05 g Magnesium Sulfate, 0.1 g	Manganese Chloride, 0.14 g Magnesium Sulfate, 0.8 g Ferrous Sulfate, 0.04 g
Osmotic balance, electrolytes		Sodium Chloride, 5 g
Thiamine source		Thiamine Hydrochloride, .0001 g
Chelating agent	Ammonium Citrate, 2 g	Sodium Citrate, 5 g
Inhibitory agent	Sodium Acetate, 5 g Ammonium citrate	

[a] Difco™ & BBL manual of microbiological culture media *(24)*

E. coli O157:H7 lag phase

The mean log values from 0 to 180 min and the curve and fitted linear function are shown in Figure 3. The lag phase was calculated from the linear function:

$$y = 0.0073x + 1.2846, (R^2 = 0.80)$$

The end of the lag phase was considered an increase on the mean log CFU/ml of 1 log value. Thus, x value was set to 2.4 log CFU/ml, and the resulting length of the lag phase was 153 min. However, given the possible variation in the samples, the length of incubation for recovery of bacteria in nonselective media was set to 120 min.

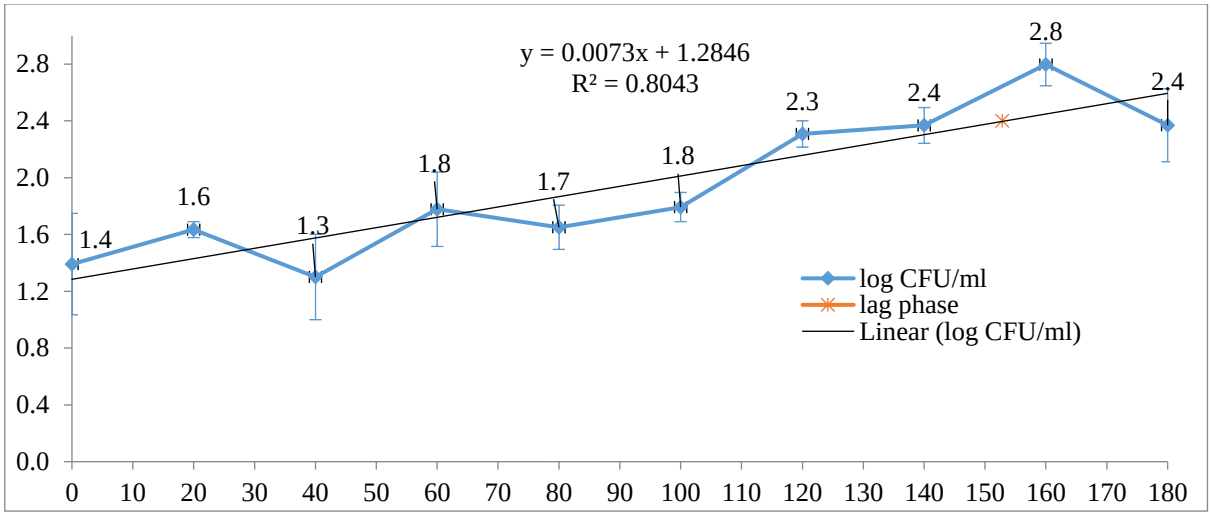

FIGURE 3. Estimation of the *E. coli* O157:H7 lag phase

Differential media to isolate *E. coli* O157:H7 from leafy green samples

Xylose lysine tergitol$_4$ (XLT-4) and Sorbitol MacConkey agar media for enumeration of *S.* Saintpaul-inoculated fruit skin samples and *E. coli* O157:H7-inoculated leaves samples were used during preliminary tests to determine their feasibility in the identification of pathogenic colonies from background microbiota colonies. When MacConkey agar was used to enumerate *E. coli* O157:H7 growth on spinach inoculated samples, the negative controls (not inoculated) presented colorless colonies, identical to the pathogen colonies from pure culture, the counts of the negative samples were: 2.6, 3.1, 2.9, 4.0, 3.2, 4.2, and 4.8 log CFU/10 cm^2 at 0, 1, 2, 4, 6, 10 and 12 h of incubation, while inoculated samples presented counts of 3.3, 3.6, 3.6, 3.5, 3.4, 4.2, and 4.3 log CFU/10 cm^2. Therefore, it was determined that this medium was not convenient for the purposes of the experiments in produce surfaces. In the case of XLT-4, the use of this media also presented some difficulties. When *S.* Saintpaul was streaked for isolation on XLT-4, some colonies

presented weak black coloration from the characteristic hydrogen-sulfide reaction. Although some colonies presented the black coloration, the results from the enumeration of black colonies from inoculated samples would likely be erroneous. Furthermore, the possible underestimation of the pathogens was a concern since highly selective media such as XLT-4 will likely inhibit the growth of stressed pathogenic bacteria after their exposure to antagonistic bacteria. Brashears et al. *(36)* reported this problem in acid-stressed *Salmonella* and *E. coli* O157:H7, where counts obtained in nonselective media (TSA) were higher than those in selective media (XLT-4 for *Salmonella* and VRBA for *E. coli* O157:H7) after stressing the bacteria using lactic acid solutions. Hence, the use of these differential media in further experiments was not considered appropriate. Subsequently, the use of an alternative nonselective media such as TSA, supplemented with an antibiotic such as RIF, along with the use of RIF-resistant derivatives of the pathogens was proposed and further tested.

Growth curves

The growth curves of *E. coli* O157:H7 K3999 RIF-resistant derivative and of *S.* Saintpaul RIF-resistant derivative demonstrated a close resemblance in their growth patterns when compared with their respective parental strains (Fig. 4 and 5). To confirm these observations, the growth curves were fitted to a Baranyi model, and growth parameters were obtained. The parameters extracted were: The initial (N_0) and final (N_{max}) bacterial concentration, expressed in log CFU/ml, the generation time (d-t) in min, and the growth rate in h^{-1} (μ_{max}), for *E. coli* O157:H7, *S.* Saintpaul and its RIF resistant derivatives.

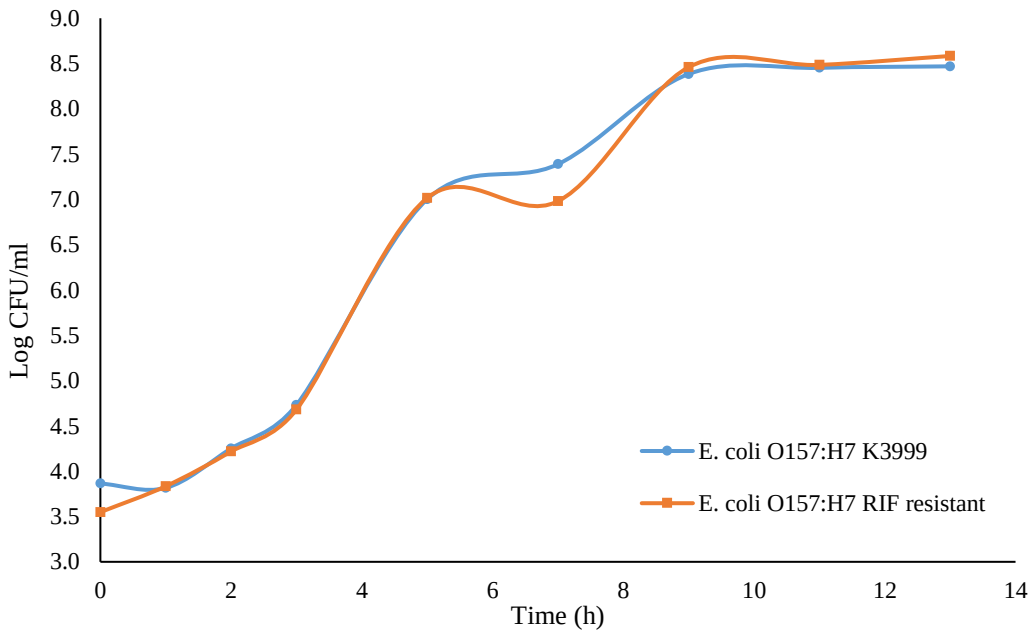

FIGURE 4. Growth curves of *E. coli* O157:H7 K3999 and its rifampicin resistant derivative in tryptic soy broth

The results of the calculated growth parameters are included in Table 4 for *E. coli* O157:H7 and its derivative and in Table 5 for *S.* Saintpaul and its derivative. In general, no significant differences were found in any of the parameters calculated when parent and derivative pathogens were compared for any of the pathogens ($P > 0.05$). Thus, the parental and derivative strains were determined indistinguishable in their growth behavior.

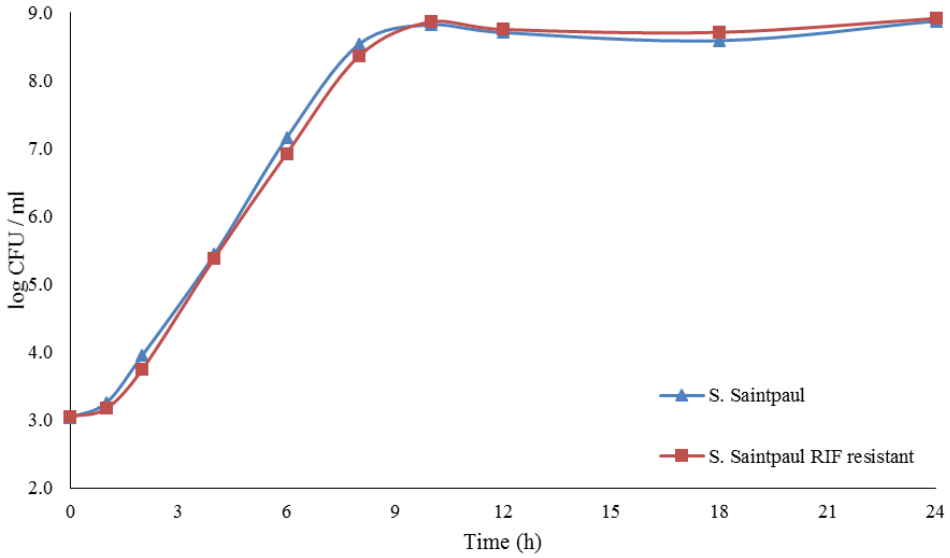

FIGURE 5. Growth curves of *S.* Saintpaul and its rifampicin resistant derivative in tryptic soy broth.

TABLE 4. Growth parameters of *E. coli* O157:H7 and rifampicin resistant derivative

Microorganism	Mean ± SD[a]			
	μ_{max}[b] h^{-1}	d-t min	N_0 Log CFU/ml	N_{max} Log CFU/ml
E. coli O157:H7	0.67 ± 0.09 A[c]	15.9 ± 0.4 A	3.9 ± 0.03 A	8.5 ± 0.09 A
E. coli O157:H7 RIF	0.60 ± 0.04 A	15.5 ± 0.2 A	3.5 ± 0.51 A	8.6 ± 0.03 A

[a] Mean ± standard deviation (SD), n=3

[b] μ_{max}: Growth rate, d-t: doubling time, N_0: initial count, N_{max}: final count

[c] Values followed by the same letter are not significantly different (P > 0.05).

TABLE 5. Growth parameters of *S.* Saintpaul and rifampicin resistant derivative

Microorganism	Mean ± SD[a]			
	μ_{max}[b] h^{-1}	d-t min	N_0 Log CFU/ml	N_{max} Log CFU/ml
S. Saintpaul	0.88 ± 0.04 A[c]	23.6 ± 0.3 A	3.0 ± 0.08 A	8.8 ± 0.07 A
S. Saintpaul RIF	0.85 ± 0.03 A	23.8 ± 0.4 A	3.0 ± 0.02 A	8.8 ± 0.04 A

[a] Mean ± standard deviation (SD), n=3

[b] μ_{max}: Growth rate, d-t: Doubling time, N_0: initial count, N_{max}: final count

[c] Values followed by the same letter are not significantly different (P > 0.05).

Effect of antagonistic bacteria toward parent and rifampicin-resistant pathogens

The inhibition of parent and RIF derivative pathogens was compared using ivAEB from fruit and leafy greens. The mean IA of the parent and RIF resistant *E. coli* O157:H7 for each leafy green *iv*AEB (31 isolates) are shown in Table 6. The mean IA of the parent and RIF resistant *S.* Saintpaul by fruit *iv*AEB (40 isolates) are shown in Table 7. The analysis by *iv*AEB and commodity for *E. coli* O157:H7 and *S.* Saintpaul are included in Tables 6 and 7, respectively.

Parent and RIF-resistant derivative *E. coli* O157:H7 were similarly susceptible ($P > 0.05$) to leafy green *iv*AEB except for four isolates: *Bacillus pumilus* and *Enterococcus gallinarum* from endives, *Aerococcus viridans* from parsley, and spinach-isolated *Kocuria kristinae*. When *B. pumilus* was used, *E. coli* O157:H7 RIF resistance was less susceptible than the parent *E. coli* O157:H7 ($P < 0.05$), showing a mean IA of 36 ± 9 mm^2, while the parent mean IA was 58 ± 12 mm^2. *E. coli* O157:H7 RIF was also more resistant to *K. kristinae* with a mean IA of 42 ± 9 mm^2, while the parent strain presented a mean IA of 72 ± 28 mm^2. In contrast, when *E. gallinarum* was used, RIF-resistant *E. coli* O157:H7 was more susceptible ($P < 0.05$), showing a mean IA of 46 ± 19 mm^2, while the parent resulted in a mean IA of 79 ± 26 mm^2. RIF-resistant *E. coli* O157:H7 was more susceptible to *A. viridans*, presenting a mean IA of 15 ± 6 mm^2, while the parent *E. coli* O157:H7 had a mean IA of 9 ± 2 mm^2.

TABLE 6. *In vitro* inhibition of *E. coli* O157:H7 parent and rifampicin resistant derivative strains using leafy green-isolated bacteria

		Mean ± SD[c]	
No.[a]	Species[b]	Parent[d,e]	RIF-resistant
Endive isolates			
3075	*Streptococcus alactolyticus*	226 ± 100 A, a	198 ± 31 A, a
3251	*Bacillus licheniformis*	164 ± 91 A, b	103 ± 40 A, cd
3152	*Streptococcus equinus*	154 ± 69 A, b	157 ± 41 A, b
3955	*Streptococcus mutans*	142 ± 33 A, b	135 ± 35 A, b
3154	*Pediococcus pentosaceus*	123 ± 37 A, bc	103 ± 24 A, cd
3302	*Staphylococcus sciuri*	85 ± 78 A, cd	108 ± 53 A, c
3756	*Enterococcus gallinarum*	79 ± 26 A, cde	46 ± 19 B, efghi
3915	*Lactobacillus rhamnosus*	73 ± 36 A, cdef	75 ± 34 A, de
3874	*Vagococcus fluvialis*	70 ± 32 A, def	62 ± 20 A, efg
3597	*Streptococcus sanguinis*	70 ± 20 A, def	64 ± 16 A, ef
3200	*Listeria grayi*	65 ± 18 A, def	74 ± 17 A, de
3953	*Lactobacillus plantarum*	64 ± 14 A, def	57 ± 12 A, efgh
2900	*Bacillus pumilus*	58 ± 12 A, def	36 ± 9 B, fghi
3622	*Staphylococcus lentus*	44 ± 24 A, def	33 ± 13 A, ghi
3277	*Gemella bergeri*	25 ± 6 A, ef	30 ± 14 A, hi
3552	*Aerococcus viridans*	24 ± 5 A, ef	26 ± 10 A, i
3554	*Leuconostoc mesenteroides* subsp. *dextranicum*	23 ± 6 A, f	21 ± 11 A, i
Parsley isolates			
5897	*Bacillus mycoides*	141 ± 75 A, a	98 ± 67 A, ab
5432	*Staphylococcus lentus*	131 ± 22 A, ab	133 ± 22 A, a
4145	*Bacillus* sp.	86 ± 27 A, bc	66 ± 32 A, bc
4094	*Pseudomonas paucimobilis*	74 ± 33 A, c	57 ± 9 A, bcd
4197	*Enterococcus casseliflavus*	58 ± 16 A, cd	39 ± 8 A, cde
4111	*Enterococcus gallinarum*	55 ± 22 A, cde	50 ± 28 A, cde
4876	*Staphylococcus intermedius*	37 ± 15 A, cde	37 ± 17 A, cde
5438	*Aerococcus viridans*	9 ± 2 A, de	15 ± 6 B, de
4075	*Gemella morbillorum*	7 ± 2 A, e	10 ± 9 A, e
Spinach isolates			
1610	*Kocuria kristinae*	72 ± 28 A, a	42 ± 9 B, b
1552	*Enterococcus cecorum*	70 ± 25 A, a	64 ± 18 A, a
1637	*Aerococcus viridans*	67 ± 16 A, a	58 ± 14 A, ab
1650	*Cupriavidus pauculus*	59 ± 17 A, a	53 ± 9 A, ab
358	*Enterococcus casseliflavus*	46 ± 13 A, a	43 ± 6 A, b

[a] Identification code from stock culture. Food Microbiology Laboratory. Texas A&M University.
[b] According to Vitek-2 system identification.
[c] Inhibition area mean ± standard deviation of *E. coli* O157:H7 parent or rifampicin-resistant derivative, n = 8.
[d] Within rows, values followed by the same uppercase letter are not significantly different (P > 0.05).
[e] Within columns, and commodity, values followed by the same lowercase letter are not significantly different (P > 0.05)

TABLE 7. *In vitro* inhibition of *S.* Saintpaul parent and rifampicin resistant derivative strains using fruit-isolated bacteria

		Mean ± SD[c]	
No.[a]	Species[b]	Parent[d,e]	RIF-resistant
Cantaloupe isolates			
13172	*Enterobacter cloacae* subsp. *dissolvens*	430 ± 97 A a	315 ± 78 B a
13510	*Enterobacter ludwigii*	231 ± 38 A b	260 ± 65 A a
12712	*Staphylococcus warneri*	217 ± 24 A bc	184 ± 16 B b
12196	*Leuconostoc mesenteroides* subsp. *cremoris*	204 ± 55 A bcd	172 ± 41 A bcd
13957	*Leuconostoc mesenteroides* subsp. *dextranicum*	167 ± 32 A bcde	124 ± 36 B bcdefg
10039	*Lactococcus pentosaceus*	163 ± 40 A bcdef	183 ± 42 A b
12871	*Lactococcus lactis* subsp. *lactis*	157 ± 42 A bcdefg	141 ± 35 A bcdef
10040	*Streptococcus thoraltensis*	157 ± 21 A bcdef	179 ± 29 A bc
13271	*Enterococcus cecorum*	154 ± 46 A bcdegf	97 ± 27 B fgh
12115	*Enterococcus casseliflavus*	147 ± 78 A cdefg	74 ± 16 B hg
13598	*Citrobacter sedlakii*	142 ± 14 A cdefg	167 ± 22 B bcde
12194	*Enterobacter kobei*	140 ± 27 A cdefg	139 ± 48 A bcdef
10240	*Staphylococcus vitulinus*	135 ± 47 A defg	111 ± 17 A efgh
12032	*Staphylococcus lentus*	132 ± 57 A defg	122 ± 36 A cdefg
12833	*Aerococcus viridans*	127 ± 24 A defg	105 ± 25 A fgh
10191	*Enterococcus faecium*	113 ± 29 A efg	103 ± 14 A fgh
13632	*Staphylococcus xylosus*	106 ± 21 A efg	109 ± 17 A efgh
10352	*Enterococcus gallinarum*	102 ± 19 A efg	105 ± 36 A fgh
13713	*Enterococcus faecalis*	99 ± 26 A efg	116 ± 17 A defgh
10199	*Lactococcus garvieae*	98 ± 23 A efg	90 ± 10 A fgh
10074	*Staphylococcus gallinarum*	95 ± 50 A efgh	58 ± 15 A hi
13755	*Staphylococcus epidermidis*	91 ± 27 A efgh	81 ± 29 A fgh
10473	*Kocuria kristinae*	87 ± 17 A fgh	85 ± 21 A fgh
13552	*Enterococcus gallinarum*	79 ± 62 A hgi	60 ± 17 A hi
12795	*Staphylococcus cohnii* subsp. *urealyticus*	19 ± 11 A hi	10 ± 5 B i
13119	*Klebsiella pneumoniae* subsp. *pneumoniae*	9 ± 4 A i	5 3 ± A i
Pepper isolates			
9072	*Enterococcus faecalis*	106 ± 22 A a	95 ± 20 A a
17038	*Staphylococcus epidermidis*	91 ± 26 A ab	103 ± 30 A a
17600	*Staphylococcus warneri*	75 ± 12 A bc	66 ± 6 A b
16759	*Staphylococcus lugdunensis*	58 ± 20 A c	80 ± 20 B ab
9191	*Staphylococcus hominis* subsp. *hominis*	24 ± 13 A d	29 ± 7 A c

TABLE 7. Continued.

No.[a]	Species[b]	Mean ± SD[c] Parent[d,e]	RIF-resistant
Tomato isolates			
15318	*Lactococcus lactis* subsp. *lactis*	171 ± 34 A a	138 ± 31 A a
15313	*Enterococcus gallinarum*	127 ± 36 A b	140 ± 27 A a
15651	*Bacillus polymyxa*	123 ± 14 A b	106 ± 14 B abc
6034	*Kocuria kristinae*	118 ± 25 A b	124 ± 53 A ab
6031	*Staphylococcus epidermidis*	73 ± 14 A c	79 ± 17 A bcd
14596	*Staphylococcus hominis* subsp. *novobiosepticus*	36 ± 11 A d	35 ± 16 A de
14594	*Staphylococcus saprophyticus*	35 ± 14 A d	61 ± 30 B cd
6392	*Staphylococcus hominis* subsp. *hominis*	33 ± 9 A d	61 ± 33 B cd
14599	*Staphylococcus lentus*	11 ± 4 A d	7 ± 4 B e

[a] Identification code from stock culture. Food Microbiology Laboratory. Texas A&M University.
[b] According to Vitek-2 system identification.
[c] Inhibition area mean ± standard deviation of *S.* Saintpaul parent or rifampicin-resistant derivative, n = 8.
[d] Within rows, values followed by the same uppercase letter are not significantly different (P > 0.05).
[e] Within columns, within commodity, values followed by the same lowercase letter are not significantly different (P > 0.05).

The RIF-resistant *S.* Saintpaul and the parent strains were equally susceptible to the inhibitory action of 28 ivAEB (P >0.05), and different from 12 ivAEB (P < 0.05). RIF resistant *S.* Saintpaul was more susceptible (P < 0.05) to the *in vitro* inhibitory action of 4 *iv*AEB including *Citrobacter sedlakii* from cantaloupe, *Staphylococcus lugdunensis* from pepper, and *Staphylococcus saprophyticus* and *Staphylococcus hominis* subsp. *hominis* isolated from tomato. On the contrary, RIF derivative was more resistant than the parent strain (P < 0.05) to the inhibitory action of 8 *iv*AEB including *Enterobacter cloacae* subsp. *dissolvens*, *Enterococcus cecorum*, *Enterococcus casseliflavus*, *Staphylococcus warneri*, *Staphylococcus cohnii* subsp. *urealyticus*, and *Leuconostoc mesenteroides* subsp.

dextranicum isolated from cantaloupe, and to *Staphylococcus lentus*, and *Bacillus polymyxa* isolated from tomato.

Ten isolates previously identified from the 41 *iv*AEB toward *E. coli* O157:H7 did not show inhibitory results during this preliminary experiment. Therefore, they were not included in the analysis. In the case of the fruit-isolated antagonistic bacteria, 55 *iv*AEB previously tested as inhibitory to *S.* Saintpaul according to *in vitro* experiments were selected; however, 15 isolates did not replicate their inhibitory activity toward S. *Saintpaul* and were not included or further tested. The lack of antagonistic activity after a demonstrated initial antagonistic activity has been previously reported. The absence of the inhibition might be related to the adaptation of the epiphytic strains to laboratory conditions in the complex media through consecutive passes during revival and proliferation, possibly changing the utilization and synthesis of nutrients and inhibitory metabolites. Although some differences were detected during the comparison of RIF-resistant and parent strains, the use of RIF-resistant bacteria for further experiments using the surfaces of fruits and vegetables is the most suitable alternative to selecting and identifying only inoculated pathogens and no other bacteria from this surfaces. The alternatives of using differential media such as XLT-4 to isolate *Salmonella* or sorbitol MacConkey agar to differentiate *E. coli* O157:H7 were previously tested and determined not suitable for these experiments.

Moreover, this experiment was able to compare the inhibition strength of different epiphytic bacteria. In general, the largest IA were shown when using *Streptococcus alactolyticus* (226 ± 100 mm^2) isolated from endives toward *E. coli* O157:H7. Similarly,

Enterobacter cloacae subsp. *dissolvens* and *Enterobacter ludwigii* from cantaloupe, and *Lactococcus lactis*, from tomato produced the largest IA of S. Saintpaul, with values of 430 ± 97 mm^2, 231 ± 38 mm^2, and 171 ± 24 mm^2.

Even when the possibility of larger IA indicated a stronger inhibition effect toward the pathogens, inferences about the inhibitory activity of these strains might not be accurate based only in these values. A smaller inhibitory area might not be directly related to a lesser amount or extent of the inhibition but rather to different mechanisms of action or different diffusions of the released inhibitory elements in the semisolid media. Nonetheless, the variations observed with different species illustrate the possibility of different substances and/or mechanisms being employed by different bacteria. This highlights the importance of the evaluation of antagonistic isolates on the actual plants, and the further analysis of mechanisms employed by these microorganisms to determine their possible application as biocontrol agents.

Determination of biofilm using the crystal violet method

In an attempt to determine the biofilm activity of pathogens and ivAEB for further challenge experiments, a preliminary experiment was conducted. From the absorbance results, it was evident that the majority (68 out of 70) of the isolates tested at 1h and after 24 h of incubation were similar ($P > 0.05$) or smaller ($P < 0.05$) than the *E. coli* O157:H7 values of 1.35 ± 0.9 AU and 0.22 ± 0.4 AU at 1 and 24 h respectively, and of *S.* Saintpaul of 2.5 ± 0.9 and 0.20 ± 0.4 AU at 1 and 24 h. This was due to the formation of biofilm with the corresponding retention of crystal violet by the *iv*AEB and the pathogens. Furthermore, media control for TSB, and MRS gave absorbance values similar to those

observed by the controls ($P < 0.05$), possibly due to unspecific retention of crystal violet by components of the media adhered to the wells.

The ability of most bacteria to form biofilms is well established. In particular, the ability of epiphytic bacteria to form biofilms has been reported on plant surfaces. Nongkhlaw and Joshi *(215)*, observed clusters of epiphytic bacteria close to the plant stomata and on the vein grooves in micrographs of 20 different plants and reported biofilm formation by numerous epiphytes as an adaptation mechanism that prevailed on the plant surface. Thus, the evaluation of the inhibition of biofilm formation by epiphytic bacteria would get altered by the isolate (applied as treatment) in biofilm formation. The evaluation of pathogen biofilm activity as a function of the presence of other microorganism requires specific markers that allow the evaluation of the pathogenic biofilm only.

Determination of biofilm formation using red Congo agar

The colonies formed in red Congo agar were evaluated to determine the specific staining of biofilm through the amyloid staining in black aggregates. Although colonies demonstrated a strong black color in media supplemented with sucrose, colonies in media not containing sucrose were weakly stained. The addition of sucrose to plant surfaces was not an alternative, neither the staining of the plant, since red Congo was highly unspecific to particular colonies and the whole media reacted to the amyloid presence in nearby colonies. Thus, this method was considered inappropriate for its application on leaf surfaces. Possibly, the detection of amyloid or other proteins during biofilm formation would be an alternative for the direct evaluation of biofilm on plant surfaces. However, this would require extensive studies to determine the specific target substance and a

method suitable to measure it. For the purpose of this study, an alternative to the plant surface biofilm evaluation was utilized, using confocal microscopy.

Evaluation of the microbiological content in fruits and leafy greens

Leafy greens

Differences by commodity

The mean and standard deviation of the microbiological content for parsley, endives, and spinach, by bacterial group (MS, CL, PY, and LAB) are shown in Table 8. In general, MS, CL, LAB, and PY were more numerous on spinach, while parsley remained as the less populated leafy green for each of the bacterial groups tested ($P < 0.05$).

TABLE 8. Bacterial content of leafy greens collected in the winter harvesting season[a]

Commodity	Mean ± SD[b]			
	MS[c]	LAB	CL	PY
Parsley	4.6 ± 0.4 A[d]	3.0 ± 0.4 A	3.0 ± 0.7 A	4.2 ± 0.8 A
Endives	6.1 ± 0.4 B	4.6 ± 0.5 B	5.2 ± 0.6 B	5.0 ± 0.5 B
Spinach	6.9 ± 0.5 C	5.2 ± 0.8 C	6.0 ± 0.6 C	6.4 ± 0.6 C

[a] Winter season in Texas: February-March.
[b] Mean of bacterial counts averaged in log CFU/g ± standard deviation, n = 50.
[c] Bacterial groups, MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs.
[d] Within each column, values followed by the same letter are not significantly different ($P > 0.05$).

The microbial counts in the samples tested in this study are slightly lower than those of Garcia-Villanova Ruiz et al. *(116)*, who reported aerobic plate counts (APC) of 8 log CFU/g, for spinach, 7.0 log CFU/g for endives, and 6.5 log CFU/g for parsley. However,

in accordance with the present study, spinach was also more populated than other leafy greens, including endives, and escarole, lettuce, and parsley.

The counts found in the current study are similar to those reported by Abadias et al. (1). In their study, spinach MS, LAB, CL, and PY content were 7.4, 5.1, 6.0, and 7.4 log CFU/g in spinach, whereas endive counts for MS, LAB, CL and PY were 6.2, 2.7, and 4.8 and 6.8, log CFU/g. Furthermore, these authors also described spinach as the most bacteria populated leafy green when comparing this to endives, arugula, and lettuce.

The spinach MS counts of the present study, were also similar to those reported by Babic et al. *(15)* of 6-7 log CFU/g, and higher than their CL and LAB counts of 3.7 log CFU/g of CL and LAB of 3-4 log CFU/ g. Ailes et al. *(10)* also found lower MS and LAB counts than those found in the present study. They reported 6.0 log CFU/g of APC and 1.5 log CFU/g of LAB on spinach. CL counts in the present study were also more numerous than those reported by Marine et al. *(180)* who studied different leafy greens including 130 samples of spinach, 203 samples of lettuce, and 36 samples of other leafy greens. They calculated APC, and CL counts of 5.7 and 2.2 log CFU/g in organic farms, and APC and CL counts of 5.4 log CFU/g and 1.3 log CFU/g, in conventional farms. In their study, no significant differences were found based on commodities in either farming system. It should be noted that in all these studies, do not include any wash or other processing after harvesting.

Johnston et al. *(144)* reported APC counts of 5.2 log CFU/g, and 1.7 log CFU/g of CL in parsley. Ailes et al. *(10)* reported similar APC counts of 6.0 log CFU/g and 2.4 log CFU/g for coliforms found on parsley. In a study involving broad leaf endives by Rediers

et al. *(235)*, the aerobic mesophilic bacteria (AMB) (incubated for 72 h at 30 ºC) and coliforms (grown in VRBA with lactose) were 6.9 and 4.5 log CFU/g, respectively. Samples were unwashed and core outer leaves were excluded. The counts obtained in the present study for MS of 6.0 log CFU/g and CL of 5.2 log CFU/g are very similar to those reported in this study, although the endive variety is different from that study.

Guisti et al. *(96)* studied curly endives, along with other leafy greens and reported counts of AMB for curly endives much larger than those found in the present study. The curly endives in their study presented AMB counts (incubated at 30 ºC for 48 h) of 8.9, 11.0, and 9.9 log CFU/g, much higher than the MS counts of 6.0 log CFU/g found in the present study. Their samples were collected from three different producers, and processed before antimicrobial treatments were applied.

Although other studies have reported initial concentrations of microbiota on spinach, parsley, or endives, the samples have been processed by washing or disinfection, possibly altering their bacterial content before the microbiological evaluation. For example, Conte et al. *(88)* reported APC of 5 log CFU/g, PY counts of 8 log CFU/g, < 2 log CFU/g of CL, and 3-4 log CFU/g of LAB in spinach samples harvested from experimental plots. The APC counts and CL counts are less than those found in the present study. However, their samples were washed with tap water, dipped in chlorinated water for one min, and immersed in tap water for one more min. An interesting finding in this study when compared to the present results, is the larger count of PY in comparison to the PY from the present study, despite their water and chlorinated baths. In another study, Lopez-Velasco et al. *(170)* reported 4.5–4.6 log CFU/g of total culturable bacteria after

rinsing spinach leaves with sterile distilled water to remove soil particles. Carlin et al. *(48)* reported APC and LAB counts, much less than those found in the present study, being 3.8 to 4.8 log CFU/g and > 2 log CFU/g for LAB in fresh broad leaf endives. However, leaves were washed with distilled water and disinfected with hydrogen peroxide with an expected bacterial reduction of 1–2 log CFU due to the disinfection. This could explain why those samples of endive found relatively lower counts of APC and LAB than the present samples. In another experiment by Carlin et al. *(46)* using similarly disinfected endives leaves, the AMB reported counts after 48 h of incubation at 30 ºC were 3.4 to 5.0 log CFU/g which are slightly less than the 6.0 log CFU/ g of MS found in the endives studied here.

In the present study, no washing step or any further treatment was followed after harvesting. Transportation of samples was carried out using frozen cooling packs. The insulated containers and samples were processed for microbiological analysis within 24 h of harvesting. Thus, minimal changes were expected on their microbiological content from harvesting to processing. The samples were pummeled in a stomacher for 1 min, and PW was used as a suspension liquid and as a diluent solution. Therefore, the counts found in the leaves of spinach, endives, and parsley, likely include bacteria in direct contact with the leaves, and those included in debris and attached soil particles. Although, visibly soiled leaves were not included in the sample composite, microscopic particles likely remained in the sampled leaves. Taking into account that the subjects studied are living microorganisms (bacteria), attached to other living organisms (leafy greens), considerable

variations were expected in their bacterial content. However, other causes could have led to the microbiological content differences between commodities.

For all the commodities employed in the experiments, the cultivating soil and irrigation water were similar. All fields were neighboring land areas within the Weslaco agricultural region. Furthermore, all the crops were irrigated using the same water source (from the Rio Grande River); thus, their differences might not be closely related to these factors especially since the irrigation method used was similar, being categorized as within a flood system. This irrigation method might not have influenced the differences seen between commodities. However, the close proximity of the harvested part of the plants with the ground could be important when considering the contamination of spinach leaves under this type of irrigation. Plant leaves might get wetted by the irrigation water, depending on their proximity to the ground. Spinach average height at harvest is approximately 15–20 cm, while parsley plants are typically 30–40 cm tall, and can reach 68–76 cm when flowering *(271, 272)*. Furthermore, parsley stems are longer than spinach, allowing harvest to take place at a height farther from the ground. Thus, spinach leaves sampled might have had more frequent contact with the ground or with organic matter carried from irrigated runoff water than parsley.

Some features inherent to the plant species can also play a role in their microbial variation. For example, the arrangement, roughness, and waxy cuticle of the leaves.

Spinach leaves are arranged closely to the center, mainly due to their high density of plants per row cultivated. Planting spinach close together increases the field yields, but also eases their harvesting, since it forces the plants to develop their leaves vertically, and

to not spread out. Curly endive leaves also arrange together, forming a well-defined head, although their outer leaves are slightly spread. In the case of parsley, the leaves arrangement on the stem shows a broader plant configuration. This might allow better ventilation, less water retention, and fewer soil particles. Debris can accumulate when leaves are close together, as seen in spinach.

The roughness of spinach might also contribute to debris and soil particle retention. It also extends the surface area; thus there are more areas for bacterial distribution. Other structures that might increase with more surface area are trichomes, vein grooves, and stomata *(112)*. Bacteria have been found commonly surrounding these areas more frequently *(302)*. However, this characteristic was not measured in this experiment; thus, the influence on the microbial variation due to surface roughness and total area cannot be determined.

Another important factor involved in the attachment of bacteria is plant wettability. The surface characteristics of the parsley cuticle has been related to its resistant to oil-solvent chemicals used for pest control *(220)*. In a study to determine the disinfection levels reached, calculated as *L. monocytogenes* reductions, using home washing treatments, parsley presented lower reductions of the pathogen than lettuce, and a plausible causative factor was the low wettability of the parsley leaves *(208)*. However, while the parsley leaves were visibly shinier than those of spinach or endives, maybe due to the presence of a thick waxy cuticle, this was not measured or determined.

Effect of environmental factors

The environmental factors of temperature, and relative humidity (RH), and the content of different bacterial groups was analyzed by harvesting day. The data utilized for this comparison is shown in Table 9. Furthermore, the environmental temperature, RH, and epiphytic bacteria content by bacterial group by fields sampled are plotted in Fig. 6.

The statistical analysis was carried out comparing the two fields set apart for sample collection, since the samples were obtained on different days, and the temperature and RH presented certain variations. In general, the MS, LAB, and CL content for leafy greens was more numerous in samples harvested during warmer days ($P < 0.05$). On the contrary, the PY content was larger in samples collected on colder days ($P < 0.05$). The effect of RH was not easily observable, since high humidity was not always related to higher bacterial load, as seen in the spinach counts.

TABLE 9. Bacterial content of leafy greens by field and environmental conditions at harvesting[a]

Commodity-field[b]	Harvesting date	RH[c]	Temp.[d]	Mean ± SD[e]			
				MS	LAB	CL	PY
endives-a	03/01/12	81	23.9	6.1 ± 0.3 A[f]	5.2 ± 0.3 A	5.5 ± 0.7 A	4.8 ± 0.5 A
endives-b	03/03/12	51	15.6	6.2 ± 0.5 A	4.0 ± 0.7 B	4.9 ± 0.5 B	5.2 ± 0.4 B
parsley-a	03/18/12	79	23.8	4.9 ± 0.4 A	3.6 ± 0.5 A	3.7 ± 0.7 A	3.6 ± 0.7 A
parsley-b	03/22/12	55	20	4.2 ± 0.3 B	2.3 ± 0.3 B	2.4 ± 0.8 B	4.8 ± 1.0 B
spinach-a	02/07/12	90	18.3	6.4 ± 0.5 A	4.1 ± 0.9 A	5.4 ± 0.9 A	5.6 ± 0.8 A
spinach-b	02/21/12	81	22.2	7.4 ± 0.5 B	6.3 ± 0.7 B	6.6 ± 0.6 B	7.3 ± 0.5 B

[a] Winter harvesting season in Texas: February-March.
[b] Samples from 2 different fields (a and b) harvested by commodity.
[c] RH: Relative humidity (RH), in %.
[d] Environmental temperature in ºC.
[e] Mean log CFU/g ± Standard deviation for bacterial counts on selective media. MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, and PY: Psychrotrophs, n=25.
[f] Within columns, and commodity, values followed by same letter are not significantly different ($P > 0.05$).

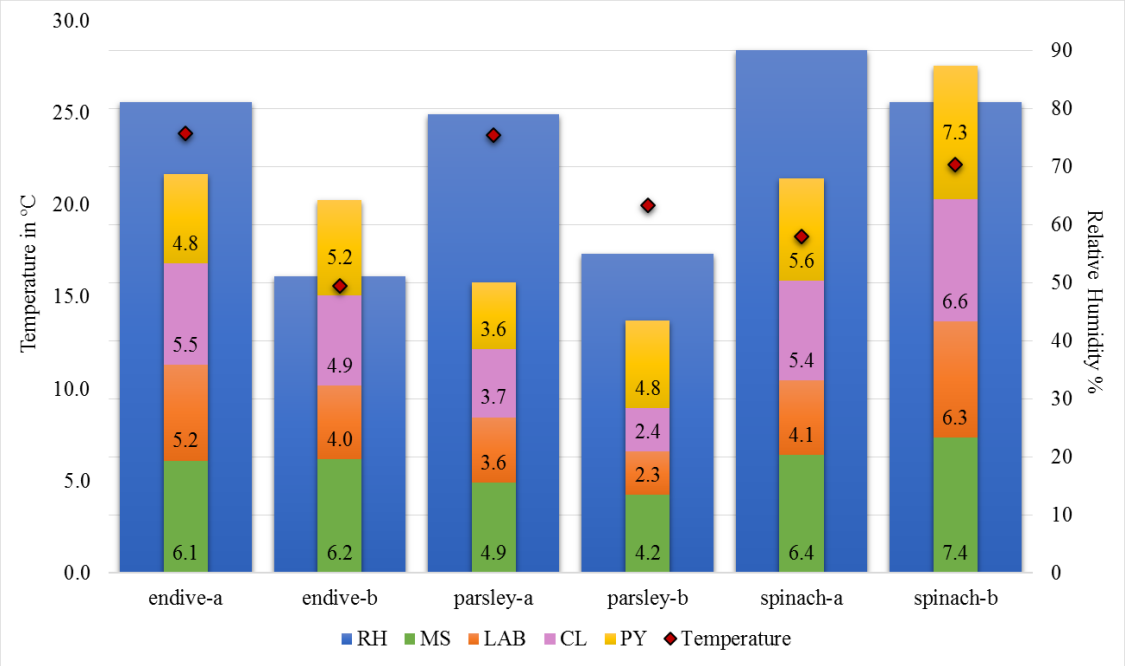

FIGURE 6. Bacterial content of endives, parsley and spinach, and environmental conditions at harvesting. All leafy greens were collected from two fields (a and b) during the winter harvesting season in Texas. Mean log CFU/g by bacterial groups (value within thin bar sections) for MS are shown for: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, and PY: Psychrotrophs. Temperature in ºC were plotted to left axis, and relative humidity (RH) in % (thick bars) were plotted to left axis during sample collection.

The effect of the temperature and humidity over the microbiological content of plants has been previously described. During a study involving Mediterranean plants, water on the plant surface was one of the first descriptors of the epiphytic bacteria variation, accounting for 55% of the variance changes *(302)*. Medina-Martinez et al. *(186)* reported a rapid change in the proliferation of epiphytic coliforms in baby lettuce toward the end of the winter that was not observed during the first days of the same season. These differences in bacterial loads, which were related to season temperature changes, could explain the differences found between the two spinach fields studied. In the case of parsley and endives, the two fields were harvested with a difference of three days. In contrast, the

time gap between harvesting the first and second field of spinach was 15 days. Thus, spinach samples collected in the second field were exposed to more days with warmer temperatures than the first one, since they were getting closer to the spring season. Marine et al. *(180)* have reported this behavior in bacterial loads from leafy greens. In their study, a significant difference was found between samples harvested on different days for spinach, lettuce, and other leafy greens and the rise in the bacterial numbers became progressively larger as the warmer season approached *(180).*

Using the averaged historical weather data for Weslaco TX, the minimum and maximum temperature in February was is 12.4–26.6 ºC from the 8th (24 h after first samples of the spinach field were collected) to the 22nd (when the second spinach field was collected), and this range was significantly higher ($P < 0.05$) than that from the previous two weeks (Jan 24th to Feb 7th) with a temperature range of 10.1–22.3 ºC. Then, it is noticeable that the harvested samples from field *b* were exposed to warmer days than field *a*, since this was harvested 15 days later.

Other factors not measured during this study include soil conditions, wildlife presence, temperature variation, and RH during the entire pre-harvest production of these commodities. All of these factors could have also influenced the presence and variability of the bacterial groups considered and caused the differences found between commodities, but the extent of their effect would remain undetermined.

The environmental conditions and the intrinsic plant characteristics possibly allowed the proliferation of epiphytic bacteria and variation by bacterial species. However, the extent of their influence requires more studies that include different varieties within

plant species. Further studies should also include measurements of leaf surface physicochemical characteristics including wettability, thickness of the waxy cuticle, roughness, number of trichomes and stomata, and nutritional content or organic matter presence to determine which of the aforementioned factors are of higher impact in the variation of the microbiological content of leafy greens within and between commodities.

Fruits

Differences by commodity

The bacterial content of the fruits collected by the bacterial group is presented in Table 10. The differences in the microbiological content by bacterial group were analyzed for each season since there were differences in the bacterial group content of similar commodities, which varied according to season. When the epiphytic bacterial counts of fruit commodities were compared, cantaloupe presented the highest bacterial content for all bacteria groups analyzed, while tomato and pepper counts were similar ($P > 0.05$), but significantly lower than those from cantaloupe ($P < 0.05$).

The comparison of the present study to other studies is difficult to analyze due to the differences found in the methodology used by the different studies. Some studies reported the enumeration of bacteria from fruit surfaces as total content by fruit pieces, ml of diluent, g of peel, etc. *(144, 245).* Only a few studies calculated the bacterial content by cm^2 of pericarp, rind, or peel of the commodities tested, as calculated in the present study. Furthermore, the methodology utilized for the procurement of these counts is different. Other studies present the counts of bacterial groups after inoculation with particular

pathogens, since their objective was to detect the pathogen and its behavior and not to assess the original microbiota content *(299).*

Tomás-Callejas et al. *(273)* reported MS, and CL counts of 4.1 and 6.5 log CFU/fruits, and although these were higher than those counts for tomatoes ads found in the present study, the sample size was not comparable since in the referred study, the researchers rinsed the whole fruit, and did not provide information about the area or size of the sampled tomatoes. Johnston et al. *(144)* reported APC, coliforms, and *E. coli* counts of 6.6 ± 1.0, 3.0 ± 1.3, and 1.5 ± 1.1 log/g on cantaloupes. Although these counts seem similar to the present study, they are expressed in g of pericarp, and not in cm^2. Thus, the comparison of the current data to previous reports would be inaccurate unless some conversion system is employed, and even then, the variation in the methodologies should be considered for these comparisons. One study published by Ukuku et al. *(275)* reported the APC of cantaloupes in units comparable to those used in the present study. In their study, the cantaloupe APC was 6.5 log CFU/cm^2. This is in agreement with the results found in the present study. Aguiló-Aguayo et al. *(9)* reported APC counts of approximately 4.7 log CFU/cm^2 on tomatoes var. Climberley. The tomato samples in this study presented 1 log CFU/cm^2 of difference from those reported in that study.

TABLE 10. Bacterial content of fruit surfaces by harvesting season in Texas

Commodity	Season[b]	Mean ± SD [a]				Mean ± SD [a]
		MS [c]	LAB	CL	PY	All bacteria[f]
Cantaloupes	Summer	6.1 ± 0.5 A[d] a[e]	4.1 ± 0.7 A a	4.3 ± 0.7 A a	5.2 ± 0.6 A a	4.9 ± 0.9 A
	Fall	5.5 ± 0.7 B a	4.9 ± 0.7 B a	5.1 ± 0.7 B a	4.3 ± 0.8 B a	
Tomatoes	Summer	3.3 ± 0.8 A b	0.7 ± 0.7 A b	1.0 ± 1.0 A b	1.6 ± 0.9 A b	2.0 ± 1.4 B
	Fall	3.6 ± 0.9 A b	2.6 ± 1.6 B b	1.2 ± 0.9 A b	1.6 ± 1.0 A b	
Peppers	Summer	3.6 ± 1.3 A b	0.6 ± 0.6 A b	1.1 ± 0.9 A b	1.6 ± 0.7 A b	2.1 ± 1.5 B
	Fall	3.7 ± 0.6 A b	2.6 ± 1.5 B b	2.1 ± 1.1 B c	1.1 ± 0.7 B b	
All commodities	Summer	4.3 ± 1.6 A	3.3 ± 1.8 A	2.2 ± 1.8 A	2.8 ± 1.9 A	2.8 ± 2.0 A
	Fall	4.3 ± 1.1 A	1.8 ± 1.7 B	2.8 ± 1.9 B	2.4 ± 1.6 B	3.2 ± 1.8 B

[a] Mean log CFU/cm^2 ± standard deviation of bacterial counts of 50 samples (two harvested fields, 25 samples each), n = 50 for bacterial groups, n = 100 for commodity

[b] Summer season: May-June, Fall season: October-December

[c] MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs

[d] Within column, within commodity, values followed by the same uppercase letter are not significantly different ($P > 0.05$)

[e] Within column, within season, values followed by the same lowercase letter are not significantly different ($P > 0.05$)

[f] Within column, values followed by the same letter are not significantly different ($P > 0.05$)

When comparing the bacterial content between cantaloupe, tomatoes, and peppers, the high content of bacteria from cantaloupes was evident. These high counts in cantaloupes have been previously reported. In a study published by Ailes et al. *(10)* cantaloupes presented higher APC counts than cabbage, arugula, celery, collards, dill, kale, parsley, spinach, Swiss chard, and turnip greens, with a mean of 6.7 log CFU/g. In the same study, coliforms, *E. coli* and *Enterococcus* spp. were also more frequently found in cantaloupes, than in the other commodities tested *(144)*.

The marked differences between the cantaloupes with respect to tomatoes and peppers bacterial content might have several origins. The cantaloupe proximity to soil can cause transference of bacteria from the soil to the fruit, principally during heavy raining episodes, and flooding. This phenomenon has been reported by Lopez-Velasco et al. *(171)* while trying to demonstrate the contamination of cantaloupes with an non-virulent strain of *Salmonella* spp. These researchers could not replicate the contamination of cantaloupes through the irrigation water, yet the *Salmonella* reached the fruit during a heavy raining episode that flooded the field. Also, under normal environmental conditions, bacteria might be transferred from the soil to the plant, since cantaloupes will lay on the ground. Regardless of the application of materials to separate the fruit from the soil, such as plastic or fabric, and the raise in the cultivar beds, cantaloupe are likely to have contact with soil particles more often than aerial fruits. On the contrary, peppers and tomatoes, are naturally kept away from the ground, since the plant conformation grows as bushes, not as trailing vines, as in cantaloupes, and when needed, vine tomatoes are kept from the ground using trellising or staking.

Other important factor that separate tomatoes and peppers from cantaloupes, are the roughness of their skin, their cell hydrophobicity and the electrical charges between the bacteria and the surface *(276)*. Cantaloupe rind presents crests and indentations on its surface. This surface might increase bacterial proliferation by different means including a greater area surface for bacterial attachment and more crevices for bacteria to allocate and be protected from external environmental conditions. The netting on the cantaloupe rind has been suggested to be one of the main factors limiting the effect of antimicrobial solutions used to reduce pathogens and spoilage bacteria *(223, 282)*. Wang et al. *(289)* studied the roughness and hydrophobicity of apples, avocados, oranges, and cantaloupe and determined that the strength of attachment of pathogenic *E. coli* on the surface of these commodities depended principally on the surface roughness and secondarily, on the surface hydrophobicity. In their study, cantaloupe rind had the roughest and least hydrophobic surface, and also showed the highest bacterial counts (after removing loosely attached bacteria). Consequently, the calculated adhesion rate was highest in comparison to other commodities. Furthermore, rind netting might allow entrapment of organic matter, nutrients, and soil particles that could contribute to the rise in the bacterial content of the cantaloupe surface.

Effect of the harvesting season

The bacterial counts on fruits affected by the harvesting season and bacterial groups, are shown in Table 10. When compared by bacterial group and season, cantaloupes remained as the most populated for MS, CL, PY, and LAB when compared to tomatoes and peppers ($P < 0.05$) in both seasons. Tomatoes and peppers showed no significant

difference ($P > 0.05$) in their bacterial content for each bacterial group, except in the case of CL, since tomatoes presented lower CL counts. However, this was only seen in the samples collected in the fall. When comparing the bacterial groups to determine the effect of the seasons, the MS content was not different for the fall and summer samples, while LAB and PY were more numerous in the summer, and CL were more numerous in the fall. To better understand this effect, counts were compared within each commodity.

In the case of cantaloupe, the bacterial populations were affected by season ($P < 0.05$). MS and PY counts in the summer were 6.1 ± 0.5 and 5.2 ± 0.6 log CFU/cm^2, and significantly higher ($P<0.05$) than those harvested in the fall, of 5.5 ± 0.7 and 4.3 ± 0.8 log CFU/cm^2, respectively. In contrast, LAB and CL counts were lower in the summer samples, with 4.1 ± 0.7 and 4.3 ± 0.7 log CFU/cm^2 than in the fall, being these 4.9 ± 0.7 and 5.1 ± 0.7 log CFU/cm^2, respectively ($P < 0.05$).

In the case of tomatoes, all the bacterial groups presented similar counts ($P > 0.05$) except for the LAB. This group was more numerous in the fall samples, with counts of 2.6 ± 1.6 log CFU/cm^2, than in the summer, being of 0.7 ± 0.7 log CFU/cm^2.

In the case of peppers, LAB, CL, and PY were affected by season ($P < 0.05$). LAB and CL counts were more numerous in the fall season, being 2.6 ± 1.5 and 2.1 ± 1.1 log CFU/cm^2 than those found in the summer samples of 0.6 ± 0.6 and 1.1 ± 0.9 log CFU/cm^2 respectively. The opposite behavior was seen for PY, since the counts were more numerous in the summer with 1.6 ± 0.7 log CFU/cm^2 while the summer samples presented less PY, with a mean of 1.1 ± 0.7 CFU log/cm^2.

Analyzing the results by bacterial group, the MS were similar in tomatoes and peppers, but not in cantaloupes, because cantaloupes collected in summer were more populated. Similarly, PY were more numerous in the summer, at least in cantaloupes and peppers, while CL and LAB were less numerous in the summer. The LAB content was noticeably changed by seasons with a fall reading of 0.9–2.0 log CFU/cm^2 showing more numerous LAB than that collected in the summer.

The effect of the cultivating season in the microbiological content of produce has been previously reported. Medina-Martinez et al. *(186)* reported a rapid decrease in the epiphytic coliforms from baby lettuce toward the end of the winter that was not observed during the first days of the same season. Ailes et al. *(10)* collected different produce commodities from 15 U.S. and Mexican farms (north of Mexico, and south of U.S.) to assess the influence of different factors influencing the variation in the microbiological content of produce. They reported a marked effect due to production season when comparing different commodities, which was show in their multivariate regression model. Ailes et al. *(9)* also found a greater content of APC, coliforms, *E. coli*, and *Enterococcus* spp. on cilantro and parsley samples collected in the fall, while samples collected in the spring and winter were less populated. Similarly, collards and spinach presented greater APC content in the fall than in the winter, although the APC content was not different for samples collected in the spring.

The season effect over the microbiological content of produce can be an indication of other factors influencing the bacteria loads in the produce. Other factors influencing produce bacteria loads include differences in climate conditions such as variations in

temperature and relative humidity, frost days, extreme cold or hot days (e.g., frost, and heatwaves), wind speed, etc. Microbiological content of produce can also be related to differences in irrigation water content disturbed by heavy raining or changes in the bacterial composition of the soils as reported by Won et al. *(298)* and Bing Zhang, et al. *(306)*, respectively. Even agricultural practices can vary with respect to soil as when an increased use of soil amendments are necessary due to a rapid depletion of soil nutrients in warmer seasons *(169)*. Furthermore, the migratory and seasonal birds, pests, and insects entering the fields can vary by season.

To further investigate the possible effect on the microbiological content of fruits and vegetables caused by variations in temperature and RH due to the cultivating season, the samples were analyzed separately according to the conditions of each harvested field, and utilizing temperature and RH of the harvesting days as benchmarks to measure variation.

Effect of the environmental conditions

To investigate the effect of temperature and humidity at harvest, statistical analyses were carried out for each bacterial group and field. Although the overall weather conditions during the cultivar development from seeds to harvesting is not included in this study, the harvesting weather conditions offer valuable information to complement the analysis of the variation of counts by season while illustrating the possible variation in bacterial numbers between fields, and permits determination of certain trends in the bacterial proliferation due to environment. The environmental conditions for fruits samples during each collection time and the comparison of bacterial content by field and

commodity are listed in Table 11. Furthermore, the environmental temperature, RH, and bacterial group counts are plotted in Fig. 7.

During the summer, the weather on harvest days at cantaloupe farms a and b presented temperatures and RH of 32.2 ºC and 37.8 ºC and 42% and 62%, respectively. During the fall, the temperature and RH at harvest were 25.6 ºC and 26.1 ºC and 65%, and 79% for fields a and b, respectively. Cantaloupes presented significant differences in their microbiological content during different seasons, although not in all bacterial groups. The MS of one field (fall-a) was less populated in 0.8–1.2 log CFU/cm^2 than the other fields. In general, LAB and PY content of cantaloupes from the two fields collected in the summer were different than those collected in the fall with LAB counts lower in the summer and PY counts higher in the same season ($P < 0.05$).

TABLE 11. Bacterial content of fruit surfaces, and environmental conditions at harvesting, by season[a]

Season[b]	Commodity-Field	Harvest date	Environ. conditions[c]		Mean ± SD[d]			
			RH	Temp	MS[e]	LAB	CL	PY
Summer	Cantaloupe-a	06/12/12	42	32.2	5.9 ± 0.6 A[f]	3.8 ± 0.4 A	4.3 ± 0.8 A	5.3 ± 0.6 A
	Cantaloupe-b	06/25/12	62	37.8	6.3 ± 0.4 A	4.3 ± 0.8 A	4.4 ± 0.6 AB	5.2 ± 0.7 A
Fall	Cantaloupe-a	10/04/12	65	25.6	5.1 ± 0.5 B	4.8 ± 0.8 B	5.2 ± 0.9 C	4.5 ± 0.7 B
	Cantaloupe-b	10/10/12	79	26.1	5.9 ± 0.6 A	4.9 ± 0.7 B	4.9 ± 0.6 BC	4.2 ± 0.9 B
Summer	Tomato-a	05/21/12	49	30.0	3.0 ± 0.8 A	0.9 ± 0.8 A	0.6 ± 0.6 A	1.4 ± 0.8 A
	Tomato-b	05/24/12	45	37.2	3.6 ± 0.8 AB	0.7 ± 0.6 A	1.5 ± 1.0 B	1.9 ± 0.9 B
Fall	Tomato-a	11/26/12	86	21.1	3.9 ± 1.1 B	3.4 ± 1.7 B	0.9 ± 0.8 AB	0.8 ± 0.6 C
	Tomato-b	12/04/12	77	22.2	3.4 ± 0.6 AB	1.8 ± 0.9 C	1.5 ± 1.0 B	2.5 ± 0.6 B
Summer	Pepper-a	06/07/12	62	28.9	2.5 ± 0.8 A	0.5 ± 0.4 A	0.8 ± 0.7 A	1.7 ± 0.7 A
	Pepper-b	06/11/12	60	36.1	4.7 ± 0.4 B	0.7 ± 0.8 A	1.5 ± 0.9 B	1.6 ± 0.7 A
Fall	Pepper-a	11/27/12	43	22.1	3.7 ± 0.8 C	3.1 ± 1.7 B	1.7 ± 1.2 B	0.9 ± 0.6 B
	Pepper-b	12/03/12	78	22.2	3.6 ± 0.4 C	2.1 ± 1.1 C	2.5 ± 0.7 C	1.3 ± 0.7 AB

[a] Harvesting seasons: Summer, May-June, and fall, October-December.
[b] Two separate fields collected per commodity, (a and b) and season (Summer or fall).
[c] RH: Relative humidity in %, Temp: Temperature in ºC.
[d] Mean log CFU/cm^2 ± Standard deviation by bacterial groups, n=25.
[e] MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs
[f] Within columns and commodity, values followed by the same letter are not significantly different (P > 0.05)

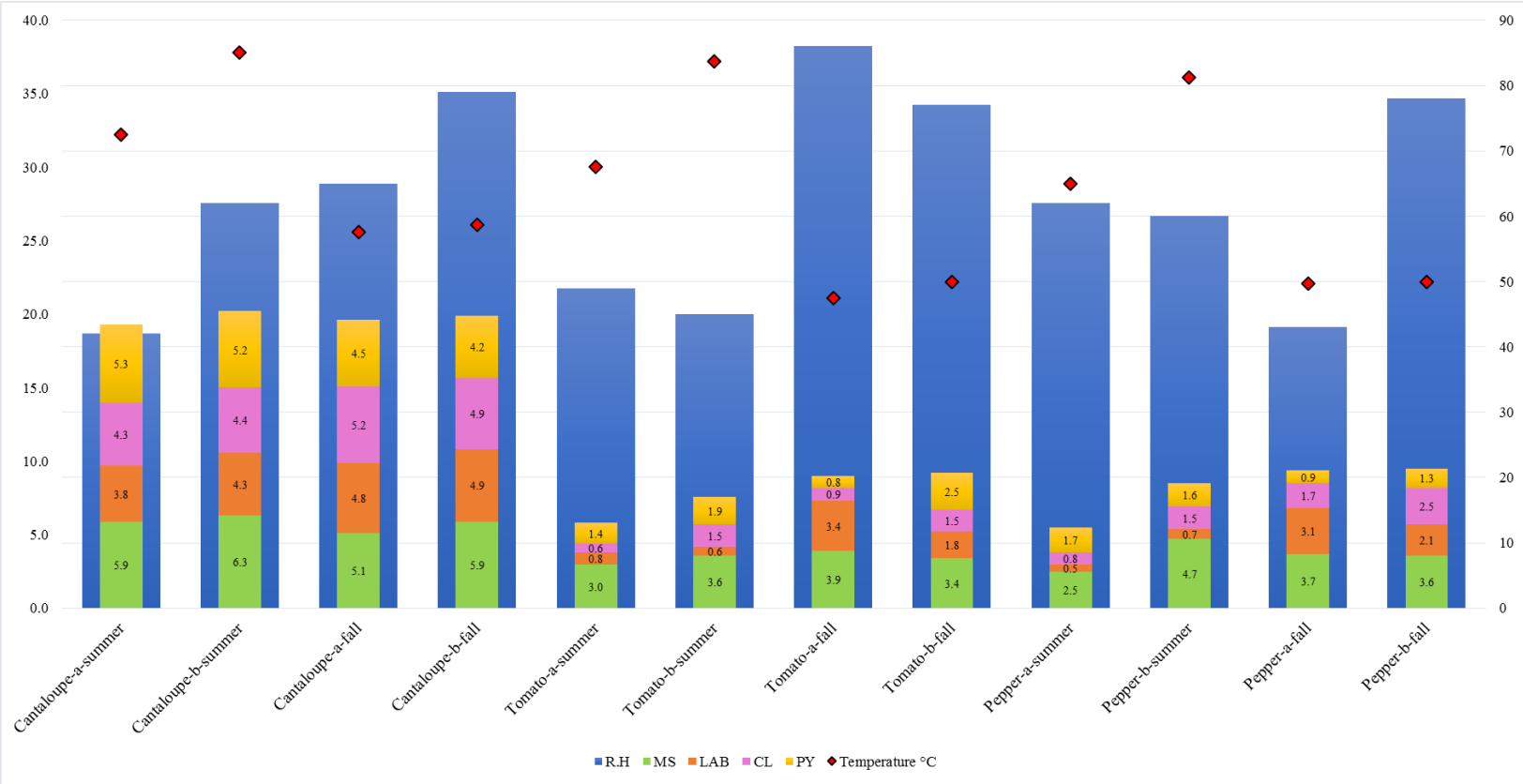

FIGURE 7. Bacterial content of cantaloupes, tomatoes, and peppers by season and field. Fruit samples collected during two seasons, from two fields each. Horizontal axis: Commodity, field (a or b) and season (summer or fall). Mean (log CFU/cm^2) for each field sampled by bacterial group (divided thin bars) for MS: mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, and PY: Psychrotrophs. Environmental temperature in ºC is plotted to the left vertical axis, and relative humidity (RH) in % (thick bars) is plotted to the right vertical axis.

The temperature, and RH during the collection of tomatoes in the summer were 30 and 37 ºC, and 49 and 45% for fields *a*, and *b*, respectively. For fields *a* and *b* collected in the fall, the temperature and RH were 21.1 and 22.2 ºC, and 88 and 77%, respectively. There were differences also found by field within and between seasons in the MS, CL, and PY counts. In the case of LAB, it was evident that both fields collected in the summer had less LAB when compared to summer samples. The samples collected in the summer had almost no detectable LAB, being 0.8 and 0.6 log CFU/cm^2, while those collected in the fall were higher at 3.4 and 1.8 log CFU/cm^2 ($P < 0.05$). An interesting finding was seen in the fall-*a* field where samples with the lowest PY content (0.8 CFU/cm^2) presented the highest LAB population (3.4 log CFU/cm^2) when compared with the other fields ($P < 0.05$).

For peppers, the temperature and RH in the summer harvesting days were 29 and 36 ºC, and 62 and 60%, for fields *a* and *b*, respectively. In the fall, the temperature and RH during the harvesting days were 22.1 and 22.2 ºC, and 43 and 78% for fields *a* and *b*, respectively. When bacterial groups were compared, LAB presented a trend similar to tomatoes where the samples collected in the summer presented a reduced LAB count of 0.5 and 0.7 log CFU/cm^2, while those collected in the fall, obtained counts significantly larger of 2.1, and 3.1 log CFU/cm^2 ($P < 0.05$). Similar to tomatoes, the field that presented the highest LAB counts, (3.4 log CFU/cm^2) was one of the fields that presented the lowest PY content (0.9 ± 0.7 log CFU/cm^2). This field was collected during the fall season.

The variation in the microbiological content of produce commodities has been related to the environmental humidity and temperature in diverse studies. Yadav et al.

(302) described water on the leaves, as the first descriptor to determine the variation on the epiphytic bacteria of diverse Mediterranean plants. Marine et al. *(180)* also found significant differences between sampling dates while studying epiphytic bacteria on spinach, lettuce and other leafy greens samples and related these results to differences in the humidity and temperature on harvesting days. In this study, the trends were more evident with the variation of temperature than humidity, yet the variation in the different bacterial groups demonstrate that the epiphytic community is likely adapting to different environmental conditions. Further studies determining the extent of the effect of all other environmental factors not included in this study, along with temperature and relative humidity would possibly confirm the marked effect of these two environmental factors.

Effect of the irrigation method

The irrigation factor effect in the variation of the microbiological content of fruits was investigated. To analyze the effect of the irrigation system, the counts of tomatoes and peppers from fall cultivars were compared since these cultivars where produced during the same season with one cultivar of each commodity using each irrigation system. Drip and flood irrigation comparison analyses for tomatoes, peppers and overall differences by irrigation method used are shown in Table 12.

For CL and PY, the populations found in the samples collected from drip irrigated fields were significantly lower than in samples from flood irrigated fields. On the contrary, samples from drip-irrigated fields presented higher LAB content than those from flood irrigated fields ($P < 0.05$). Such a tendency was similar in the MS counts; however, the differences between drip and flood irrigated samples were less than 1 log/cm^2.

TABLE 12. Number of epiphytic bacteria in peppers and tomatoes using two different irrigation systems

Commodity	Irrigation[a]	Mean ± DS[b]			
		MS[c]	LAB	CL	PY
Tomatoes	Drip	3.9 ± 1.1 A	3.4 ± 1.7 A	0.9 ± 0.8 A	0.8 ± 0.6 A
	Flood	3.4 ± 0.6 B	1.8 ± 0.9 B	1.5 ± 1.0 B	2.5 ± 0.6 B
Peppers	Drip	3.7 ± 0.8 A	3.1 ± 1.7 A	1.7 ± 1.2 A	0.9 ± 0.6 A
	Flood	3.6 ± 0.4 A	2.1 ± 1.1 B	2.5 ± 0.7 B	1.3 ± 0.7 B
Both commodities	Drip	3.8 ± 0.9 A	3.3 ± 1.7 A	1.3 ± 1.1 A	0.9 ± 0.6 A
	Flood	3.5 ± 0.5 B	1.9 ± 1.0 B	2.0 ± 1.0 B	1.9 ± 0.9 B

[a] Irrigation system reported by the producer

[b] Mean log CFU/cm^2 ± standard deviation of bacterial counts averaged n = 25 or n =50 for both commodities.

[c] Bacterial groups, MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs. Within each column, within each commodity, values followed by the same letter are not significantly different ($P > 0.05$)

The variation in the content of different groups of bacteria might be influenced by the source of bacteria being affected by irrigation. For example, plants irrigated by flood might have a higher chance of contacting water from irrigation than those drip irrigated. Thus, bacteria in flood samples might be more related to soil epiphytes than drip irrigated plants. Splashed irrigation water can reach peppers and tomatoes that grow above the ground. If epiphytic bacteria populations shift according to predominant groups present, then it is possible that the higher content of LAB limited the population of CL on the samples. Yet, for the CL source, it is likely that the cultivating soil served as a supplier of CL to the fruits based on flood samples. One more cause for the variation given by irrigation is the use of different agricultural practices in these fields. Drip irrigation is considered an improved irrigation method, but it requires a higher investment for its application. Thus, it is possible that those fields irrigated by drip might also have access

to newer technologies besides irrigation, possibly due to higher investments from producers. The health state of the plant, due to these implemented technologies might promote the growth of certain bacteria, in this case LAB. Further studies that determine the extent of all the possible causes influencing the epiphytic bacteria variation should include all practices during production, temperature and humidity, rain, flood episodes, and other environmental factors, as well as include different varieties of the same species of tomatoes, peppers, and melons. A study evaluating the influence of the level of netting, possibly using different hybrids of cantaloupes, could help to elucidate the actual impact of the netting level over the microbiological content of cantaloupe rinds.

In vitro antagonistic effect of epiphytic bacteria against enteric pathogens

Effect of leafy green-epiphytic bacteria on *E. coli* O157:H7 and *S*. Saintpaul

The number of epiphytic isolates originally selected, recovered, tested, and found antagonistic toward one or both pathogens by leafy green commodity, bacterial group isolated, and by pathogen inhibited are shown in Table 13. In total, from 3,426 leafy green isolates tested, 397 (11.6%) tested antagonistic toward *E. coli* O57:H7, *Salmonella* or toward both pathogens. One-hundred-ninety isolates were antagonistic toward both pathogens; 174 was antagonistic only towards *E. coli* O157:H7, and 32 was antagonistic only towards *S.* Saintpaul. The different leafy green commodities presented similar proportions of antagonistic bacteria ($P>0.05$). Overall, 13.8% (134) of the 971 spinach-isolated bacteria, 10% (109) of 1,088 endive-isolated bacteria, and 11.2% (153) of 1,367 parsley-isolated bacteria tested as antagonistic toward one or both pathogens. The isolates considered LAB (recovered from MRS) from parsley and endives were antagonistic more

frequently than other bacterial groups within each commodity for parsley and endives. The number of antagonistic LAB was similar to that of PY in the spinach isolates.

From 89 endive LAB-isolates tested, and from 109 parsley LAB-isolates, 58.4%, and 33.9% gave antagonistic results toward one or both pathogens. From the 264 spinach-LAB isolates, 16.3% (43) were antagonistic. Similarly, from 495 spinach PY isolates, 18.4% (91) tested antagonistic. Antagonistic isolates from CL and MS were infrequently detected in all commodities. Only five out of 144 CL isolates from endives and seven out of 407 CL isolates from parsley tested antagonistic toward one or both pathogens. Furthermore, none of the 84 CL spinach isolates tested antagonistic. For MS, three out of 355, and three out of 368 isolates were positive for antagonism, while none of the 128 MS isolates from spinach tested antagonistic to any of the pathogens.

TABLE 13. Number of epiphytic isolates from leafy greens, total and antagonistic toward *E. coli* O157:H7, and/or *S.* Saintpaul

Commodity[a]	Isolated	Tested	Epiphytic bacteria antagonistic toward[b]:			Total antagonistic (%)[c]
			E. coli O157:H7	*S.* Saintpaul	both pathogens	
Spinach						
MS[d]	500	128	-	-	-	0
LAB	489	264	5	3	35	43 (16.3) a[e]
CL	500	84	-	-	-	0
PY	500	495	67	5	19	91 (18.4) a
Total	*1,989*	*971*	*72*	*8*	*54*	*134 (13.8) A*[f]
Endives						
MS	500	355	1	-	2	3 (0.8) a
LAB	498	89	-	-	52	52 (58.4) b
CL	500	144	1	1	3	5 (3.5) c
PY	500	500	37	6	6	49 (9.8) d
Total	*1,998*	*1,088*	*39*	*7*	*63*	*109 (10.0) A*
Parsley						
MS	500	368	-	1	5	6 (1.6) a
LAB	473	109	1	1	36	38 (34.9) b
CL	424	407	-	1	6	7 (1.7) a
PY	483	483	62	15	26	103 (21.3) c
Total	*1,880*	*1,367*	*63*	*17*	*73*	*153 (11.2) A*
Grand total	5,867	3,426	174	32	190	397 (11.6)

[a] Produce samples collected during the winter (February-March) from two fields per commodity in Texas.
[b] Isolates resulting antagonistic during *in vitro* spot agar test.
[c] Counts and percentages (%) of antagonistic bacteria from isolates tested.
[d] Bacterial groups, MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs.
[e] Within columns within commodity, values showing same lowercase letter are not significantly different ($P > 0.05$).
[f] Within columns between commodities, values showing same uppercase letter are not significantly different ($P > 0.05$).

TABLE 14. Number of epiphytic isolates from leafy greens, antagonistic toward *E. coli* O157:H7, and/or *S.* Saintpaul by bacterial group, and commodity

Bacterial group[a]	Number of antagonistic isolates per commodity												
	Spinach				Endives				Parsley				Grand total (%)
	EC[b]	SS	Bo	*Tot*	EC	SS	Bo	*Tot*	EC	SS	Bo	*Tot*	
MS	-	-	-	-	1	-	2	*3*	-	1	5	*6*	9 (2.3) A[c]
LAB	5	3	35	*43*	-	-	52	*52*	1	1	36	*38*	133 (33.5) B
CL	-	-	-	-	1	1	3	*5*	-	1	6	*7*	12 (3.1) A
PY	67	5	19	*91*	37	6	6	*49*	62	15	26	*103*	243 (61.2) C
Total	72	8	54	*134*	39	7	63	*109*	63	18	73	*154*	397 (100)

[a] MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs.

[b] Isolates antagonistic toward *E. coli* O157:H7 (EC), toward *S.* Saintpaul (SS), toward both pathogens (Bo), and total antagonistic isolates (Tot).

[c] Within columns, percentage values showing same letter are not significantly different ($P > 0.05$).

The number of antagonistic isolates from leafy greens of each bacterial group are shown in Table 14. From the 397 isolates testing antagonistic, 61.2% (243) were isolated from the PY group, and this was the group which presented the greatest proportion of antagonistic isolates ($P < 0.05$). The second largest percentage was 33.5% (133) from isolates that belonged to the LAB group ($P < 0.05$). Fewer antagonistic isolates came from the MS, and CL groups ($P < 0.05$). Only 2.3% (9), and 3.1% (12) of the antagonistic isolates originated from MS and CL, respectively. Further biochemical identification of the isolates allowed comparison of the frequency of each species by commodity and season. The different antagonistic isolates were biochemically identified and their percentages of occurrence by bacterial species are shown in Table 15.

From spinach, 26 PY, and six presumptive LAB antagonistic isolates were biochemically identified, while 19 PY, and three MS isolates were not identified by the VITEK-2 system. From endives, 39 presumptive LAB, four presumptive CL, 15 PY, and three MS antagonistic isolates were biochemically identified, while it was not possible to identify 14 presumptive LAB isolates. From parsley, 16 presumptive LAB, seven presumptive CL, 44 PY and five MS antagonistic isolates were biochemically identified, while three isolates of PY, one of MS, and three presumptive LAB isolates could not be identified.

The most frequently identified species from the PY group was *Alcaligenes faecalis* sbsp. *faecalis*. This species represented 50%, 24.6%, and 47.4% of the total antagonistic isolates identified from spinach, endives, and parsley, respectively. This species was mainly isolated from the PY group. However, one strain was isolated from a VRBA plate.

TABLE 15. Number of leafy green-epiphytic isolates antagonistic toward *E. coli* O157:H7 and/or *S.* Saintpaul and frequency of identification

Isolation medium[a]	Bacterial species	Isolates	%
Spinach			
TSA (PY)	*Alcaligenes faecalis* sbsp. *faecalis*	16	50.0 A
TSA (PY)	*Enterococcus casseliflavus*	4	12.5 B
TSA (PY)	*Cupriavidus pauculus*	2	6.3 B
TSA (PY)	*Kocuria kristinae*	2	6.3 B
TSA (PY)	*Pseudomonas stutzeri*	1	3.1 B
TSA (PY)	*Pseudomonas pseudoalcaligenes*	1	3.1 B
MRS	*Enterococcus casseliflavus*	1	3.1 B
MRS	*Enterococcus cecorum*	1	3.1 B
MRS	*Enterobacter cloacae* sbsp. *dissolvens*	1	3.1 B
Total		*32*	*100*
Endives			
TSA (PY)	*Alcaligenes faecalis* sbsp. *faecalis*	14	23.0 A
TSA (PY)	*Myroides* ssp.	1	1.6 C
MRS	*Streptococcus alactolyticus*	9	14.8 AB
MRS	*Enterococcus gallinarum*	8	13.1 ABC
MRS	*Pediococcus pentosaceus*	4	6.6 BC
MRS	*Lactobacillus plantarum*	3	4.9 BC
MRS	*Streptococcus equinus*	3	4.9 BC
MRS	*Streptococcus mutans*	3	4.9 BC
MRS	*Streptococcus sanguini*	3	4.9 BC
MRS	*Aerococcus viridans*	1	1.6 C
MRS	*Lactobacillus rhamnosus*	1	1.6 C
MRS	*Vagococcus fluvialis*	1	1.6 C
MRS	*Leuconostoc mesenteroides* sbsp. *dextranicum*	1	1.6 C
MRS	*Listeria grayi*	1	1.6 C
MRS	*Gemella bergeri*	1	1.6 C
TSA (MS)	*Bacillus licheniformis*	1	1.6 C
TSA (MS)	*Bacillus pumilus*	1	1.6 C
TSA (MS)	*Staphylococcus lentus*	1	1.6 C
VRBA	*Alcaligenes faecalis* sbsp. *faecalis*	1	1.6 C
VRBA	*Pantoea* ssp.	1	1.6 C
VRBA	*Staphylococcus lentus*	1	1.6 C
VRBA	*Staphylococcus sciuri*	1	1.6 C
Total		*61*	*100*

TABLE 15. Continued.

Isolation medium[a]	Bacterial species	Isolates	%
Parsley			
TSA (PY)	*Alcaligenes faecalis* sbsp. *faecalis*	36	47.4 A
TSA (PY)	*Myroides* ssp.	5	6.6 BC
TSA (PY)	*Enterococcus casseliflavus*	1	1.3 C
TSA (PY)	*Providencia rettgeri*	1	1.3 C
TSA (PY)	*Serratia plymuthica*	1	1.3 C
MRS	*Enterococcus casseliflavus*	5	6.6 BC
MRS	*Enterococcus gallinarum*	3	3.9 BC
MRS	*Aerococcus viridans*	1	1.3 C
MRS	*Lactobacillus plantarum*	1	1.3 C
MRS	*Staphylococcus gallinarum*	4	5.3 BC
MRS	*Staphylococcus intermedius*	3	3.9 BC
MRS	*Staphylococcus lentus*	2	2.6 BC
MRS	*Gemella morbillorum*	1	1.3 C
TSA (MS)	*Bacillus* ssp.	3	3.9 BC
TSA (MS)	*Bacillus mycoides*	1	1.3 C
TSA (MS)	*Sphingomonas paucimobilis*	1	1.3 C
VRBA	*Bacillus* ssp.	7	9.2 B
Total		*76*	*100*

[a] TSA (PY): Tryptic soy agar, incubated at 7 ºC for 7 d. TSA (MS). Tryptic soy agar, incubated at 37 ºC for 48 h. MRS: De Man, Rogosa, and Sharpe, VRBA: violet red bile agar.

Other antagonistic isolates identified and included in the PY group were *Cupriavidus pauculus*, *Kocuria kristinae*, *Pseudomonas stutzeri*, and *Pseudomonas pseudoalcaligenes* from spinach, *Enterococcus casseliflavus* from spinach and parsley, *Myroides* spp. from endives and parsley, and *Providencia rettgeri* and *Serratia plymuthica* from parsley.

The presumptive LAB group of antagonistic isolates contained similar proportions of *Aerococcus viridans, Enterococcus cecorum,* and *E. casseliflavus*. One isolate of *Enterobacter cloacae* sbsp. *dissolvens* was also isolated from MRS.

In the case of endives, the antagonistic LAB identified included isolates of *Streptococcus alactolyticus, Enterococcus gallinarum, Pediococcus pentosaceus, Lactobacillus plantarum,* and *Streptococcus equinus*, and one isolate of *A. viridans, Lactobacillus rhamnosus*, *Vagococcus fluvialis* and *Leuconostoc mesenteroides* sbsp *dextranicum.* Non LAB isolates obtained from MRS included one *Listeria grayi* and one *Gemella bergeri*.

From parsley, antagonistic LAB included several isolates of *E. casseliflavus,* and *Enterococcus gallinarum,* and one isolate of *A. viridans* and *L. plantarum*. Antagonistic isolates of *Staphylococcus* species were also obtained from MRS, including four isolates of *S. gallinarum,* three of *S. intermedius*, and two of *Staphylococcus lentus*. Also from MRS, one isolate of *Gemella morbillorum* was also identified.

The MS strains isolated from endives were identified as *Bacillus licheniformis, Bacillus pumilus,* and *Staphylococcus lentus.* In the case of those MS antagonistic isolates obtained from parsley, three isolates were identified as *Bacillus* spp., one as *Bacillus mycoides,* and one as *Sphingomonas paucimobilis*.

Although only a few isolates were identified from the total of antagonistic isolates obtained from leafy greens, those identified might be considerable constituents of the total population of antagonistic epiphytic bacteria found on each commodity. The determination of the actual occurrence of these species using selective conditions adapted

to each species or genera could allow a more precise determination of their occurrence in the leafy greens surfaces.

Effect of fruit-epiphytic bacteria on *E. coli* O157:H7 and *S*. Saintpaul

The total number of epiphytic isolates originally selected, recovered, tested, and deemed positive (antagonistic) by commodity, isolated bacterial group, and inhibited pathogens from fruits are shown in Tables 16.

From a total of 5,881 fruit isolates tested, 499 (8.5 %) tested antagonistic toward *E. coli* O57:H7, *Salmonella* Saintpaul or toward both pathogens. In total, 402 fruit isolates resulted antagonistic to both pathogens, 33 only toward *S*. Saintpaul, and 64 only toward *E. coli* O157:H7. Most of the antagonistic isolates originated from cantaloupes (312), while tomatoes and peppers presented fewer antagonistic isolates (133), and peppers presented the lesser amount of antagonistic bacteria (54) ($P<0.05$).

The LAB group had the largest percentages of antagonistic isolates among the bacterial groups within commodity, being 43.2% out of 658, 47.9% out of 58, and 38.8% out of 103 LAB isolates from cantaloupe, tomatoes, and peppers, all testing antagonistic toward one or both pathogens. The PY antagonistic isolates were also significantly large, after LAB, in peppers and tomatoes, being 9.5% and 12.4% antagonistic to the pathogens tested, respectively. From the MS and CL groups, only a few tested antagonistic. For example, 0.7% and 0.9% of the MS from cantaloupe, and tomatoes were antagonistic and none of the 653 MS isolates from peppers were antagonistic toward any of the pathogens. Similarly, 2.5% and 1.2% of the CL from cantaloupes and tomatoes were antagonistic, and none of the 179 CL isolates tested tested positive for antagonistic effect.

TABLE 16. Number of epiphytic isolates from fruits, total testing antagonistic toward *E. coli* O157:H7, and/or *S.* Saintpaul

Commodity[a]	Isolated	Tested	Epiphytic bacteria antagonistic toward[b]:			Total antagonistic (%)[c]
			E. coli O157:H7	*S.* Saintpaul	Both pathogens	
Cantaloupe						
MS[d]	1,000	737	3	1	1	5 (0.7) ad [e]
LAB	1,000	655	7	15	261	283 (43.2) b
CL	1,000	568	7	-	7	14 (2.5) c
PY	997	769	2	-	8	10 (1.3) cd
Total	*3,997*	*2,729*	*19*	*16*	*277*	*312 (11.4) A*[f]
Pepper						
MS	932	653	-	-	-	0
LAB	557	58	1	-	21	22 (37.9) a
CL	757	179	-	-	-	0
PY	696	338	11	3	18	32 (9.5) b
Total	*2,942*	*1,228*	*12*	*3*	*39*	*54 (4.4) B*
Tomato						
MS	999	919	4	-	4	8 (0.9) a
LAB	563	103	-	1	39	40 (38.8) b
CL	580	243	1	-	2	3 (1.2) a
PY	794	659	28	13	41	82 (12.4) c
Total	*2,936*	*1,924*	*33*	*14*	*86*	*133 (6.9) C*
Grand total	9,875	5,881	64	33	402	499 (8.5)

[a] Three commodities sampled during two harvesting seasons from two fields in Texas.
[b] Isolates resulting antagonistic during *in vitro* spot agar test.
[c] Counts and percentages (%) of antagonistic bacteria from isolates tested.
[d] Bacterial groups, MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs.
[e] Within columns within commodity, values showing same lowercase letter are not significantly different (P > 0.05).
[f] Within columns between commodities, values showing same uppercase letter are not significantly different (P > 0.05).

The total number of antagonistic isolates by commodity and bacterial group are shown in Table *17*. When the overall percentages of antagonistic isolates per bacterial groups were compared, LAB isolates represent the group with the highest number of antagonistic isolates (345 isolates) followed by PY (124 isolates). The CL and MS isolates were represented by only 17 and 13 of the 499 total isolates identified as antagonistic. To further examine the differences found by commodity and bacterial groups, the biochemical identification of some antagonistic isolates is included.

The different antagonistic isolates were biochemically identified, and their percentages of occurrence by bacterial species are shown in Table *18*. From cantaloupes, 176 presumptive LAB, 10 presumptive CL, and 1 MS isolate were biochemically identified, while 39 LAB isolates were not identified.

From tomatoes, 11 PY, 23 presumptive LAB, 5 MS, and 4 presumptive CL isolates were biochemically identified, although it was not possible to identify two presumptive LAB, one MS, and one PY antagonistic isolate. From peppers, 6 PY and 17 presumptive LAB isolates were identified, although it was not possible to identify one LAB isolate.

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TABLE 17. Number of epiphytic isolates from fruits antagonistic toward *E. coli* O157:H7 and/or *S.* Saintpaul by bacterial group, and commodity

Bacterial group[a]	Number of antagonistic isolates per commodity												
	Cantaloupes				Peppers				Tomatoes				Grand total (%)
	EC[b]	SS	Bo	*Tot*	EC	SS	Bo	*Tot*	EC	SS	Bo	*Tot*	
MS	3	1	1	*5*	-	-	-	-	4	-	4	*8*	13 (3.6) C[c]
LAB	7	15	261	*283*	1	-	21	*22*	-	1	39	*40*	345 (69.1) A
CL	7	-	7	*14*	-	-	-	-	1	-	2	*3*	17 (3.4) C
PY	2	-	8	*10*	11	3	18	*32*	28	13	41	*82*	124 (24.8) B
Total	19	16	277	*312*	12	3	39	*54*	33	14	86	*133*	499 (100)

[a] MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs.
[b] Isolates antagonistic toward *E. coli* O157:H7 (EC), toward *S.* Saintpaul (SS), toward both pathogens (Bo), and total antagonistic isolates (Tot).
[c] Within columns, percentage values showing same letter are not significantly different ($P > 0.05$).

TABLE 18. Number of fruit-epiphytic isolates antagonistic toward *E. coli* O157:H7 and/or *S.* Saintpaul and frequency of identification

Isolation medium[a]	Bacterial species	Isolates	%
Cantaloupe			
MRS	*Leuconostoc mesenteroides* sbsp. *dextranicum*	32	17.4 A
MRS	*Leuconostoc pseudomesenteroides*	30	16.3 A
MRS	*Enterococcus faecalis*	16	8.7 B
MRS	*Enterococcus gallinarum*	12	6.5 BC
MRS	*Enterococcus casseliflavus*	9	4.9 BCD
MRS	*Leuconostoc citreum*	8	4.3 BCDE
MRS	*Aerococcus viridans*	6	3.3 CDEF
MRS	*Streptococcus thoraltensis*	5	2.7 CDEF
MRS	*Lactobacillus plantarum*	4	2.2 DEF
MRS	*Lactococcus garvieae*	4	2.2 DEF
MRS	*Lactococcus lactis* sbsp. *lactis*	3	1.6 DEF
MRS	*Pediococcus pentosaceus*	2	1.1 E F
MRS	*Enterococcus cecorum*	1	0.5 F
MRS	*Enterococcus spp.*	1	0.5 F
MRS	*Lactococcus pentosaceus*	1	0.5 F
MRS	*Streptococcus* ssp.	1	0.5 F
MRS	*Leuconostoc mesenteroides* sbsp. *cremoris*	1	0.5 F
MRS	*Leuconostoc mesenteroides* sbsp. *mesenteroides*	1	0.5 F
MRS	*Staphylococcus gallinarum*	8	4.3 BCDE
MRS	*Staphylococcus lentus*	4	2.2 DEF
MRS	*Staphylococcus epidermidis*	2	1.1 EF
MRS	*Staphylococcus sciuri*	2	1.1 EF
MRS	*Staphylococcus vitulinus*	2	1.1 EF
MRS	*Staphylococcus warneri*	2	1.1 EF
MRS	*Staphylococcus cohnii* sbsp. *urealyticus*	1	0.5 F
MRS	*Staphylococcus xylosus*	1	0.5 F
MRS	*Citrobacter sedlakii*	3	1.6 DEF
MRS	*Enterobacter hormaechei*	3	1.6 DEF
MRS	*Enterobacter kobei*	2	1.1 EF
MRS	*Enterobacter cloacae* sbsp. *dissolvens*	1	0.5 F
MRS	*Enterobacter ludwigii*	1	0.5 F
MRS	*Klebsiella pneumoniae* sbsp. *pneumoniae*	2	1.1 EF
MRS	*Kocuria kristinae*	1	0.5 F
MRS	*Kocuria rosea*	1	0.5 F
TSA (MS)	*Bacillus vallismortis*	1	0.5 F
VRBA	*Enterobacter ludwigii*	4	2.2 DEF
VRBA	*Klebsiella pneumoniae* sbsp. *pneumoniae*	4	2.2 DEF
VRBA	*Enterobacter aerogenes*	1	0.5 F
VRBA	*Enterobacter hormaechei*	1	0.5 F
Total		187	100

TABLE 18. Continued.

Isolation medium[a]	Bacterial species	Isolates	%
Tomato			
TSA (PY)	*Alcaligenes faecalis faecalis*	5	11.6 AB
TSA (PY)	*Kitococcus sedentarius*	5	11.6 AB
TSA (PY)	*Enterococcus casseliflavus*	1	2.3 B
MRS	*Lactococcus lactis* sbsp. *lactis*	2	4.7 B
MRS	*Enterococcus gallinarum*	1	2.3 B
MRS	*Kocuria kristinae*	8	18.6 A
MRS	*Staphylococcus epidermidis*	8	18.6 A
MRS	*Staphylococcus hominis* sbsp. *hominis*	1	2.3 B
MRS	*Staphylococcus hominis* sbsp. *novobiosepticus*	1	2.3 B
MRS	*Staphylococcus lentus*	1	2.3 B
MRS	*Bacillus mycoides*	1	2.3 B
TSA (MS)	*Bacillus licheniformis*	2	4.7 B
TSA (MS)	*Bacillus polymyxa*	1	2.3 B
TSA (MS)	*Bacillus pumilus*	1	2.3 B
TSA (MS)	*Bacillus* ssp.	1	2.3 B
VRBA	*Pantoea* ssp.	3	7.0 B
VRBA	*Alcaligenes faecalis faecalis*	1	2.3 B
Total		43	100
Peppers			
TSA (PY)	*Alcaligenes faecalis faecalis*	5	21.7 AB
TSA (PY)	*Cronobacter* ssp.	1	4.3 B
MRS	*Enterococcus faecium*	4	17.4 AB
MRS	*Enterococcus faecalis*	1	4.3 B
MRS	*Staphylococcus epidermidis*	6	26.1 A
MRS	*Staphylococcus lugdunensis*	2	8.7 AB
MRS	*Staphylococcus warneri*	2	8.7 AB
MRS	*Staphylococcus hominis* sbsp. *hominis*	1	4.3 B
MRS	*Staphylococcus saprophyticus*	1	4.3 B
Total		23	100

[a] TSA (PY): Tryptic soy agar, incubated at 7 ºC for 7 d. TSA (MS). Tryptic soy agar, incubated at 37 ºC for 48 h. MRS: De Man, Rogosa, and Sharpe, VRBA: violet red bile agar.

From those antagonistic isolates found in cantaloupe, the most frequently identified species were from the LAB group including *Leuconostoc mesenteroides* sbsp. *dextranicum* and *Leuconostoc pseudomesenteroides* representing 32 and 30% of the total antagonistic isolates identified from cantaloupes. Several other isolates from the LAB group were identified as *Enterococcus faecalis* (16 isolates), *E. gallinarum* (12 isolates),

Leuconostoc citreum (eight isolates) and *E. casseliflavus* (nine isolates) while other 12 LAB species were represented by fewer identified isolates.

Other species were recovered from MRS, although they do not belong to LAB, including eight species of *Staphylococcus* spp., species belonging to the *Enterobacteriaceae* family, such as *Citrobacter sedlakii* and *Klebsiella pneumoniae*, and one each of *Kocuria kristinae*, and *Kocuria rosea*. From the MS group, only one isolate was identified, corresponding to *Bacillus vallismortis*. From the CL group, four isolates were identified as *Enterobacter ludwigii*, one as *Enterobacter aerogenes*, one as *Enterobacter hormaechei*, and one as *Klebsiella pneumoniae*.

From the antagonistic isolates derived from tomatoes, the PY group included five isolates identified as *A. faecalis*, five as *Kytococcus sedentarius*, and one isolate of *E. casseliflavus*. Most of the tomato antagonistic isolates recovered from MRS were not LAB. In the case of the isolates recovered from MRS, the only definite LAB were two isolates of *Lactococcus lactis* sbsp. *lactis*, and one isolate of *Streptococcus gallinarum*. Other non-LAB bacterial isolates, recovered from MRS plates of tomato samples included eight isolates of *Kocuria kristinae*, eight of *Staphylococcus epidermidis*, and one each of *Staphylococcus hominis* sbsp. *hominis*, *Staphylococcus hominis* sbsp. *novobiosepticus*, *Staphylococcus lentus*, and *Bacillus mycoides* were recovered from MRS. Antagonistic isolates from the tomato MS group consisted of four different species of *Bacillus* spp. From the CL isolates, three strains of *Pantoea* spp. and one isolate of *A. faecalis* was identified.

In the case of pepper-isolated antagonistic bacteria, the PY group contained five isolates of *A. faecalis*, and one isolate of *Cronobacter* ssp. From the isolates recovered from MRS, five were LAB, and consisted of four isolates of *Enterococcus faecium* and one of *Enterococcus faecalis*, while 12 isolates were different *Staphylococcus* species, including *S. epidermidis*, *S. lugdunensis*, *S. warneri*, *S. hominis* sbsp. *hominis*, and *S. saprophyticus*.

The isolates of the same species were possibly the same strain isolated several times, although this was not determined. The broad distribution, or a large amount of a particular species, could also influence the likelihood of isolation. Further assessment studies that determine the presence of a particular species and their population could help clarify their distribution in the surfaces of different commodities.

Effect of season in the occurrence of antagonistic bacteria

The total number of antagonistic isolates by season, field collected, commodity, and bacterial group are shown in Table 19. The occurrence of antagonistic epiphytic bacteria was influenced by the harvesting season ($P < 0.05$). In total, 180 of the 499 antagonistic isolate toward one or both pathogens derived from fruit samples collected during the summer harvesting season, while 319 were isolated from those coming from the fall harvested fields. A probable explanation of the variation in the antagonistic population by season is the population variation in number, wherein the more populated the fruit surfaces are, the higher the probability of finding antagonistic bacteria. However, this trend is not observable when analyzing the data obtained in the present study, as shown in Table 20. For example, the mean LAB population of cantaloupes in the summer was 0.8

log CFU/cm^2 fewer than the LAB population in the fall, while the proportion of antagonistic LAB was almost seven times smaller in the summer than in the fall. The population of PY for tomatoes from the summer was similar ($P > 0.05$) for both seasons, while the antagonistic isolated bacteria was much different. Only two antagonistic isolates resulted from the PY group in the fall as opposed to 80 isolates recovered in the summer. The causes of the variation in the occurrence of antagonistic bacteria in different seasons would remain undetermined. Further analysis of the behavior of particular populations of potential antagonistic species, as those identified in the present study, during the production and harvesting of these fruits, could help determine the possible reasons for this marked variation in the content of antagonistic bacteria, and the extent of the season as a causative effect of this variation.

TABLE 19. Number of epiphytic isolates from fruits antagonistic toward *E. coli* O157:H7 and/or *S.* Saintpaul by bacterial group, season, and commodity

Bacterial group[a]	Season[b]	Antagonistic isolates by commodity												Grand total (%)[d]
		Cantaloupes				Peppers				Tomatoes				
		EC[c]	SS	Bo	*Tot*	EC	SS	Bo	*Tot*	EC	SS	Bo	*Tot*	
MS	Summer	2	-	-	*2*	-	-	-	-	4	-	3	*7*	9 (1.8) BC
	Fall	1	1	1	*3*	-	-	-	-	-	-	1	*1*	4 (0.8) AB
LAB	Summer	-	1	35	*36*	1	-	4	*5*	-	-	7	*7*	48 (9.6) D
	Fall	7	14	226	*247*	-	-	17	*17*	-	1	32	*33*	297 (49.5) F
CL	Summer	-	-	1	*1*	-	-	-	-	1	-	-	*1*	2 (0.4) A
	Fall	7	-	6	*13*	-	-	-	-	-	-	2	*2*	15 (3.0) C
PY	Summer	2	-	8	*10*	11	3	17	*31*	28	13	39	*80*	121 (24.2) E
	Fall	-	-	-	-	-	-	1	*1*	-	-	2	*2*	3 (0.6) AB
Total	Summer	4	1	44	*49*	12	3	21	*36*	33	13	49	*95*	180 (36.1) a[e]
	Fall	15	15	233	*263*	0	0	18	*18*	0	1	37	*38*	319 (63.9) b
	Both seasons	19	16	277	*312*	12	3	39	*54*	33	14	86	*133*	499 (100)

[a] MS: Mesophiles, LAB: Lactic acid bacteria, CL: Coliforms, PY: Psychrotrophs.
[b] Summer season: May-June, Fall Season: October-December
[c] Isolates antagonistic toward *E. coli* O157:H7 (EC), toward *S.* Saintpaul (SS), toward both pathogens (Bo), and total antagonistic isolates (Tot).
[d] Percentage values showing same uppercase letter are not significantly different (P > 0.05).
[e] Percentage values showing same lowercase letter are not significantly different (P > 0.05)
.

TABLE 20. Microbiological content of fruits and antagonistic bacteria isolates by bacterial group, commodity and season

Commodity	Season[a]	Bacterial group[b]							
		MS		LAB		CL		PY	
		Mean ± SD[c]	Isolates[d]	Mean ± SD	Isolates	Mean ± SD	Isolates	Mean ± SD	Isolates
Cantaloupes	Summer	6.1 ± 0.5 A[e] a[f]	2	4.1 ± 0.7 A a	36	4.3 ± 0.7 A a	1	5.2 ± 0.6 A a	10
	Fall	5.5 ± 0.7 B a	3	4.9 ± 0.7 B a	247	5.1 ± 0.7 B a	6	4.3 ± 0.8 B a	0
Tomatoes	Summer	3.3 ± 0.8 A b	7	0.7 ± 0.7 A b	7	1.0 ± 1.0 A b	1	1.6 ± 0.9 A b	80
	Fall	3.6 ± 0.9 A b	1	2.6 ± 1.6 B b	33	1.2 ± 0.9 A b	2	1.6 ± 1.0 A b	2
Peppers	Summer	3.6 ± 1.3 A b	0	0.6 ± 0.6 A b	5	1.1 ± 0.9 A b	0	1.6 ± 0.7 A b	31
	Fall	3.7 ± 0.6 A b	0	2.6 ± 1.5 B b	17	2.1 ± 1.1 B c	0	1.1 ± 0.7 B b	1

[a] Summer: May-June, Fall: October-December

[b] MS: Mesophiles, LAB: lactic acid bacteria, CL: coliforms, PY: Psychrotrophs

[c] Mean and standard deviation (log CFU/cm^2) of bacterial counts averaged from 25 samples collected in two fields per season, n=50

[d] Number of isolates recovered, resulting antagonistic *in vitro* (Inhibition area > 1 mm^2 in spot agar test) toward *E. coli* O157:H7 and/or *S.* Saintpaul

[e] Within column within commodity, values followed by the same uppercase letter are not significantly different ($P > 0.05$)

[f] Within column within season, values followed by the same lowercase letter are not significantly different ($P > 0.05$)

Variation in the antagonistic effect toward one or two enteric pathogens

Although most of the antagonistic bacteria from fruits were inhibitory toward both pathogens (402, out of 499) and about 50% of leafy greens were also antagonistic to both pathogens (190 out of 397), in those cases where the inhibitory action of an isolate was only effective toward one of the pathogens, more frequently, isolates were antagonistic toward *E.coli* O157:H7. For example, 174 isolates from leafy greens were antagonistic toward *E. coli* O157:H7, but *Salmonella* was not inhibited by those isolates. On the other hand, only 33 isolates were antagonistic to *S.* Saintpaul and had no effect toward *E. coli* O157:H7. Similarly, 27 isolates could inhibit *S.* Saintpaul and could not inhibit *E. coli* O157:H7, while 64 isolates from fruits presented antagonistic activity toward *E. coli* O157:H7, and *S.* Saintpaul was not sensitive to this activity. This higher susceptibility to the inhibitory action of native microbiota by *E. coli* O157:H7 when compared to *Salmonella* has been previously described in fresh manure *(244)*. In their study, *E. coli* O157:H7 and *S.* Typhimurium showed similar survival rates when inoculated in sterile manure. However, when these pathogens were separately inoculated in fresh, non-sterile manure, *E. coli* O157:H7 counts declined more rapidly than *S.* Typhimurium. Although some nutrients and the pH slightly varied between raw and sterile manure, their effect was not significant, and it was determined that the decrease of *E. coli* O157:H7 was due to the presence and possible inhibitory action of the native microbiota contained by the non-sterile manure *(244)*.

Biochemical identification of antagonistic epiphytic bacteria

In total, from the 896 isolates classified as antagonistic toward one or two pathogens, 519 isolates were randomly selected and processed for their biochemical identification. The VITEK system did not identify 16.8% (87) of the isolates. Furthermore, 13 selected isolates did not proliferate on propagation media for further studies; thus, their identification was not possible. In total, 419 isolates were identified, 24 of them by genera, and 395 by genera and species. The taxonomical classification of the antagonistic species was identified. Their commodity source, the total number of antagonistic isolates from each species, and their averaged *in vitro* IA inhibited by pathogen are shown in Table 21.

Overall, *A. faecalis* sbsp. *faecalis* was the most frequently identified antagonistic bacteria, recognized on 78 occasions. The second largest proportion of identified isolates were *L. mesenteroides dextranicum*, with 33 isolates identified, and *L. pseudomesenteroides* with 30 isolates identified, both in cantaloupes isolated from cantaloupe rinds. Also numerous bacteria were isolates of *Enterococcus gallinarum, E. casseliflavus, E. faecalis, S. epidermidis* and *Bacillus licheniformis*, which led to the identification of more than 10 isolates at the very least.

TABLE 21. Taxonomical identification and isolation frequency of antagonistic epiphytic isolates toward *E. coli* O157:H7 and/or *S.* Saintpaul isolated from different leafy greens and fruits

Taxonomical classification	Number of isolates by source [a]							Number of isolates by pathogen inhibited [b]			
	Leafy greens			Fruits			Total[c]		IA Mean ±		IA Mean ±
	SP	EN	PA	CA	TO	PE		EC[d]	SD[e]	SS[f]	SD[g]
Gram positive bacteria											
Phylum: Actinobacteria											
Class: Actinobacteridae											
Order: Actinomycetales											
Family: *Intrasporangiaceae*											
Species: *Kytococcus sedentarius*	-	-	-	-	5	-	5 GHIJ	4	47.8 ± 3.9	3	48.1 ± 18.5
Family:*Micrococcaceae*											
Species: *Kocuria kristinae*	2	-	-	1	8	-	11 EFGH	11	79.4 ± 91.0	10	43.3 ± 91.0
Family:*Micrococcaceae*											
Species: *Kocuria rosea*	-	-	-	1	-	-	1 J	1	4.6	1	6.7
Phylum: Firmicutes											
Class: Bacilli											
Order: Bacillales											
Family: *Bacillaceae*											
Species: *Bacillus* ssp.	-	-	10	-	1		11 EFGH	9	50.1 ± 15.8	10	48.3 ± 20.0
Bacillus licheniformis	-	1	-	-	2		3 IJ	3	64.7 ± 26.5	1	51.1
Bacillus mycoides	-	-	1	-	1		2 IJ	1	114.3	2	104.8 ± 2.7
Bacillus polymyxa	-	-	-	-	1		1 J	1	151.1	1	101.6
Bacillus pumilus	-	1	-	-	1		2 IJ	2	83 ± 0.6	1	42.0
Bacillus vallismortis	-	-	-	1	-	-	1 J	1	42.2	0	
Family: *Listeriaceae*											
Species: *Listeria grayi*	-	1	-	-	-	-	1 J	1	167.1	1	210.2
Family: Not assigned											
Species: *Gemella bergeri*	-	1	-	-	-	-	1 J	1	35.8	1	44.9
Gemella morbillorum	-	-	1	-	-	-	1 J	1	4.6	1	4.9
Order: Lactobacillales											
Family: *Aerococcaceae*											
Species: *Aerococcus viridans*	3	1	1	6	-	-	11 EFGH	11	59.6 ± 28.0	11	69.1 ± 29.4
Family: *Enterococcaceae*											

TABLE 21. Continued.

Taxonomical classification	Number of isolates by source [a]							Number of isolates by pathogen inhibited [b]			
	Leafy greens			Fruits			Total[c]		IA Mean ±		IA Mean ±
	SP	EN	PA	CA	TO	PE		EC[d]	SD[e]	SS[f]	SD[g]
Species: *Enterococcus ssp.*	-	-	-	1	-	-	1 J	1	71.6	1	54.2
Enterococcus casseliflavus	5	-	6	9	1	-	21BCDE	21	77.6 ± 20.6	17	59.5 ± 42.9
Enterococcus cecorum	1	-	-	1	-	-	2 IJ	2	73.7 ± 6.6	2	118.0 ± 39.0
Enterococcus faecalis	-	-	-	16	-	1	17 CDEF	17	120.4 ± 71.5	17	120.4 ± 46.6
Enterococcus faecium	-	-	-	4	-	-	4 HJI	4	123.8 ± 73.5	4	142.1 ± 30.5
Enterococcus gallinarum	-	8	3	12	1	-	24 BCD	24	72.8 ± 35.9	23	79.2 ± 30.6
Vagococcus fluvialis	-	1	-	-	-	-	1 J	1	68.0	1	143.6
Family: *Lactobacillaceae*											
Species: *Lactobacillus plantarum*	-	3	1	4	-	-	8 FGHI	7	54.5 ± 37.5	8	64.0 ± 45.7
Lactobacillus rhamnosus	-	1	-	-	-	-	1 J	1	13.4	1	9.3
Pediococcus pentosaceus	-	4	-	2	-	-	6 GHIJ	6	104.6 ± 61.8	6	118.4 ± 66.9
Family: *Leuconostocaceae*											
Species: *Leuconostoc citreum*	-	-	-	8	-	-	8 FGHI	8	181.1 ± 62.3	8	198.0 ± 80.0
Leuconostoc mesenteroides sbsp. *cremoris*	-	-	-	1	-	-	1 J	1	132.3	1	89.1
Leuconostoc mesenteroides sbsp. *dextranicum*	-	1	-	32	-	-	33 B	33	109.0 ± 73.2	33	93.6 ± 55.3
Leuconostoc mesenteroides sbsp. *mesenteroides*	-	-	-	1	-	-	1 J	1	12.8	0	
Leuconostoc pseudomesenteroides	-	-	-	30	-	-	30 BC	28	68.4 ± 34.6	28	55.1 ± 36.4
Family: *Staphylococcaceae*											
Species: *Staphylococcus cohnii* sbsp. *urealyticus*	-	-	-	1	-	-	1 J	1	36.7	1	67.9
Staphylococcus epidermidis	-	-	-	2	6	8	16 DEF	16	90.2 ± 53.6	16	82.4 ± 52.2

TABLE 21. Continued.

Taxonomical classification	Number of isolates by source [a]							Number of isolates by pathogen inhibited [b]			
	Leafy greens			Fruits			Total[c]		IA Mean ±		IA Mean ±
	SP	EN	PA	CA	TO	PE		EC[d]	SD[e]	SS[f]	SD[g]
Staphylococcus gallinarum	-	-	4	8	-	-	12 DEFG	11	33.7 ± 20.3	12	40.9 ± 38.8
Staphylococcus hominis sbsp. *hominis*	-	-	-	-	1	1	2 IJ	2	78.3 ± 59.6	2	129.1 ± 19.3
Staphylococcus hominis sbsp. *novobiosepticus*	-	-	-	-	1	-	1 J	1	20.1	1	23.6
Staphylococcus intermedius	-	-	3	-	-	-	3 IJ	3	75.1 ± 53.2	3	37.6 ± 17.2
Staphylococcus lentus	-	2	2	4	1	-	9 FGHI	7	48.2 ± 40.5	7	40.1 ± 24.3
Staphylococcus lugdunensis	-	-	-	-	-	2	2 IJ	2	116.7 ± 34.3	2	86.0 ± 16.9
Staphylococcus saprophyticus	-	-	-	-	1	-	1 J	1	8.7	1	12.2
Staphylococcus sciuri	-	1	-	2	-	-	3 IJ	2	65.6 ± 14.5	2	40.6 ± 37.0
Staphylococcus vitulinus	-	-	-	2	-	-	2 IJ	1	112.2	2	79.6 ± 50.1
Staphylococcus warneri	-	-	-	2	-	2	4 HJI	4	45.4 27.3	4	40.8 16.4
Staphylococcus xylosus	-	-	-	1	-	-	1 J	1	15.0	1	19.5
Family: *Streptococcaceae*											
Species: *Streptococcus* ssp.	-	-	-	1	-	-	1 J	1	91.1	1	112.0
Streptococcus alactolyticus	-	9	-	-	-	-	9 FGHI	9	134.0 ± 148.8	9	128.7 ± 122.7
Streptococcus equinus	-	3	-	-	-	-	3 IJ	3	156.4 19.3	3	229.6 103.4
Streptococcus mutans	-	3	-	-	-	-	3 IJ	3	94.2 ± 27.8	3	148.2 ± 77.2
Streptococcus sanguini	-	3	-	-	-	-	3 IJ	3	14.6 ± 8.4	3	21.7 ± 10.5
Streptococcus thoraltensis	-	-	-	5	-	-	5 GHIJ	5	80.4 ± 54.4	5	104.1 ± 61.2
Lactococcus garvieae	-	-	-	4	-	-	4 HJI	4	81.5 ± 17.1	4	121.5 ± 53.8
Lactococcus lactis	-	-	-	3	2	-	5 GHIJ	5	65.2 ± 34.3	5	59.2 ± 21.6
Lactococcus pentosaceus	-	-	-	1	-	-	1 J	1	54.9	1	53.1

TABLE 21. Continued.

Taxonomical classification	Number of isolates by source [a]							Number of isolates by pathogen inhibited [b]			
	Leafy greens			Fruits			Total[c]	EC[d]	IA Mean ± SD[e]	SS[f]	IA Mean ± SD[g]
	SP	EN	PA	CA	TO	PE					
Gram negative bacteria											
Phylum: Bacteroidetes											
Class: Flavobacteria											
Order: Flavobacteriales											
Family: *Flavobacterium odotatum*											
Species: *Myroides* ssp.	-	1	5	-	-	-	6 GHIJ	5	35.7 ± 12.0	5	66.8 ± 60.8
Phylum: Proteobacteria											
Class: Alphaproteobacteria											
Order: Sphingomonadales											
Family: *Sphingomonadaceae*											
Species: *Sphingomonas paucimobilis*	-	-	1	-	-	-	1 J	1	169.8	1	103.6
Class: Betaproteobacteria											
Order: Burkholderiales											
Family: *Alcaligenaceae*											
Species: *Alcaligenes faecalis* sbsp. *faecalis*	16	15	36	-	6	5	78 A	73	41.8 ± 19.3	28	45.2 ± 27.5
Family: *Burkholderiaceae*											
Species: *Cupriavidus pauculus*	2	-	-	-	-	-	2 IJ	2	35.5 ± 1.3	0	
Class: Gammaproteobacteria											
Order: Enterobacteriales											
Family: *Enterobacteriaceae*											
Species: *Citrobacter sedlakii*	-	-	-	3	-	-	3 IJ	3	9.6 ± 3.2	3	11.5 ± 1.3
Cronobacter ssp.	-	-	-	-	-	1	1 J	1	19.8	1	29.0
Enterobacter aerogenes	-	-	-	1	-	-	1 J	1	212.1	1	119.4
Enterobacter cloacae sbsp. *dissolvens*	1	-	-	1	-	-	2 IJ	1	57.7	2	27.8 ± 20
Enterobacter hormaechei	-	-	-	4	-	-	4 HIJ	3	120.3 ± 55.8	3	86.7 ± 35.9
Enterobacter kobei	-	-	-	2	-	-	2 IJ	2	106 ± 19.0	2	132.1 ± 8.7
Enterobacter ludwigii	-	-	-	5	-	-	5 GHIJ	5	83.9 44.4	5	72.7 ± 44.4

TABLE 21. Continued.

Taxonomical classification	Number of isolates by source [a]							Number of isolates by pathogen inhibited [b]			
	Leafy greens			Fruits			Total[c]		IA Mean ±		IA Mean ±
	SP	EN	PA	CA	TO	PE		EC[d]	SD[e]	SS[f]	SD[g]
Klebsiella pneumoniae sbsp. *pneumoniae*	-	-	-	6	-	-	6 GHIJ	5	17.5 ± 2.7	2	26.1 ± 21
Pantoea ssp.	-	1	-	-	3	-	4 HIJ	3	31.9 ± 10.4	4	44.2 ± 21.1
Providencia rettgeri	-	-	1	-	-	-	1 J	1	181.5	1	177.1
Serratia plymuthica	-	-	1	-	-	-	1 J	1	73.0	0	
Order: Pseudomonadales											
Family: *Pseudomonadaceae*											
Species: *Pseudomonas stutzeri*	-	1	-	-	-	-	1 J	1	28.7	0	
Pseudomonas pseudoalcaligenes	-	1	-	-	-	-	1 J	1	23.7	0	

[a] Leafy greens include isolates from spinach (SP), endives (EN), and parsley (PA). Fruits include isolates from cantaloupes (CA), tomatoes (TO), and peppers (PE).

[b] Bacterial antagonistic isolates with inhibition area (IA) > 1.0 mm^2 against *E. coli* O157 and/or *S.* Saintpaul during *in vitro* test

[c] Total isolates by each species identified. Numbers followed by the same letter are not proportionately different (P > 0.05)

[d] Number of isolates antagonistic toward *E. coli* O157:H7 *in vitro*

[e] Mean ± Standard deviation of inhibition area (IA, mm^2) averaged for all positive results by isolate species towards *E. coli* O157:H7

[f] Number of isolates antagonistic toward *S.* Saintpaul *in vitro*

[g] Mean ± Standard deviation of inhibition area (IA, mm^2) averaged for all positive results by isolate species towards *S.* Saintpaul

Other studies have analyzed the occurrence of different bacterial species in produce surfaces. Al Kharousi et al. *(11)* reported the occurrence of different bacterial genera in 105 samples of fruits including watermelons, mangoes, bananas, dates, papayas, pomegranates, and tomatoes; and vegetables including cabbage, carrots, peppers, cucumbers, lettuce, and radishes. In their study they found *Enterobacteriaceae* species in 60% of the fruits and 91% of the vegetables. Species of the genera *Enterococcus* were isolated in 20% and 42% of the fruit and vegetables, and they also identified several isolates of *Klebsiella pneumoniae, E. casseliflavus,* and *E. cloacae*. Some of the species identified were also found in the present study and include *E. cloacae, E. hormaechei, E. ludwigii E. casseliflavus, E. faecium, E. faecalis, E. raffinose, K. pneumoniae,* and *Pantoea agglomerans.* Interestingly in this study, they paired the identification of isolates using VITEK-2 to PCR and found some discrepancies. For example, isolates not identified by VITEK-2 were found as isolates of *E. mundii, E. sulfureus,* and *E. gilvus*. Furthermore, samples identified as *K. pneumoniae,* were found as *K. oxytoca* in PCR, and *Pantoea* ssp. in VITEK resulting in *Erwinia aphidicola* on PCR. These discrepancies can also be present in this study, although they cannot be recognized until further studies are completed.

Scientific evidence of antagonistic activity by identified bacteria

Gram positive antagonistic bacteria

The species *Kytococcus sedentarius* is known for the production of oligoketide antibiotics monensin a and b, and has been reported as an opportunistic pathogen causing valve endocarditis, hemorrhagic pneumonia, and pitted keratolysis *(227, 254).*

The species *Kocuria kristinae* and *Kocuria rosea* have been previously identified as an antagonistic bacteria capable of restricting the growth of plant root nematodes *(190, 261)*. *Kocuria kristinae* has also been studied and recognized as antagonistic toward pathogenic bacteria from the cow reproductive system, including *Arcanobacterium pyogenes, Fusobacterium necrophorum, Streptococcus equi,* and *Gardnerella vaginalis*. *Kocuria kristinae* antagonistic action has been related to its ability to form different organic acids including acetic, acetoacetic, acetic, propionic, formic, and succinic acid *(262)*.

In the case of *Bacillus* spp., their antagonistic activity has been attributed to the production of antibiotic substances. Several species have been studied for their biocontrol activity toward plant pathogens *Bacillus* spp has also been documented as able to compete with pathogens for nutrients and space and can induce the plant defense response *(257)*.

Multiple species from the Lactobacillales order have been described as antagonistic towards human pathogens. The antibacterial effect of this family has been attributed to their organic acid production and consecutive pH drop, and competition for nutrients. They also have been related to the production of antibacterial proteins known as bacteriocins *(12, 26, 158, 167, 222, 300)*. The antagonistic activity of *Leuconostoc mesenteroides* has been recognized in the past. Several subspecies are able to produce bacteriocins known as mesenterocins. These proteins are synthetized during the exponential growth phase of the bacteria and can inhibit a broad variety of microorganisms, including *L. monocytogenes* and *E. coli* O157:H7 (*300)*. Species of *Enterococcus* have also been studied due to their antagonistic effect in previous

investigations. In a study published by Line et al. *(167)*, *E. durans*, *E. faecium*, and *E. hirae* demonstrated the production of bacteriocins known as enterocins, and were able to antagonize multiple pathogens including *Campylobacter jejuni, S. enterica, E. coli* O157:H7, *Yersinia enterocolitica, Citrobacter freundii, Klebsiella pneumoniae, Shigella dysenteriae, Pseudomonas aeruginosa, Proteus mirabilis, Morganella morganii, Staphylococcus aureus, Staphylococcus epidermidis*, and *Listeria monocytogenes*.

The antagonistic effect and potential use as biocontrol agents of nonpathogenic *Staphylococcus* species have been previously identified. Their antagonistic activity towards other bacteria has been related to their ability to produce siderophores, known as staphyloferrins, which chelate the iron required by other bacteria for their proliferation *(232)*.

Gram negative antagonistic bacteria

In a previous study, one isolate of *Myroides odoratimimus*, demonstrated antagonistic activity toward a soil borne pathogen of tobacco plants; however, the mechanism of action was not determined *(143)*. This species is particularly resistant to antimicrobials, although the causative agents are unknown. Dharne et al. *(101)* demonstrated the antagonistic activity of *Mycoides* spp. toward *Aeromonas hydrophila, A. culicicola, Morganella morganii* subsp. *sibonii, Ochrobactrum anthropi, Escherichia coli, Ochrobactrum* spp., *Serratia* spp., *Kestersia* spp., I*gnatzschineria* spp., and *Bacillus* spp.

The psychrotrophs *Sphingomonas* spp. were previously studied to determine their antagonistic activity toward *S. aureus, B. subtilis, E. faecium*, *Citricoccus* sp. and *Candida*

albicans (238). They are believed to produce argimicin and other antimicrobial compounds including the terpene astaxanthin *(177).*

The importance of *Alcaligenes faecalis* as an antagonistic toward other microorganisms has been previously reported. Strains of this species have been identified as antagonistic toward plant fungi, plant pathogens, human pathogens, and insects *(230, 305).* Its antagonistic activity has been attributed to different compounds produced including ammonia, and hydroxylamine *(304).* Xu et at.*(301)* studied six bioactive compounds produced by one strain of *A. faecalis* and indicated that at least three of six bioactive compounds produced by this strain inhibited *E. coli* and *S. aureus* proliferation in agar, similar to the results found in the present study.

Effect of antagonistic epiphytic bacteria over the growth of *E. coli* O157:H7 on leafy greens and of *S.* Saintpaul on fruits

Leafy greens

From the leafy greens isolates, 17 from endives, nine from parsley, and five isolates from spinach were used to determine their inhibitory effect against *E. coli* O157:H7 on their respective commodities of origin. The isolates selected for this test corresponded to one isolate of the different species identified by commodity, which obtained the largest IA from their species during the antagonistic *in vitro* test toward *E. coli* O157:H7.

Spinach

Growth curves were constructed using the averaged counts of *E. coli* O157:H7 on spinach leaf circles, in the presence of each selected antagonistic isolate (Fig. 8). The

growth parameters calculated from the *E. coli* O157:H7 by treatment are shown in Table 22.

The growth rate, doubling time, and overall growth of *E. coli* O157:H7 on the spinach leaf surface were 0.021 ± 0.017 h^{-1}, 333 ± 160 min, and 1.0 ± 0.6 log CFU/cm^2, with the initial counts at 0 h of 4.0 log CFU/10 cm^2.

When *E. coli* O157:H7 was inoculated on spinach containing the epiphytic isolates, no growth inhibition effect was observed. On the contrary, on samples treated with *Aerococcus viridans* and *Enterococcus cecorum*, the pathogen presented a higher overall growth of 2.9 ± 0.4 log and 2.5 ± 0.5, and CFU/10cm^2, respectively ($P < 0.05$).

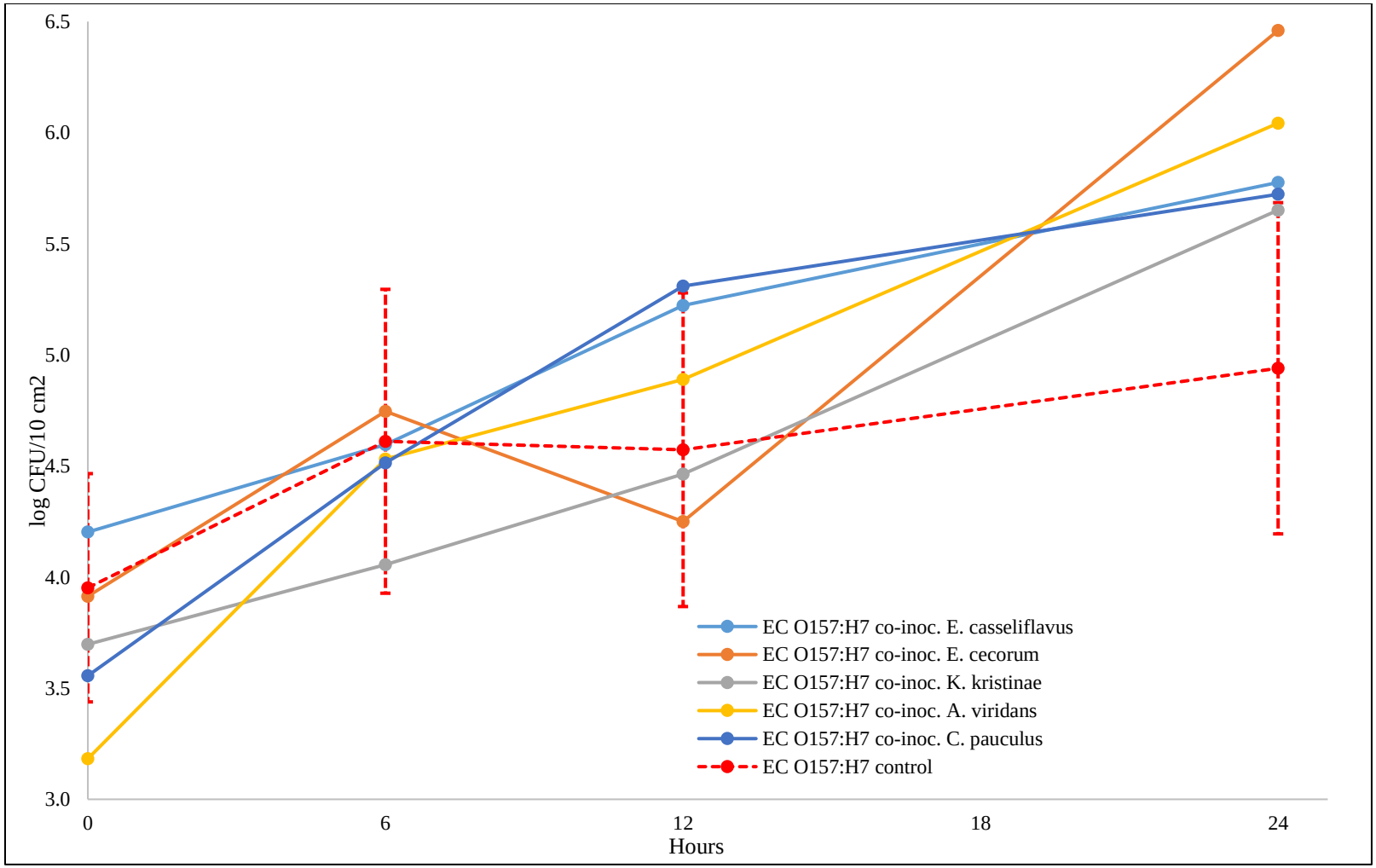

FIGURE 8. Growth curves of *E. coli* O157:H7 in the presence of antagonistic bacteria in spinach leaf samples

TABLE 22. Growth parameters of *E. coli* O57:H7 on spinach inoculated with antagonistic epiphytic bacteria

ID code	Treatment[a]	Mean ± SD[b]		
		μ_{max}[c]	d-t[d]	Overall growth[e]
Control	*EC* + sterile PBS	0.021 ± 0.017	333 ± 160	1.0 ± 0.6
Sp 1637	*EC* + *Aerococcus viridans*	0.025 ± 0.010	161 ± 56	2.9 ± 0.4 *
Sp 1650	*EC* + *Cupriavidus pauculus*	0.020 ± 0.02	185 ± 36	2.2 ± 0.8
Sp 0358	*EC* + *Enterococcus casseliflavus*	0.020 ± 0.008	209 ± 68	1.6 ± 0.5
Sp 1552	*EC* + *Enterococcus cecorum*	0.026 ± 0.010	147 ± 63	2.5 ± 0.5 *
Sp 1610	*EC* + *Kocuria kristinae*	0.030 ± 0.018	165 ± 60	2.0 ± 0.7

[a] *E. coli* O157:H7 (*EC*) inoculation in previously inoculated (2 h before) leaf circles (10 cm^2) with epiphytic isolate.
[b] Mean ± Standard deviation of three replicates by treatment, n = 3.
[c] $\mu_{max:}$ Growth rate (in h^{-1}).
[d] d-t: Doubling time (or generation time, in min).
[e] Overall growth, in log CFU/10 cm^2 = Growth at t_{max} (24 h) – Growth at t_0 (0 h).
[f] * Indicates significant difference ($P < 0.05$) with respect to control.

Endives

In the case of endives, the growth curves presented by *E. coli* O157:H7 in presence of antagonistic epiphytic bacteria and control are shown in Figure 9. The growth parameters calculated from the *E. coli* O157:H7 by treatment (antagonistic isolate) are shown in Table 23.

The growth rate, doubling time, and overall growth of *E. coli* O157:H7 on the endive without any treatment were 0.025 ± 0.009 h^{-1}, 184 ± 66 min, and 1.6 ± 0.2 log CFU/10 cm^2, being the initial counts (at 0 h) of 3.8 ± 0.5 log CFU/10 cm^2. The results of the treated samples were not different from the control ($P > 0.05$) except for one treatment and one parameter. In the samples inoculated with *Aerococcus viridans*, the overall growth of *E. coli* O157:H7 was 0.9 log CFU/10 cm^2 greater than the control ($P < 0.05$).

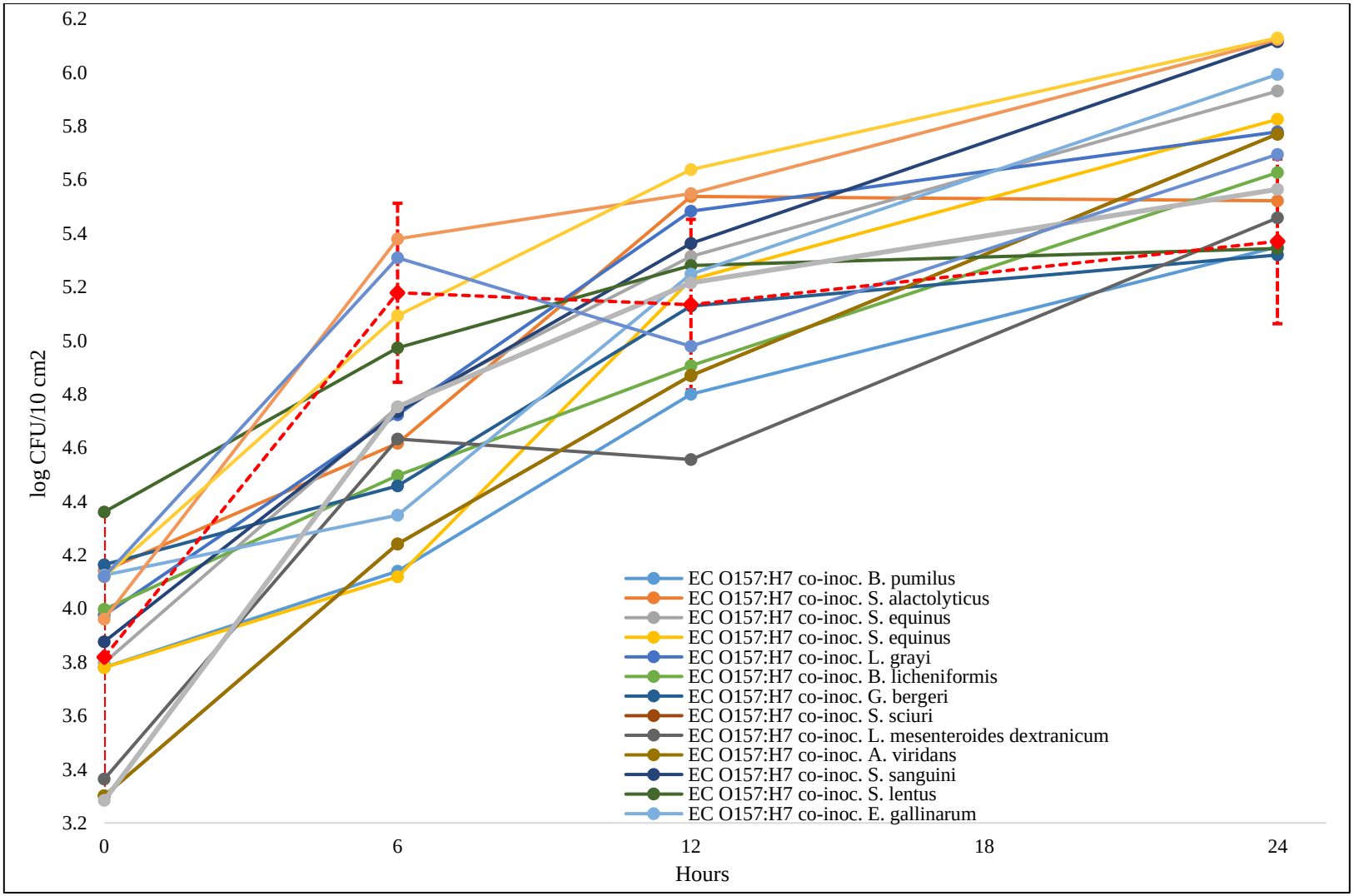

FIGURE 9. Growth curves of *E. coli* O157:H7 in the presence of antagonistic bacteria in endive leaf samples

TABLE 23. Growth parameters of *E. coli* O57:H7 on endives inoculated with antagonistic epiphytic bacteria

ID code	Treatment[a]	Mean ± SD[b]		
		μ_{max}	d-t[d]	Overall growth[e]
Control	*EC* + sterile PBS	0.025 ± 0.009	184 ± 66	1.6 ± 0.2
Le 3552	*EC* + *Aerococcus viridans*	0.034 ± 0.005	139 ± 10	2.5 ± 0.2 *
Le 3251	*EC* + *Bacillus licheniformis*	0.018 ± 0.013	334 ± 161	1.6 ± 1.2
Le 2900	*EC* + *Bacillus pumilus*	0.021 ± 0.008	229 ± 57	1.6 ± 0.3
Le 3756	*EC* + *Enterococcus gallinarum*	0.022 ± 0.014	263 ± 133	1.9 ± 0.7
Le 3277	*EC* + *Gemella bergeri*	0.017 ± 0.009	290 ± 120	1.2 ± 0.5
Le 3953	*EC* + *Lactobacillus plantarum*	0.027 ± 0.008	150 ± 31	2.0 ± 0.5
Le 3915	*EC* + *Lactobacillus rhamnosus*	0.039 ± 0.008	121 ± 35	2.3 ± 0.6
Le 3554	*EC* + *Leuconostoc mesenteroides* sbsp. *dextranicum*	0.028 ± 0.015	216 ± 75	2.1 ± 0.5
Le 3200	*EC* + *Listeria grayi*	0.028 ± 0.006	147 ± 22	1.8 ± 0.6
Le 3622	*EC* + *Staphylococcus lentus*	0.016 ± 0.009	315 ± 133	1.0 ± 0.7
Le 3302	*EC* + *Staphylococcus sciuri*	0.021 ± 0.015	314 ± 163	1.2 ± 0.2
Le 3075	*EC* + *Streptococcus alactolyticus*	0.025 ± 0.008	166 ± 45	1.4 ± 0.2
Le 3152	*EC* + *Streptococcus equinus*	0.030 ± 0.012	150 ± 30	2.1 ± 0.3
Le 3154	*EC* + *Streptococcus equinus*	0.028 ± 0.006	151 ± 14	2.0 ± 0.5
Le 3955	*EC* + *Streptococcus mutans*	0.016 ± 0.008	352 ± 225	1.6 ± 0.5
Le 3597	*EC* + *Streptococcus sanguini*	0.027 ± 0.017	224 ± 139	2.2 ± 0.6
Le 3874	*EC* + *Vagococcus fluvialis*	0.029 ± 0.012	165 ± 78	2.2 ± 0.6

[a] *E. coli* O157:H7 (*EC*) inoculation in previously inoculated (2 h before) leaf circles (10 cm^2) with epiphytic isolate

[b] Mean ± Standard deviation of three replicates by treatment, n = 3.

[c] $\mu_{max:}$ Growth rate (in h^{-1}).

[d] d-t: Doubling time (or generation time, in min).

[e] Overall growth, in log CFU/10 cm^2 = Growth at t_{max} (24 h) – Growth at t_0 (0 h).

[f] * Indicates significant difference ($P < 0.05$) with respect to control.

Parsley

For parsley samples, the growth curves presented by *E. coli* O157:H7 in presence of antagonistic epiphytic bacteria, and control are shown in Figure 10. The growth parameters calculated from the *E. coli* O157:H7 by treatment (antagonistic isolate) are shown in Table *24*.

The mean growth rate, doubling time, and overall growth of *E. coli* O157:H7 on the control samples of parsley were 0.033 ± 0.004 h^{-1}, 134 ± 14 min, and 1.6 ± 0.2 log CFU/leaflet. The initial count at 0 h was 3.4 ± 0.6 log CFU/leaflet. Four isolates affected the growth behavior of *E. coli* O157:H7 on parsley leaflets. The *E. coli* O57:H7 from samples inoculated with *B. mycoides*, *E. gallinarum*, and *G. morbillorum* presented a slower growth rate than the control ($P < 0.05$) of 0.008 ± 0.002, 0.014 ± 0.008, and 0.014 ± 0.007 h^{-1}, respectively. Moreover, *E. coli* O157:H7 inoculated with *Bacillus mycoides* presented an extended doubling time of 439 ± 97 min, which is greater than the control ($P < 0.05$). On the contrary, in the samples inoculated with *Aeromonas viridans*, *E. coli* O157:H7 presented a significantly larger overall growth of 2.5 log CFU/10 cm^2, which was 1.1 log CFU/10 cm^2 larger than the control ($P < 0.05$).

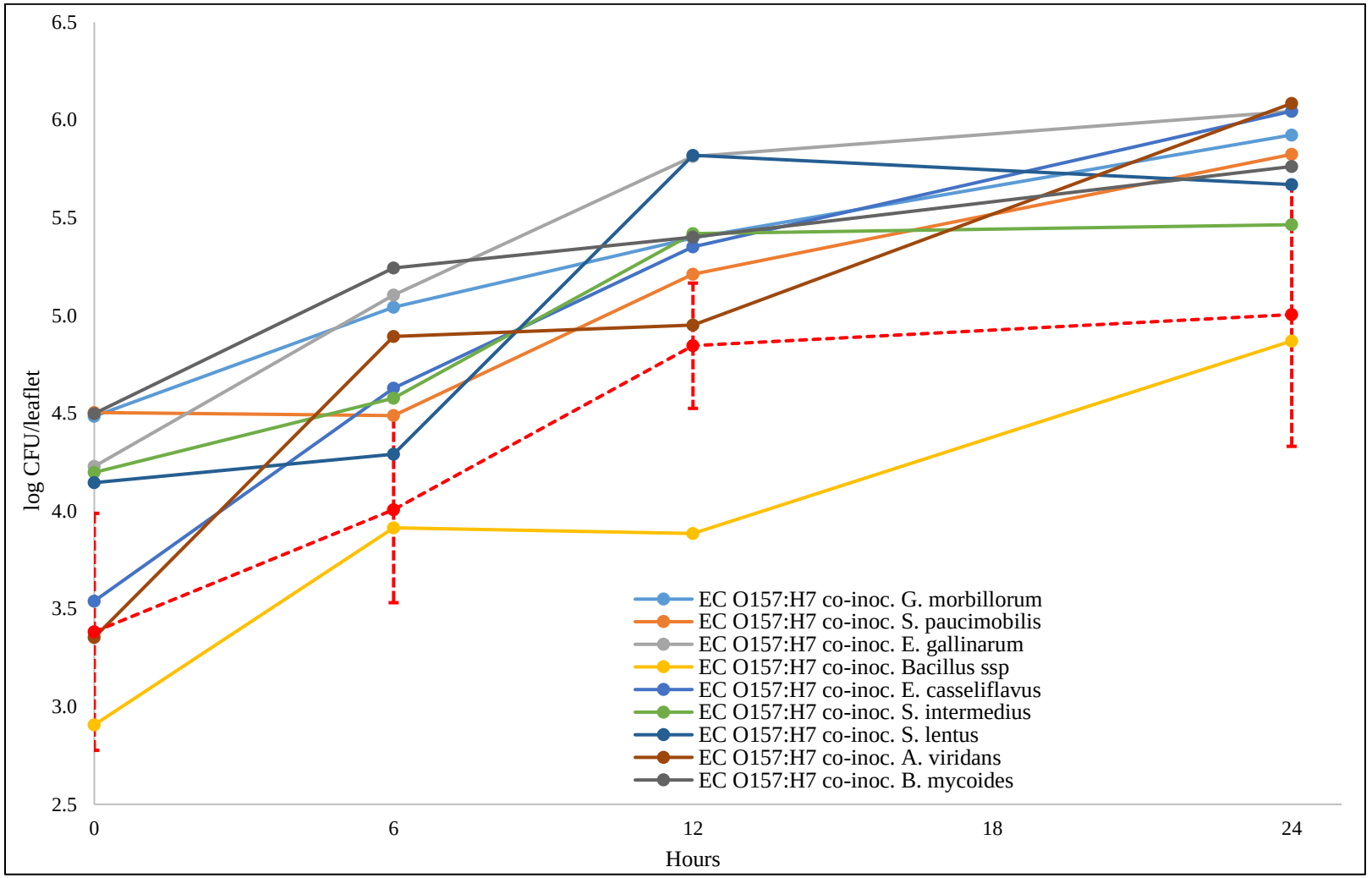

FIGURE 10. Growth curves of *E. coli* O157:H7 in the presence of antagonistic bacteria in parsley leaves

TABLE 24. Growth parameters of *E. coli* O57:H7 on parsley inoculated with antagonistic epiphytic bacteria.

ID code	Treatment[a]	Mean ± SD[b]		
		μ_{max}[c]	d-t[d]	Overall growth[e]
Control	*EC* + sterile PBS	0.033 ± 0.004	134 ± 14	1.6 ± 0.2
Pa 5438	*EC* + *Aerococcus viridans*	0.019 ± 0.010	262 ± 167	2.7 ± 0.5*
Pa 5897	*EC* + *Bacillus mycoides*	0.008 ± 0.002*	439 ± 97*	1.3 ± 0.2
Pa 4145	*EC* + *Bacillus* ssp.	0.019 ± 0.009	308 ± 188	2.0 ± 0.6
Pa 4197	*EC* + *Enterococcus casseliflavus*	0.023 ± 0.006	162 ± 40	2.5 ± 0.7
Pa 4111	*EC* + *Enterococcus gallinarum*	0.014 ± 0.008*	450 ± 395	1.8 ± 0.7
Pa 4075	*EC* + *Gemella morbillorum*	0.014 ± 0.007*	317 ± 161	1.4 ± 0.2
Pa 4094	*EC* + *Sphingomonas paucimobilis*	0.022 ±0.008	178 ± 56	1.3 ± 0.4
Pa 4876	*EC* + *Staphylococcus intermedius*	0.017 ± 0.015	390 ± 192	1.3 ± 0.1
Pa 5432	*EC* + *Staphylococcus lentus*	0.025 ± 0.016	224 ± 112	1.5 ± 0.2

[a] *E. coli* O157:H7 (*EC*) inoculation in previously inoculated (2h before) leaflets with epiphytic isolate.
[b] Mean ± Standard deviation of three replicates by treatment, n = 3.
[c] μ_{max}: Growth rate (in h^{-1}).
[d] d-t: Doubling time (or generation time, in min).
[e] Overall growth, in log CFU/leaflet = Growth at t_{max} (24 h) – Growth at t_0 (0 h).
* Indicates significant difference ($P < 0.05$) with respect to control.

Fruits

In total, 24 antagonistic isolates from cantaloupe, six from peppers, and eight isolates from tomatoes were studied to determine their inhibitory effect toward *S.* Saintpaul on the surface of corresponding fruits skin/rind. The isolates selected for this test corresponded to one isolate of each different species identified, which obtained the largest IA from their species during the antagonistic *in vitro* test toward *S.* Saintpaul.

Cantaloupes

The growth curves presented by *E. coli* O157:H7 on cantaloupe rind, pre-inoculated with different antagonistic epiphytic bacteria are shown in Figure 11 and 12. The growth parameters calculated from *S.* Saintpaul by treatment (antagonistic isolate) are shown in Table 25.

The mean growth rate, doubling time, and overall growth of *S.* Saintpaul in the control samples were 0.17 ± 0.07 h^{-1}, 73 ± 22 min, and 3.9 ± 0.7 log CFU/10 cm^2.

The initial count at 0 h, was 2.7 ± 0.4 log CFU/10 cm^2. From the 24 isolates tested, only *Enterococcus kobei*, and *Enterococcus casseliflavus* had a significant effect over the growth of *S.* Saintpaul. The overall growth of *S.* Saintpaul was significantly lesser than the control, with values of 2.2 ± 0.2, and 2.0 ± 0.3 log CFU/10 cm^2, respectively.

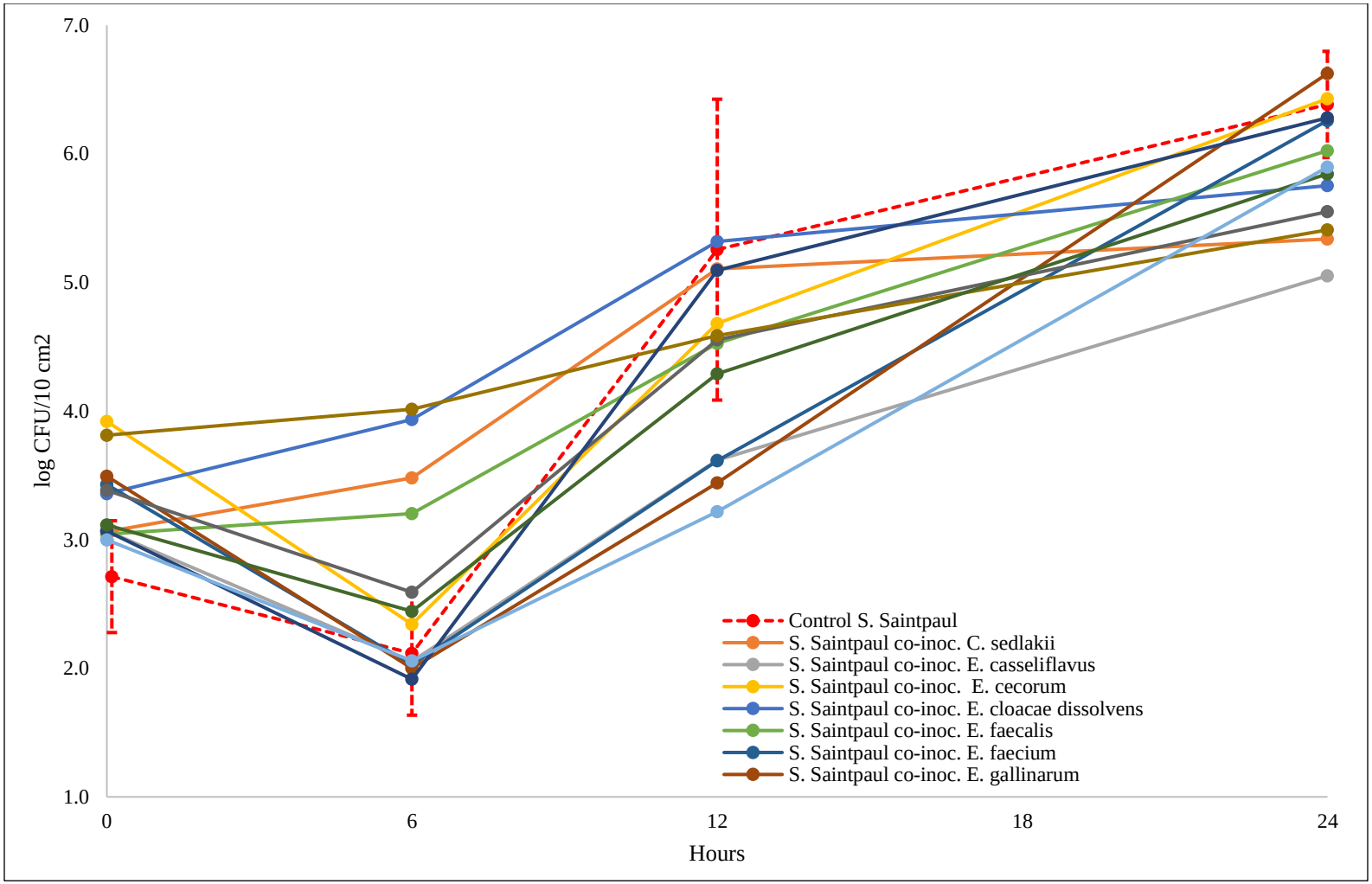

FIGURE 11. Growth curves of *S.* Saintpaul in the presence of antagonistic bacteria in cantaloupe rind (Part a)

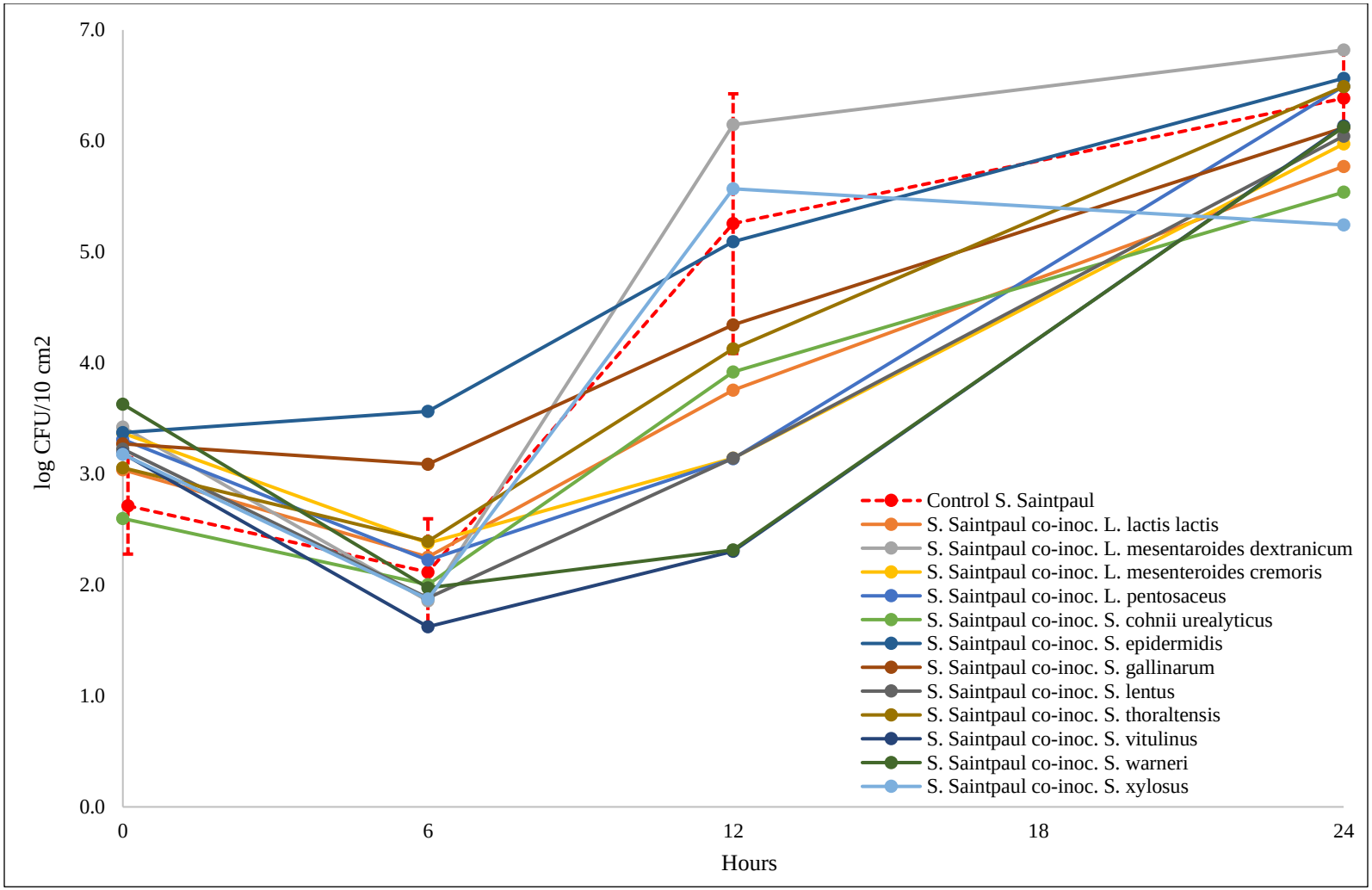

FIGURE 12. Growth curves of *S.* Saintpaul in the presence of antagonistic bacteria in cantaloupe rind (Part b)

TABLE 25. Growth parameters of *S.* Saintpaul on cantaloupe rind inoculated with antagonistic epiphytic bacteria

ID code	Treatment[a]	Mean ± SD[b]		
		μ_{max}[c]	d-t[d]	Overall growth[e]
Control	*S*.S. + sterile PBS	0.17 ± 0.07	73 ±22	3.9 ± 0.7
Ca 13598	*S*.S. + *Citrobacter sedlakii*	0.065 ± 0.044	219 ± 236	2.3 ± 0.7
Ca 13172	*S*.S. + *Enterobacter cloacae* sbsp. *cloacae*	0.050 ± 0.027	155 ± 139	2.4 ± 1.8
Ca 12194	*S*.S. + *Enterobacter kobei*	0.111 ± 0.043	58 ± 14	2.2 ± 0.2 *
Ca 13510	*S*.S. + *Enterobacter lugdwigii*	0.015 ± 0.084	21 ± 49	1.6 ± 1.5
Ca 13119	*S*.S. + *Klebsiella pneumoniae sbsp. pneumoniae*	0.089 ± 0.040	119 ± 108	2.7 ± 1.2
Ca 12115	*S*.S. + *Enterococcus casseliflavus*	0.107 ± 0.113	227 ± 193	2.0 ± 0.3 *
Ca 13271	*S*.S. + *Enterococcus cecorum*	0.115 ± 0.158	29 ± 7	2.5 ± 1.1
Ca 13713	*S*.S. + *Enterococcus faecalis*	0.064 ± 0.040	192 ± 189	3.0 ± 0.9
Ca 10191	*S*.S. + *Enterococcus faecium*	0.110 ± 0.043	74 ± 22	2.8 ± 0.6
Ca 10352	*S*.S. + *Enterococcus gallinarum*	0.098 ± 0.079	49 ± 1	3.1 ± 0.2
Ca 10473	*S*.S. + *Kocuria kristinae*	0.186 ± 0.054	35 ± 6	3.2 ± 0.5
Ca 10199	*S*.S. + *Lactococcus garviae*	0.080 ± 0.043	158 ± 126	2.9 ± 0.2
Ca 12871	*S*.S. + *Lactococcus lactis* sbsp. *lactis*	0.135 ± 0.116	168 ± 143	2.7 ± 0.5
13957	*S*.S. + *Leuconostoc mesenteroides* sbsp. *dextranicum*	0.231 ± 0.055	25 ± 2	3.4 ± 1.6
12196	*S*.S. + *Leuconostoc mesenteroides* sbsp. *cremoris*	0.078 ± 0.033	101 ± 34	2.6 ± 0.4
10039	*S*.S. + *Leuconostoc pentosaceus*	0.057 ± 0.048	75 ± 2	3.2 ± 0.2
12795	*S*.S. + *Staphylococcus cohii* sbsp. *urealyticus*	0.137 ± 0.072	68 ± 30	2.9 ± 1.4
13755	*S*.S. + *Staphylococcus epidermidis*	0.056 ± 0.018	126 ± 104	3.2 ± 0.4

TABLE 25. Continued.

ID code	Treatment[a]	Mean ± SD[b]		
		μ_{max}[c]	d-t[d]	Overall growth[e]
10074	*S*.S. + *Staphylococcus gallinarum*	0.064 ± 0.034	103 ± 46	2.8 ± 0.5
12032	*S*.S. + *Staphylococcus lentus*	0.077 ± 0.078	294 ± 192	2.8 ± 0.6
10040	*S*.S. + *Staphylococcus thoraltensis*	0.093 ± 0.053	130 ± 115	3.4 ± 0.1
10240	*S*.S. + *Staphylococcus vitulinus*	0.056 ± 0.045	1178 ± 37	3.0 ± 0.7
12712	*S*.S. + *Staphylococcus warneri*	0.026 ± 0.099	35 ± 86	2.5 ± 1.1
13632	*S*.S. + *Staphylococcus xylosus*	0.223 ± 0.080	30 ± 6	2.1 ± 1.8

[a] *Salmonella* Saintpaul (*S*.S.) inoculation in previously inoculated (2 h before) intact cantaloupe rind (10 cm^2) with epiphytic isolate.
[b] Mean ± Standard deviation of three replicates by treatment, n = 3.
[c] $\mu_{max:}$ Growth rate (h^{-1}).
[d] DT: Doubling time (min).
[e] Overall growth, in log CFU/10 cm^2 = Growth at t_{max} (24 h) – Growth at t_0 (0 h).
* Indicates significant difference ($P < 0.05$) with respect to control.

Peppers

The growth curves constructed based on the growth of *S.* Saintpaul on pepper intact skin are shown in Figure 13. The growth parameters calculated for S. Saintpaul growth per treatment are included in Table *26*.

The growth rate, doubling time, and overall growth of the control (non-treated) *S.* Saintpaul on peppers were 0.21 ± 0.05 h^{-1}, 144 ± 33 min, and 1.4 ± 0.6 log CFU/10 cm^2. From the isolates tested, three had some effect on the growth parameters of *S.* Saintpaul. In samples inoculated with *E. faecalis* sbsp. *faecalis, S.* Saintpaul showed a significantly smaller growth rate of 0.002 ± 0.10 h^{-1} compared to the control ($P < 0.05$). On the contrary, when *S.* Saintpaul was inoculated in samples previously inoculated with *S. epidermidis*, and *S. warneri*, the growth rate significantly increased to 0.042 ± 0.007 and 0.033 ± 003 h^{-1}, respectively.

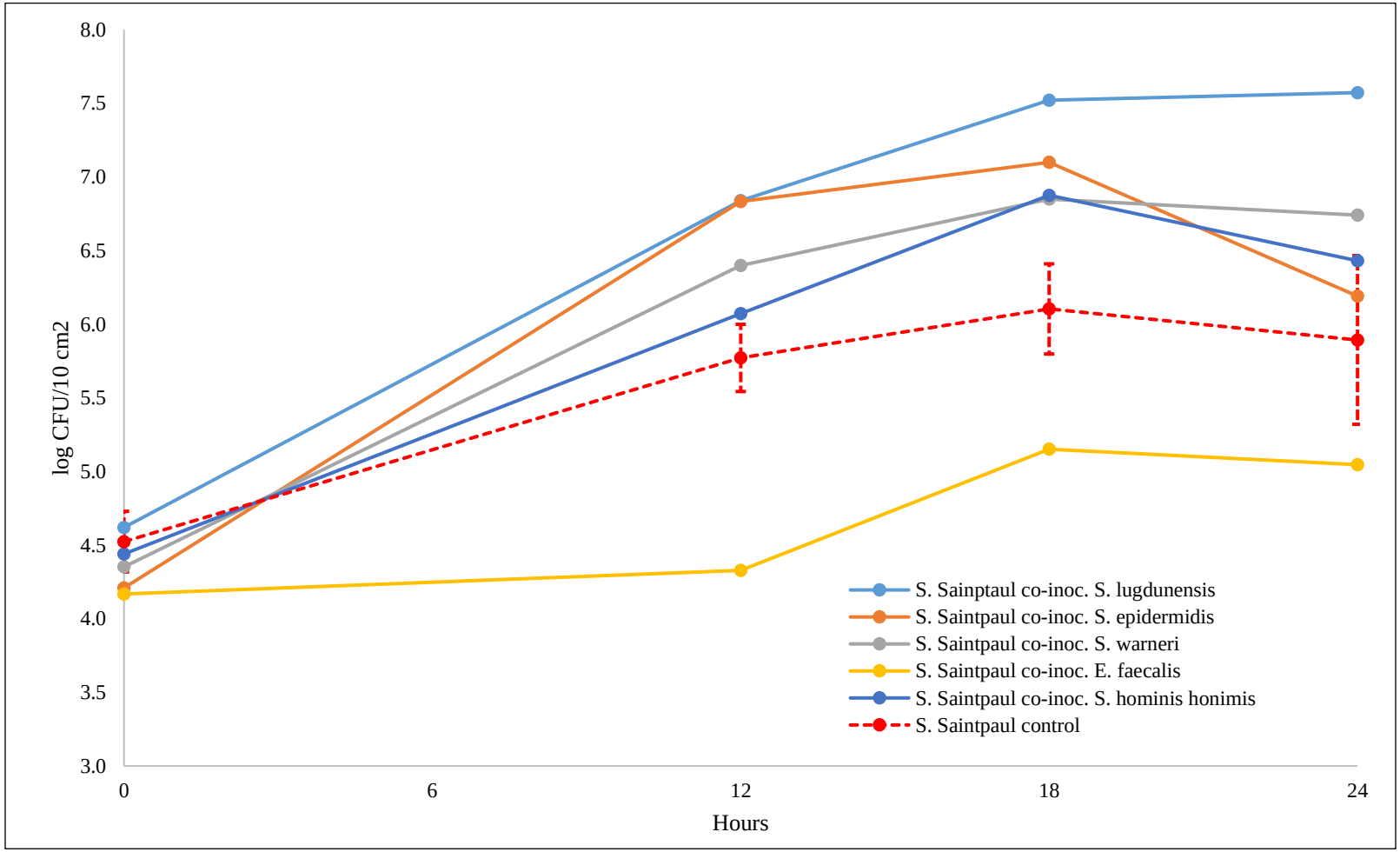

FIGURE 13. Growth curves of *S.* Saintpaul in the presence of antagonistic bacteria in peppers skin

TABLE 26. Growth parameters of *S.* Saintpaul on pepper intact skin inoculated with antagonistic epiphytic bacteria

ID code	Treatment[a]	Mean ± SD[b]		
		μ_{max}[c]	d-t[d]	Overall growth[e]
Control	*S.*S. + sterile PBS	0.21 ± 0.05	144 ± 33	1.4 ± 0.6
Pe 16759	*S.*S. + *Staphylococcus lugdunensis*	0.033 ± 0.002	79 ± 18	3.0 ± 0.6
Pe 17038	*S.*S. + *Staphylococcus epidermidis*	0.042 ± 0.007*	76 ± 8*	2.0 ± 0.6
Pe 17600	*S.*S. + *Staphylococcus warneri*	0.033 ± 0.003*	88 ± 9	2.4 ± 0.6
Pe 9072	*S.*S. + *Enterococcus faecalis*	0.002 ± 0.010*	233 ± 58	0.9 ± 0.2
Pe 9191	*S.*S. + *Staphylococcus hominis sbsp. hominis*	0.026 ± 0.05	89 ± 6	2.0 ± 0.3

[a] *Salmonella* Saintpaul (*S.*S.) inoculation in previously inoculated (2h before) intact pepper skin (10 cm^2) with epiphytic isolate

[b] Mean ± Standard deviation of three replicates by treatment, n = 3.

[c] μ_{max}: Growth rate (in h^{-1})

[d] d-t: Doubling time (or generation time, in min).

[e] Overall growth, in log CFU/10 cm^2 = Growth at t_{max} (24 h) – Growth at t_0 (0 h)

[f] * Indicates significant difference ($P < 0.05$) with respect to control

Tomatoes

The growth of *S.* Saintpaul for the different treatments (isolates) are illustrated in Figure 14 . The growth parameters calculated for *S.* Saintpaul for each treatment, are shown on Table 27. The growth rate mean of *S.* Saintpaul on tomatoes was 0.125 ± 0.034 h^{-1}. The mean doubling time was 61 ±7 min, and the overall growth was 2.3 ± 0.2 log CFU/10 cm^2.

Three of the tested bacterial epiphytic isolates affected the growth of *S.* Saintpaul on the tomatoes surface. *S.* Saintpaul on samples inoculated with *S. hominis* had a slower growth rate than the control, of 0.042 ± 0.010 ($P < 0.05$). On the other hand, *S.* Saintpaul inoculated on samples containing *B. polymyxa* and *S. epidermidis* demonstrated a greater overall growth than the control, of 3.4 ± 0.5, and 2.8 ± 0.1 log CFU/10 cm^2 ($P < 0.05$).

The growth parameters calculated for all other treatments were not different than the control ($P > 0.05$).

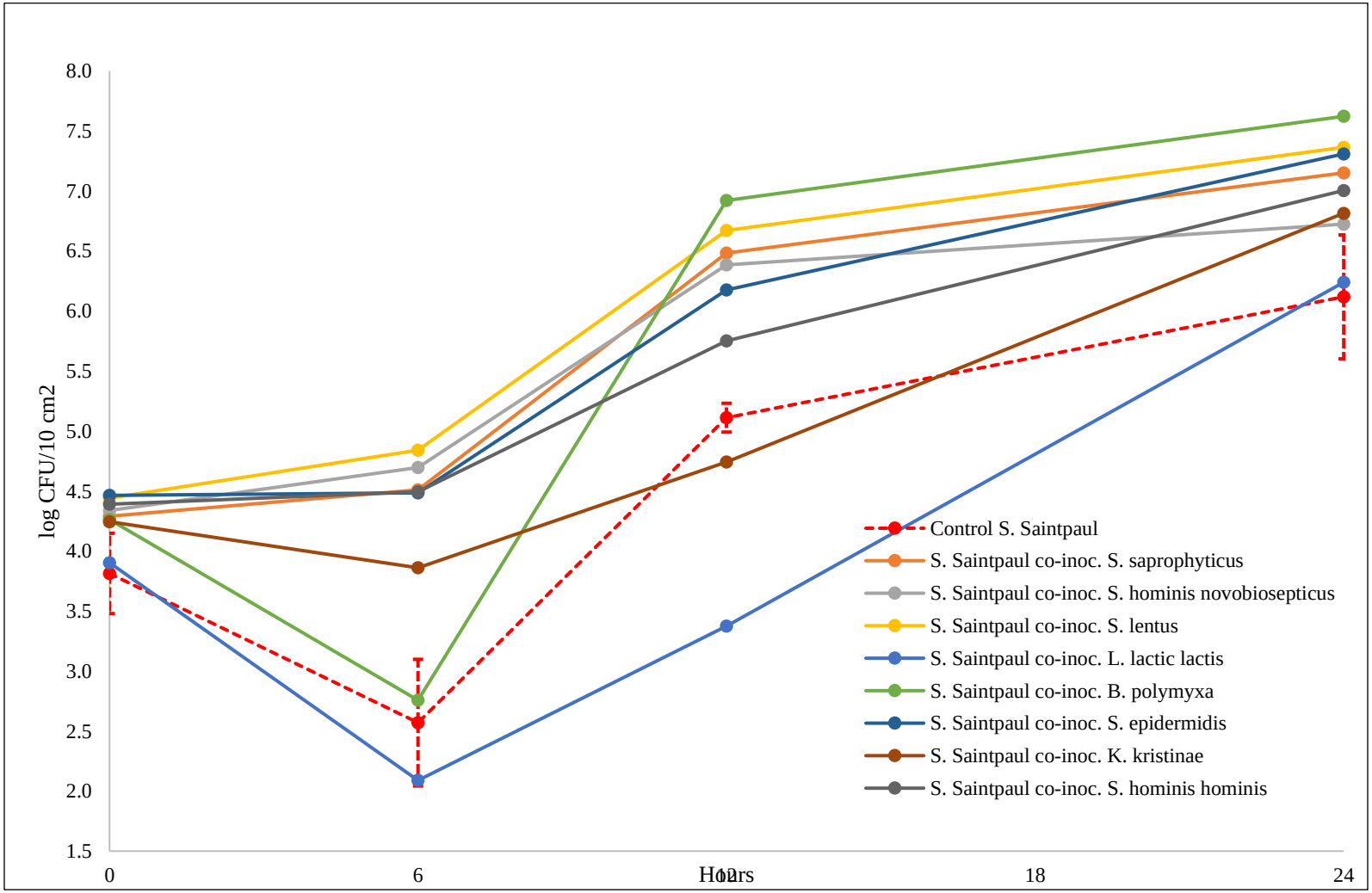

FIGURE 14. Growth curves of *S.* Saintpaul in the presence of antagonistic bacteria on tomatoes skin

TABLE 27. Growth parameters of *S.* Saintpaul in tomatoes intact skin inoculated with antagonistic epiphytic bacteria

ID code	Treatment[a]	Mean ± SD[b]		
		μ_{max}[c]	d-t[d]	Overall growth[e]
Control	None	0.125 ± 0.034	61 ±7	2.3 ± 0.2
To 14594	*S.*S. + *Staphylococcus saprophyticus*	0.062 ± 0.019	64 ±16	2.9 ± 0.3
To 14596	*S.*S. + *Staphylococcus hominis sbsp. novobiosepticus*	0.052 ± 0.014	64 ±28	2.4 ± 0.8
To 14599	*S.*S. + *Staphylococcus lentus*	0.055 ± 0.010	63 ± 13	2.9 ± 0.3
To 15318	*S.*S. + *Lactococcus lactis* sbsp. *lactis*	0.066 ± 0.08	63 ± 99	2.3 ± 0.7
To 15651	*S.*S. + *Bacillus polymyxa*	0.195 ± 0.105	51 ± 8	3.4 ± 0.5*
To 6031	*S.*S. + *Staphylococcus epidermidis*	0.054 ± 0.026	60 ± 47	2.8 ± 0.1*
To 6034	*S.*S. + *Kocuria kristinae*	0.033 ± 0.045	50 ± 178	2.6 ± 0.4
To 6392	*S.*S. + *Staphylococcus hominis* sbsp. *hominis*	0.042 ± 0.010*	94 ± 30	2.6 ± 0.2

[a] *Salmonella* Saintpaul (*S.*S.) inoculation in previously inoculated (2h before) intact tomato skin (10 cm^2) with epiphytic isolate.

[b] Mean ± Standard deviation of three replicates by treatment, n = 3.

[c] μ_{max}: Growth rate (in h^{-1}).

[d] d-t: Doubling time (or generation time, in min).

[e] Overall growth, in log CFU/10 cm^2 = Growth at t_{max} (24 h) – Growth at t_0 (0 h).

[f] * Indicates significant difference ($P < 0.05$) with respect to control.

Overall, the antagonistic effect of the studied epiphytic bacteria on fruits and leafy green leaves was not as easily reproduced as with the *in vitro* tests. This is not surprising since the experiment settings are controlled during *in vitro* tests. The researcher sets all the parameters including the nutrients and environment provided to the bacteria for proliferation. Furthermore, all materials are sterile; thus, no other bacteria is involved in these studies. However, when the study involves the use of actual living organisms, in this cases, leaves and fruits, many other factors come into play. Factors associated with the fruits and vegetables as well as the native microbiota of the surfaces can interfere with the inhibition action of some epiphytes. The nutritional conditions of the surfaces as well as the quality and quantity of epiphytic bacteria interacting with the pathogen and the epiphytic isolate is unknown. Although a reduction in the MS count of 1-2 log CFU/cm^2 was achieved using water washing and 95% alcohol to remove some bacteria that would interfere the experiment, a considerable amount of bacteria remained in the surface and was likely present when both the epiphytic isolate and the pathogen were inoculated.

Disinfection treatments of the leaves represent a modification since it significantly decreased the availability of surface nutrients giving the epiphytic bacteria less opportunity to synthetize substances and thereby inhibit the pathogens. Under normal conditions these bacteria can easily counteract the growth of pathogens in highly nutritious media such as TSA and MRS.

Even when the moisture was kept semi-controlled by keeping the fruits and leaf pieces inside capped containers possessing water for easy supply of vapor to the surrounding atmosphere, this water availability for metabolic functions of the bacteria is

not comparable to the high Aw present on the semisolid media. Therefore, even the moisture control could have affected the metabolism of antagonistic bacteria preventing their adaptation to and establishment on the surfaces of the produce. Considering all the factors that were likely negatively affecting the inhibition activity of the epiphytic bacteria on the produce surface, it is remarkable that some isolates still demonstrated a reasonable antagonistic action toward *E. coli* and *S.* Saintpaul.

On the other hand, some epiphytic bacteria do not produce *in vitro* inhibitory action, and even appear to benefit from the pathogen growth on the produce surface. One important species that fits this profile is *Aerococcus viridans*. Ramírez-Chavarin et al. *(233)* studied thermotolerant LAB as potential probiotics and described *A. viridans* as an easily aggregating species. This characteristic was related to their ability to form biofilms that could prevent pathogens from attaching to epithelial cells. Furthermore, the strain easily tolerated bile and gastric juices, maintained a relatively low pH (of 3–5), and did not easily co-aggregate with *E. coli* and *Salmonella*. Thus, it is unlikely that the isolates tested here were helping the pathogens attach to the surface. However, those isolates representing the epiphytic microorganisms on endive might have developed mechanisms that forced the plant to release nutrients or that inhibited the antibacterial activity of the endive, as some plant pathogens do. The actual causes of these agonistic interactions will remain undetermined until further studies determine the variables involved in pathogen-epiphyte interactions at the plant surface level. The use of sterile or gnotobiotic plants can control possible confounding factors involved.

Evaluation of *E. coli* O157:H7 growth and stomata invasion on endives in the presence of antagonistic epiphytic bacteria

The effect of epiphytic bacteria toward the growth of *E. coli* O157:H7 was studied using 10 micrographs taken after 12 h and 3 d of incubation to evaluate the possible growth effect of the antagonistic bacteria. Samples of each treatment were simultaneously processed to enumerate *E. coli* O157:H7 onto TSA-Amp at 0, 12 h, and 60 h.

The growth behavior of *E. coli* O157:H7 in the presence of the epiphytic isolates on endive leaf surface is shown in Figure 15. When the growth of the *E. coli* O157:H7 GFP was evaluated by time point, the mean counts of the treated samples were similar to those of the control (untreated) samples ($P > 0.05$). In the case of samples not treated with antagonistic bacteria, *E. coli* O157:H7 GFP counts at 0, 12, and 60 h of incubation were 4.2 ± 0.0, 3.8 ± 0.4, and 4.0 ± 0.1 log CFU/cm^2. The treated samples mean values were 4.3 ± 0.1, 4.2 ± 0.4, and 4.5 ± 0.2 log CFU/cm^2 at the same incubation times as control samples. The calculation of growth parameters was not possible since *E. coli* O157:H7 GFP growth was minimal during the 60 h of incubation.

The determination of the stomata presence and open/close stoma status, the degree of invasion by *E. coli* O157:H7 in open stomata, and the observed growth degree of *E. coli* O157:H7 (in 10 microscopic images of 45 mm^2) are shown on Table 28 . Samples of the images evaluated at 1 and 3 d are included in Figure 16.

TABLE 28. Microscopic analysis of endive surface invasion of stomata and growth of *E. coli* O157:H7 in the presence of antagonistic epiphytic isolates using confocal images

Treatment[a]	Incubation[b]	Number of stomata[c]			Invasion Deg[e]	Growth Deg.[d]
		Open (%)	Closed	Total		
EC + *Streptococcus alactolyticus*	12 h	10 (71.4) A[f]	4	14	0.2 ± 0.4 A	0.8 ±0.4 A [h]
	60 h	13 (100) B	0	13	0.4 ± 0.5 A [h]	2.5 ±0.5 B
EC + *Bacillus licheniformis*	12 h	12 (52.2) A	11	23	0.2 ± 0.4 A	1.1 ±0.3 A [h]
	60 h	9 (75.0) A	3	12	0.1 ± 0.3 A	2.3 ±0.5 B
EC + *Gemella bergeri*	12 h	3 (25.0) A [g h]	9	12	0.1 ± 0.3 A	1.1 ±0.3 A [h]
	60 h	14 (63.6) B [g]	8	22	0.9 ± 0.9 B [h]	2.3 ±0.5 B
EC + *Staphylococcus sciuri*	12 h	7 (58.3) A	5	12	0 A [h]	0.8 ±0.6 A [h]
	60 h	3 (100) B	0	3	0.7± 0.8 B [h]	2.4 ±0.5 B
EC + *Enterococcus gallinarum*	12 h	6 (28.6) A [g h]	15	21	0.3 ± 0.5 A	1.1 ±0.3 A [h]
	60 h	19 (67.9) B [g]	9	28	1.0 ± 0.9 A [h]	2.3 ±0.5 B
EC + sterile PBS	12 h	10 (71.4) A	4	14	0.4 ± 0.5 A	1.7 ±0.6 A
	60 h	16 (84.2) A	3	19	0 B	2.2 ±0.6 A
Neg. control	12 h	13 (68.4) A	6	19		
	12 h	8 (100) B	0	8		

[a] *E. coli* O157:H7 (EC) inoculation in previously inoculated (2 h before) endive pieces (1 cm^2) with epiphytic isolate.

[b] Incubation at 20 ºC in closed petri dishes with moisten filter paper.

[c] Number of stomata open, closed, and total observed in 2.125 mm of leave surfaces under the confocal microscope.

[d] Mean and standard deviation of degree of invasion in opens stomata, according to visual evaluation categories where 0 = no invasion, 1= low, 2= moderate, and 3 = high invasion, n = 10.

[e] Mean and standard deviation of the growth in observed leaf surface according to visual evaluation categories where 0=no growth, 1=low growth, 2=moderate growth, 3=high growth.

[f] Within column within treatment values followed by the same uppercase letter are not significantly different ($P > 0.05$).

[g] Indicates significant difference between measured value and corresponding value of the negative control (not inoculated) ($P<0.05$).

[h] Within column indicates significant difference between measured value and corresponding value of the EC control (not treated) ($P<0.05$).

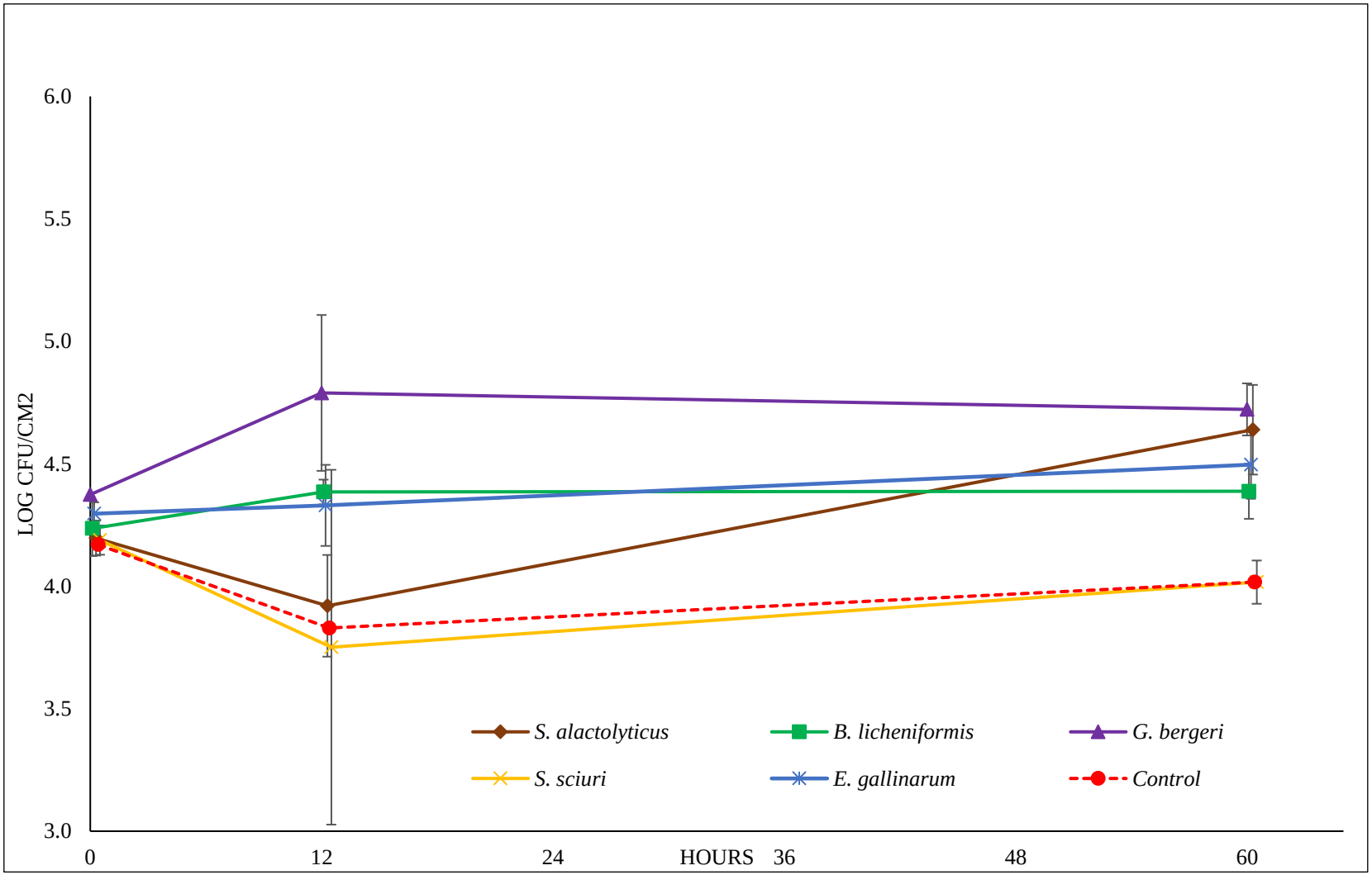

FIGURE 15. Growth of fluorescent *E. coli* O157:H7 GFP in the presence of epiphytic bacteria on endive leaf samples

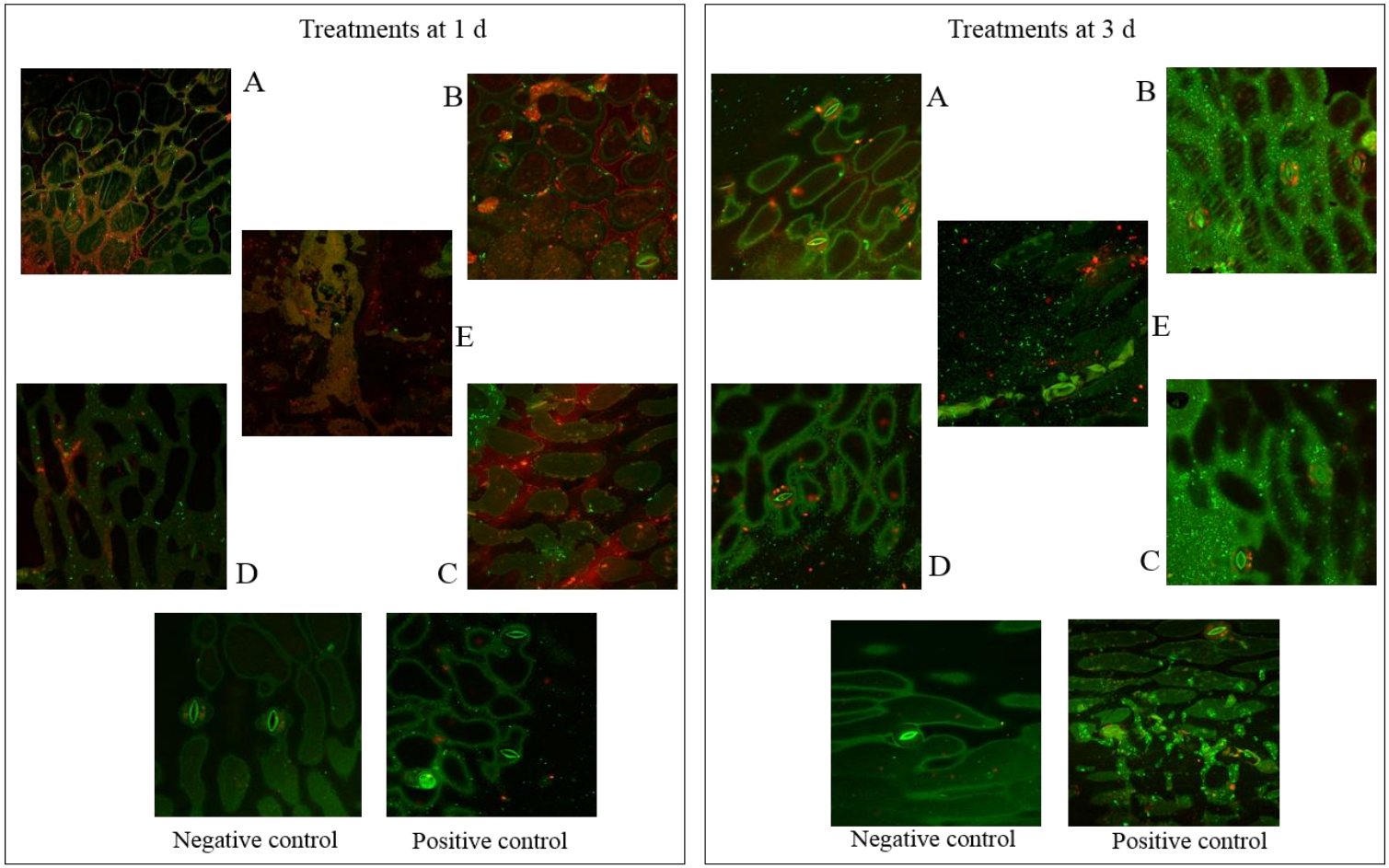

FIGURE 16. Confocal images of endive surface inoculated with fluorescent *E. coli* O157:H7 GFP (EC) and epiphytic isolates at 12 h (day 1), and 60 h (3 d) of incubation. Images observed using red and green laser excitation wavelength. Total size: 212.5 x 212.5 µm. Treatments include EC + *Streptococcus alactolyticus* (A), EC + *Bacillus licheniformis* (B), EC + *Gemella bergeri* (C), EC + *Staphylococcus sciuri* (D), and EC + *Enterococcus gallinarum* (E).

During the analysis of confocal micrographs, the number of stomata observed were highly variable, and ranged from 3 to 22 stomata in the total area surveyed per treatment and incubation time (4.51 cm^2, corresponding to 10 images of 45.156 mm^2 each). There was a significant difference in the number of open stomata by incubation period for the negative control and for four of the five treatments.

When the differences were significant, the number of open stomata were higher in the samples observed after 60 h of incubation ($P < 0.05$). Moreover, all stomata observed in the negative control at 60 h of incubation were open. When comparing stomata numbers between the treated samples and the negative control, *Gemella bergeri* and *Staphylococcus gallinarum* treated samples presented less open stomata for both incubation times, compared to the negative control ($P < 0.05$). Furthermore, these samples also presented less open stomata than the positive (untreated) control at 24 h.

When the images were captured, the stomata structures were used as reference of depth in the image, to assure the image was from the surface of the leaf, and not from the interior. Thus, images were commonly taken when one or more stomata were observed.

All treatments showed a significantly lower mean evaluation grade for the growth level of *E. coli* O157:H7 in the samples evaluated after 24 h of incubation. The average grade obtained by the control was 1.7 ±0.6, while the treatment evaluation ranked between 0.8 ± 0.4 and 1.1 ± 0.3 ($P<0.05$). *Streptococcus alactolyticus* and *Staphylococcus sciuri* obtained the lowest grades in the visual evaluation for the *E. coli* O157:H7 growth of 0.8 ± 0.4 and 0.8 ± 0.6, respectively. Interestingly, these isolates also presented the lowest counts when the samples were grown onto TSA-Amp, although in this last case,

differences between the control and treatments for their log CFU/cm^2 values were not significantly different (P>0.05). After 3 d of incubation, no significant difference was observed in the visual evaluation of the *E. coli* O157:H7 growth with respect to the control (untreated) (P > 0.05).

The internalization of enterica pathogens in fruits and vegetables has been considered an important factor contributing to their survival and resistance to antimicrobial treatments *(121, 187, 269)*. Kroupitski et al. *(159)* reported the internalization of *S. enterica* on lettuce stomata, and related this invasion to the ability of the pathogen to locate nutrients produced during the photosynthesis, being located on the stomata opening.

The evaluation of stomata invasion by *E. coli* O157:H7 was given a low to null status; however, it was observed at least one time per sample. It is interesting to notice that *E. coli* O157:H7 control (untreated with epiphytic bacteria) experienced no invasion of stomata, while some treated samples did. Thus, it is possible that the epiphytic bacteria presence stimulated *E. coli* O157:H7 to move into the stomata by unknown mechanisms.

While qualitatively measured, it was possible to evaluate the effect of some isolates over the colonization of *E. coli* O157:H7. Furthermore, the observation of invasion of stomata in all treatments confirmed the results obtained by previous studies. *E. coli* O157:H7 was capable of invading the stomata openings, and these could probably serve to protect the bacteria from external exposure and/or provide nutrients. Hence, open stomata in the plant represents a risk for the internalization of bacteria. One isolate of *G. bergeri* and one of *S. sciuri* will likely stimulate the leaf to close its stoma pores. This

phenomenon has been triggered in plants by some plant pathogens *(277)*. Further studies that elucidate the plant response to the presence of the epiphytic bacteria can lead to a better understanding of the interactions given between plant-epiphyte-enteric pathogen interactions.

CONCLUSIONS

The microbiological content of different fruits and leafy greens presented variable content of epiphytic bacteria, and this variation was influenced by external conditions, including agricultural practices such as irrigation, and environmental factors such as temperature and humidity. Furthermore, the harvesting season played an important role in the variation of epiphytic and antagonistic bacteria. The influence extent of environmental conditions and the intrinsic plant characteristics require more studies that include different varieties within plant species. It is also necessary to determine and include measurements of leaf surface physicochemical characteristics including wettability, thickness of the waxy cuticle, roughness, number of trichomes and stomata, and nutritional content or organic matter presence to determine which of these factors are of higher impact in the variation of the microbiological content of leafy greens within and between commodities. Similarly in fruits, further studies should consider the epiphytic bacteria variation due to season, temperature and humidity, rain, flood episodes, and other environmental factors, as well as include different varieties of the same species of tomatoes, peppers, and melons. A study evaluating the influence of the level of netting, possibly using different cultivars of cantaloupes, could help to elucidate the actual impact of the netting level over the microbiological content of cantaloupe rinds.

The epiphytic community of fruits and vegetables was found to comprehend a vast variety of bacterial species capable of counteracting the proliferation of enteric pathogens, and their occurrence was variable by production season and commodity of isolation.

Although numerous isolates were determined antagonistic toward *E. coli* O157:H7 and *S.* Saintpaul, their inhibitory action in the leaves and fruits surfaces was not straightforwardly determinable as it was in the *in vitro* experiments. Although only some isolates were biochemically identified from the total of antagonistic isolates obtained from all commodities, those identified might be considerable constituents of the total population of antagonistic epiphytic bacteria found on each commodity. The determination of the actual occurrence of these species using selective conditions adapted to each species or genera could allow a more precise determination of their occurrence in fruits and vegetables. The isolates of the same species were possibly the same strain isolated several times, although this was not determined. The broad distribution, or a large amount of a particular species, could also influence the likelihood of isolation. Further assessment studies that determine the presence of a particular species and their population could help clarify their distribution in the surfaces of different commodities. The possible discrepancies between the biochemical identification and results using other methods of identification should be obtained to ensure clear identification of the antagonistic bacteria with potential use as biocontrol agents.

Confocal microscopy allowed a closer study of the epiphytic bacteria and the pathogen interaction directly in the surface of the leaves. However, the evaluation of the biofilm formation by the pathogen and the antagonistic bacteria would require further investigation, possibly using florescent markers to detect specific biofilm matrix components such as amyloids. Although improbable, the agonistic interaction between pathogens and epiphytic isolates previously recognized as antagonistic to the same

pathogens might be occurring. Thus, these interaction modifications should be further analyzed to determine the causes at the plant surface level. The use of sterile or gnotobiotic plants can control possible confounding factors involved. Furthermore, microorganisms genetically modified, suppressing synthesis of particular acids, bacteriocins or other substances, might allow the determination of the factors involved in the antagonistic effect toward the enteric pathogens.

The objectives of the study were achieved since the microbiological content of fruits and vegetables was assessed, and it was demonstrated that epiphytic microbial communities of fruit and leafy greens contain numerous species with promising characteristics as biocontrol agents to counteract the colonization of important enteric pathogens such as *E. coli* O157:H7 and *S. enterica* on produce surfaces.

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