PERSISTENCE OF *CRYPTOSPORIDIUM PARVUM*, *CYCLOSPORA CAYETANENSIS* AND *SALMONELLA TYPHIMURIUM* ON CILANTRO AND PARSLEY IN THE FIELD WHEN INTRODUCED BY SPRAY INOCULATION

by

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(Under the Direction of Ynés R. Ortega)

ABSTRACT

Fresh produce and common herbs have been often linked to contamination by coccidian protozoan parasites. In this study, the persistence of *Cyclospora cayetanensis* and *Cryptosporidium parvum* and the bacterium *Salmonella enterica* Typhimurium was evaluated on the popular herbs cilantro (*Coriandrum sativum*) and parsley (*Petroselinum crispum*) under natural environmental conditions on an experimental field in Tifton, Georgia and controlled conditions in a growth chamber, with environmental parameters modeling those in agricultural growing areas associated with products linked to outbreaks of cyclosporiasis. Two separate plots with 10 replicate subplots each of cilantro and parsley were used in this study, and pathogens were introduced on the crops by spray irrigation. Samples were collected within a period of 23 days and evaluated for pathogen persistence. *Cryptosporidium* and *Cyclospora* were analyzed by molecular assays and *Salmonella* Typhimurium by a culture assay. The parasites were detected in 100% of all samples tested up to day 23 in both crops held in a growth chamber. No significant difference ($P > 0.05$) was observed in persistence on plants from the field evaluated in the spring of 2018 and in the spring of 2019. *Salmonella* was isolated up to day 15 on both

herb types in the field in the two trials with variations in persistence pattern. All the pathogens were detected in the growth chamber until day 23. There was no significant difference between the persistence of the parasites on both herbs, however, the persistence of *Salmonella* Typhimurium differed significantly between cilantro and parsley under field conditions. Our study demonstrates for the first time that *Cyclospora cayetanensis* and *Cryptosporidium parvum* are resilient pathogens and conditions in a controlled environment could be more conducive for the persistence of foodborne pathogens.

INDEX WORDS: *Coriandrum sativum*, *Petroselinum crispum*, *Cyclospora cayetanensis*, *Cryptosporidium parvum,* foodborne

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DEDICATION

This is dedicated to my parents who have been highly supportive of my goals.

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I wish to acknowledge my major professor and mentor Dr. Ynés Ortega for her continued support and belief in my abilities, Maria Patricia Torres, Alison Payton, and Alexander Studebaker for all the technical assistance in putting this work together.

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CHAPTER 1

INTRODUCTION

Throughout history, plant extracts have been either ingested internally, applied topically or inhaled by fumigation or vapor inhalation for medical purposes. For example, the seeds and leaves of cilantro (*Coriandrum sativum)* have been used internally as a spasmolytic, digestive and a carminative; topically to relieve joint pain, and as a cosmetic in Iran, India, and Pakistan (Abascal, 2012) The great philosopher Hippocrates said, "Let your food be your medicine, and your medicine be your food"(Craig, 1999). This holds very true for herbs. The medicinal properties of herbaceous plants have been corroborated by scientific research. Herbs contain a wide variety of active phytochemicals like coumarins, flavonoids, polyphenolics, and sterols, which aid in the elimination of oxidative radicals in the system (Craig, 1999; Farzaei, 2013; Yano et al, 2006; Unkle et al, 2012). They are also good sources of various vitamins and minerals. Accordingly, herbs have been said to stimulate the immune system, reduce cholesterol levels and confer some protection against cancer. Antimicrobial properties have also been attributed to them with the isolation of compounds like eugenol from basil and cloves that have inhibitory effects on a wide range of bacteria (Gonzalez-Lamothe et al, 2009; Lai, 2004).

Despite the recent discovery of synthetic drugs mainly inspired by the compounds found in herbs, many people still prefer the natural, organic herbaceous plants as the treatment of choice. According to a United States National Health interview survey conducted between 2002 and 2007, the number of adults who used herbs increased from 50.6 million to 55.1 million (Wu et al, 2011). In 2016, the American Botanical Council reported that the sale of herbs increased by 7.7 percent, surpassing $7 billion in revenue. Coupled to the medicinal properties ascribed to herbs, cultures all over the world utilize them as flavor enhancers in cuisines. Cilantro, basil,

oregano, rosemary, sage, thyme, cumin, dill, and other herbs are used as flavorings, doubling as a potential replacement for salt in foods.

In recent times, there has been an increased re-emphasis on not just the consumption of herbs but on the health benefits of leafy greens in general. Although not technically classified as a leafy green, herbs are also considered to be ready to eat (RTE) food commodities. In most cases, they are consumed raw or are added to already prepared dishes. We have witnessed an increase of foodborne outbreak cases associated with leafy greens and herbs; In 2006, a foodborne outbreak involving the bacteria *E. coli O157: H7* hit the spinach industry in the US resulting in over 199 clinically confirmed cases of infection in 26 states and 3 deaths (CDC, 2006). *E. coli* has also been detected on sweet basil and coriander from different regions (Delbeke et al, 2015). Between 1998 and 2016, nearly 3,000 people got sick from the consumption of leafy greens contaminated with pathogenic organisms (Johnson, 2019). Every year, cilantro, parsley, and basil are common herbs implicated in foodborne outbreaks and according to the Center for Disease Control and Prevention (CDC) National Outbreak Reporting System (NORS); more than 100 outbreaks have occurred between 2006 and 2017 involving these herbs either alone or in complex food matrices or salad mixes. The challenge with the correlation of foodborne outbreaks to a specific fresh produce commodity arises because most outbreaks are associated with salad mixes (complex foods) that contain a range of different leafy greens and herbs. For example, the first reported case of a *Cyclospora* outbreak linked to leafy greens in Europe occurred in the year 2000. The implicated product was a salad mix composed of lettuce and fresh leafy green herbs affecting four independent groups who attended a luncheon in Southwest Germany (Doller et al, 2002). Salad mixes accounted for multiple outbreaks that occurred in 2018 in the United States as well. An outbreak of *Cyclospora* infection was linked to

salad mix sold by the McDonalds restaurant chain and produced by Fresh Express, and vegetable trays sold by Del Monte. Another outbreak of *Salmonella* infection was linked to pasta salad containing fresh herbs (CDC, 2018). Advancements in traceback technology and new food safety regulations have been essential in improving pathogen detection and identification of contamination sources on fresh produce linked to outbreaks. In the new FSMA regulations section 204, the FDA was required to establish pilot projects geared towards improving its capacity to effectively and rapidly trace and track foods in events of outbreaks. This undoubtedly requires extensive record-keeping (Johnson, 2019).

Contamination of produce and herbs by pathogenic organisms can occur at any point in the continuum of crop production both at pre- and post-harvest and could vary for different production zones (Alegbeleye, 2018). For example, farms located close to animal production areas might be more susceptible to contamination by pathogenic and zoonotic microorganisms than those located far away from livestock zones. A study conducted in Poland between 2006 and 2007, showed that farmlands located in districts with homesteads possessing high numbers of cattle resulted in a higher number of foods contaminated with *Cryptosporidium* oocysts compared to districts with lower numbers of livestock (Rzezutka et al, 2010). In the same vein, the proximity of vegetable packing stations to wash water areas could impact contamination of packaged products. Established sources of contamination include contaminated manure, soil, wild or domestic animals and insects (flies), irrigation water, and infected workers. It has also been noted that pre-harvest contamination of produce in the field can be challenging to decontaminate (Alegbeleye, 2018).

Several pathogenic organisms have been implicated in numerous outbreaks associated with the consumption of fresh leafy greens and herbs and have been detected in food production systems

(Ricke et al, 2018; Rose and Slifko, 1999). In recent years, *E. coli*, *Salmonella,* and *Cyclospora cayetanensis* have been most often responsible for these outbreaks. Although contamination of fresh produce (RTE) is a pertinent challenge, herbs exhibit antimicrobial activities that may confer some resistance to contamination by pathogenic bacteria (Yano et al, 2005; González-Lamothe et al, 2009). Foodborne outbreaks associated with herbs might connote that these pathogenic bacteria have either developed mechanisms to overcome natural antimicrobials of these plants or, the antimicrobials found on herbs and leafy greens are present in small concentrations or in forms that might require some biochemical activation. In one study, it was observed that pathogenic bacteria like *Salmonella* spp. might possess an innate survival mechanism that results in the formation of biofilms that enhance persistence; a phenomenon referred to as a community-based strategy for bacterial persistence (Kumar et al, 2017). Consequently, GRAS (Generally Regarded As Safe) antimicrobials, UV treatment, breeding for pathogen-resistant plant varieties and proper hygienic practices, are being studied to control or eliminate bacterial contamination on produce. Beyond contamination by pathogenic bacteria of which many studies have focused on parasites like *Cyclospora cayetanensis* and *Cryptosporidium* and viruses like the *Norovirus* are becoming an increasing threat to food safety. Some studies have explored and zeroed in on these organisms to understand their microbiological and biochemical properties including challenge studies against known pathogens both in laboratory and food production settings (Erickson and Ortega, 2006; Eberhard et al, 2000; Sathyanarayanan and Ortega, 2006; Ortega and Robertson, 2017; Utaakar et al, 2017). These properties or characteristics could, however, vary in the field under natural environmental conditions. Generally, several factors may influence survival and, or growth of pathogenic organisms on leafy greens and herbs including physiological states of plants and pathogen,

environmental conditions including temperature, light intensity, and relative humidity, and plant produce type. The objective of this study was therefore to determine the impact of changing environmental conditions on the persistence of *Cyclospora cayetanensis*, *Cryptosporidium parvum,* and *Salmonella* Typhimurium on cilantro and parsley.

CHAPTER 2

LITERATURE REVIEW

THE BURDEN OF FOODBORNE OUTBREAKS ON LEAFY GREENS AND HERBS BY COMMON FOODBORNE PATHOGENS

Fresh leafy greens and herbs are ready to eat (RTE) food commodities hence undergo little or no processing steps. As pointed out earlier, a critical point for pathogenic microbial control occurs in the fields at pre-harvest. Widespread demand for leafy greens increases the chances of exposure to produce that may result in acquiring foodborne illness especially if the source of the produce is contaminated. In 2012, a Mexican-style fast-food restaurant chain in the US was linked to a widespread outbreak of *Salmonella* Enteritidis. A total of 68 persons from 12 states were affected but no deaths were recorded. Investigations could not specifically associate the outbreak to a specific food source, but mixed salads were part of the meals consumed among infected individuals (CDC, 2012). Between June to August of 2013, mixed salads were implicated in multiple outbreaks of *Cyclospora cayetanensis* which affected a total of 631 persons in 25 states leading to the hospitalization of 49, but no deaths were reported. Traceback investigations confirmed that the outbreaks were linked to mixed salads from Taylor Farms de Mexico and fresh cilantro from Puebla, Mexico (CDC, 2013). In 2014, fresh cilantro was implicated in yet another outbreak that occurred in Texas. About 304 people were affected during this outbreak, 7 were hospitalized and no deaths were recorded (CDC, 2014). In 2018, several outbreaks involving *Cyclospora cayetanensis* were reported. The most prominent were linked to Fresh Express salad mix sold at McDonald's restaurants and Del Monte vegetable trays containing carrots, cauliflowers and broccoli and dill dip. Together, these outbreaks resulted in

760 laboratory-confirmed cases of cyclosporiasis, 32 hospitalizations and no deaths (CDC, 2018).

The economic impact of foodborne outbreaks caused by microbial pathogens was evaluated by the USDA and according to the USDA Economic Research Service report, outbreaks linked to *Cyclospora cayetanensis, Cryptosporidium parvum, Salmonella spp.* has cost the US a combined $2,888,559,257 (USDA, 2019). The outbreaks on salad mix both at McDonald's and Del Monte in 2018 led to massive recalls of produce commodities resulting in losses of millions of dollars. Del Monte recalled 6 oz, 12 oz and 28 oz vegetable trays from stores in Iowa, Illinois, Indiana, Michigan, Minnesota, and Wisconsin while McDonald's stopped the sale of salads in over 3,000 restaurant locations in 14 states (CDC, 2018).

In 1996, the first widespread outbreak of cyclosporiasis occurred in the United States in which raspberries imported from Guatemala were implicated as the food source of infection (Palumbo et al, 2013). There were 976 laboratory-confirmed cases of *Cyclospora* infection during the Summer and Spring seasons. Guatemalan raspberry exports began in 1988 and by 1996, it had peaked to about 500 metric tons in exports to the United States. This nascent industry was hit hard by the outbreak; export to the U.S had dwindled to about 2 metric tons by the year 2000 and billions of dollars lost (Calvin et al, 2003). The possible sources of the outbreak were unknown although, given the host specificity of the parasite (Alfano-Sobsey et al, 2004; Eberhard et al, 2000), contamination by infected field workers or irrigation water was likely.

Table 2.1: Some foodborne outbreaks associated with leafy greens and herbs

Leafy green and herb	Country	Pathogen	Number of cases	Ref
RTE salads	US	*E. coli*	33	**CDC, 2017**
Spinach and spring mix	US	*E. coli*	33	**CDC, 2017**
Lettuce	US	*E. coli*	84	**CDC, 2017**
Mixed vegetable tray (broccoli, cauliflower, dill, carrot)	US	*C. cayetanensis*	250	**CDC, 2017**
Cilantro	US	*C. cayetanensis*	304	**CDC, 2014**
Fresh parsley in sauce	Sweden	*Cryptosporidium spp.*	21	**Insulander et al, 2008**
Basil	US UK	*C. cayetanensis* *Salmonella spp.*	205 32	**CDC, 2019** **Pezzoli et al, 2008**
Salad Mix	US	*C. cayetanensis*	631	**CDC, 2013**
Packaged Salads	US	*Listeria monocytogenes*	19	**CDC, 2017**

CILANTRO AND PARSLEY

Cilantro (*Coriandrum sativum*)

History and use

Cilantro or coriander is an economically important herbaceous species of the Apiaceae family and is a culinary herb in many tropical and subtropical cuisines. With expanding globalization and popularity of ethnic cuisines like the Mexican and Thai foods, cilantro has gained and continues to gain widespread popularity as a key ingredient in these dishes (Morales-Payan, 2011). There are uncertainties around the history of cilantro, but records indicate that it may have originated from the coastal regions North and East of the Mediterranean Sea and then spread to Asia, Africa, and Europe. References to coriander were made in Biblical texts by ancient

Israelites and some evidence exists that the herb was grown in Assyria around 9,000 years ago. Cilantro has been grown in India in the past 5,000 years and in ancient Egypt, about 4,500 years ago, where it was grown and used as a medicinal plant and condiment. In China, cilantro has been used for centuries, arguably making its way to the East from the West through ancient trade routes. The herb was brought to the Americas from Europe around the 1600s probably by the Spanish conquistadores (Berry, 2005; Diederichsen, 1996; Grubben, 2004).

Cilantro (*Coriandrum sativum*) is used both as a medicine and a culinary herb based on its primary products, seeds, roots and leaves (Diederichsen, 1996). It is known to be rich in phytonutrients, flavonoids, and active phenolic acid which are antioxidants with the capacity to fight free radicals. Coriander is rich in essential oils and fatty acids; it can aid in digestion and is used as a carminative and digestive tonic in India, properties which the ancient Egyptians also explored 4,500 years ago (Diederichsen,1996). In culinary spheres, the fruits which possess a pleasant flavor when ripe, have been used as a condiment in the preparation of pickling spices, sausages, seasonings, and for flavoring pastries and cookies. The leaves of cilantro are also widely consumed and contain high levels of vitamin C (Diederichsen, 1996).

Varieties

Two species of Coriandrum exist, *Coriandrum sativum* and *Coriandrum tordylium*. Generally, the classification of cilantro varieties is based on their sensitivity to heat and photoperiod. Accordingly, there are the slow bolting varieties which are less sensitive to heat and photoperiod, and other varieties that flower after a few weeks of germination due to their sensitivity to high temperature and photoperiod. Desirable varieties in the market are bred for slow bolting, high concentration of essential oils, resistance to diseases and large leaves/stem biomass. Some

improved varieties in the market include: ‘Santo’, ‘Long-standing’, ‘Jantar’, ‘Caribe’, and ‘Slo-bolt’ (Laemmlen et al, 1998; Morales-Payan, 2011).

Growth conditions

Cilantro is propagated by seeds usually on beds 40 to 80 inches wide. They can also be grown on other substrates, hydroponic systems or most often by direct seeding in open fields. Soil temperatures above 15 ºC allow for quick germination of seeds and temperatures close to 25 ºC are ideal for faster germination. Many soil types support the growth of cilantro although they prefer deep soil with high water holding capacity, pH 6.5 to 7.5 and adequate drainage. Appropriate levels of mineral nutrients are required for cilantro to accumulate a large amount of biomass, given its rapid growth and short period between germination and harvest. Therefore, soil nutrients like potassium, nitrogen, calcium, and phosphorus are required in ample amounts for proper growth of cilantro. Irrigation is usually done through overhead sprinkler systems or drip irrigation; frequent short irrigations are required to maintain uniform moisture in the soil. Prolonged water deficiency may lead to yield reduction, and the most critical time for irrigation is during germination and 2 to 3 weeks after.

Harvest

Cilantro is harvested when leaves are ready to be used. This might be achieved by either pulling the entire plant from the soil or cutting the stem near the soil level. Bunches are formed and tied together with a twist tie. Mechanical harvesting is also employed especially for commercial cilantro production (Morales-Payan, 2011; Laemmlen et al, 1996).

Parsley (*Petroselinum crispum*)

History and use

Parsley has been referenced in many ancient civilizations and folklore. In ancient Greece, gardens often had borders of parsley and the plant was supposedly said to have sprung from the blood of Archemorus whose name meant "forerunner of death". Garlands worn in ancient Rome during feasts were made from parsley. Parsley was believed to ward off intoxication however it has been reported to cause epilepsy in children. In rituals, parsley was sprinkled over dead bodies to remove the bad smell. Old English folklore also had references to parsley, ascribing it to death, and in Jewish culture, it was used to celebrate the Passover. Parsley belongs to the family *Apiaceae* or *Umbelliferae* and the genus *Petroselinum* (Daradkeh, 2016). The name *Petroselinum* is derived from the Greek word 'Petros' meaning stone because the plant grows in rocky hillsides and 'selinon' from celery (Daradkeh, 2016).

Petroselinum crispum is believed to be originally grown in Sardinia (Mediterranean area) where it is found in its wild form and was cultivated from the 3rd century BC. Linnaeus stated its wild habitat to be Sardinia, whence it was brought to England and apparently first cultivated in Britain in 1548 (Farzaei et al, 2013). It was rumored that Catherine de'Medici (Queen consort of France) was responsible for popularizing parsley in the 16th century when she brought it back to France from Italy.

Parsley seeds and leaves have been reported to contain a wide range of phytochemicals and essential oils of medicinal importance. As a result, seed and leaf extracts have been observed to have an immunomodulating effect, analgesic, and spasmolytic activity, gastrointestinal and cardiovascular activity and therapeutic effects on the genitourinary system (Farzaei et al, 2013). Parsley seed powder and juice have been reported to stimulate hair growth and the plant

possesses high antimicrobial properties as a result of its photoactive coumarin composition (Manderfeld et al, 1997).

Varieties

There are three main varieties of parsley, the curly leafed or common parsley with the cultivars Extra Curled Dwarf, Green River and Forest Green, the Italian or flat-leafed parsley with the cultivars, Plain Leaf Single, Giant of Italy and Dark Green Italian; and the root parsley with the cultivar Hamburg (Daradkeh, 2018).

Table 2.2: Cultivars of parsley (*Petroselinum crispum*). (Source: Craig R. Andersen, Agriculture, and Natural Resource, University of Arkansas Division of Agriculture)

Crop	**Cultivar**	**Days to Maturity**	**Seed per 100 feet/ row**	**Remarks**
Curled	Extra Curled Dwarf	60	1/8 ounce	Dark green, deeply cut, curled leaves.
	Forest Green	75	1/8 ounce	Combination of double-and-triple curled variety. Holds color well, good flavor and very productive
	Green River	75	1/8 ounce	Double-curled leaves stay curly even in the heat. Great for drying.
Plain leaf	Plain Leaf, single	60	1/8 ounce	Dark green, deeply cut. Celery-leaf type
	Giant of Italy	75	1/8 ounce	Big, dark green leaves with strong stems, sweet flavor
	Dark Green Italian	75	1/8 ounce	Strong-tasting celery-leaf type. Use dry or fresh. Stiff, upright stems for bunching.
Root	Hamburg	85	1/8 ounce	Slow to germinate, produce smooth white roots.

Growth conditions

Parsley is a cool weather plant and can be grown in mid-April in the central Mediterranean region and Europe. It typically flowers in warmer months. Ideal temperatures for pollination and seed production are between 29-30ºC. Parsley grows best in humid soils and ideal soil pH levels are between 5.3 and 7.3. Parsley seeds are short-lived and are typically planted at 10 to 15 seeds per inch row of soil at no more than a 0.6 cm deep. Plants can also be grown in a bed or mass planted in a small area. It is shallow-rooted and requires regular fertilization and watering for best results (Daradkeh, 2018)

Harvest

Parsley leaves are usually picked for daily use after the plant has become well established. The leaves can be washed, drip-dried and stored in plastic bags or wide-mouthed jars for storage. They can also be covered lightly with straw and picked continuously over much colder months of the year or during the winter. Parsley is typically hand-harvested to minimize crop damage (Simon et al, 2019)

POSSIBLE ROUTES OF CONTAMINATION

Contamination of herbs and produce can occur at any point in the continuum of production from the field during pre-harvest operations to consumption after post-harvest operations. The general supply chain for fresh culinary herbs is outlined in Fig 2.1

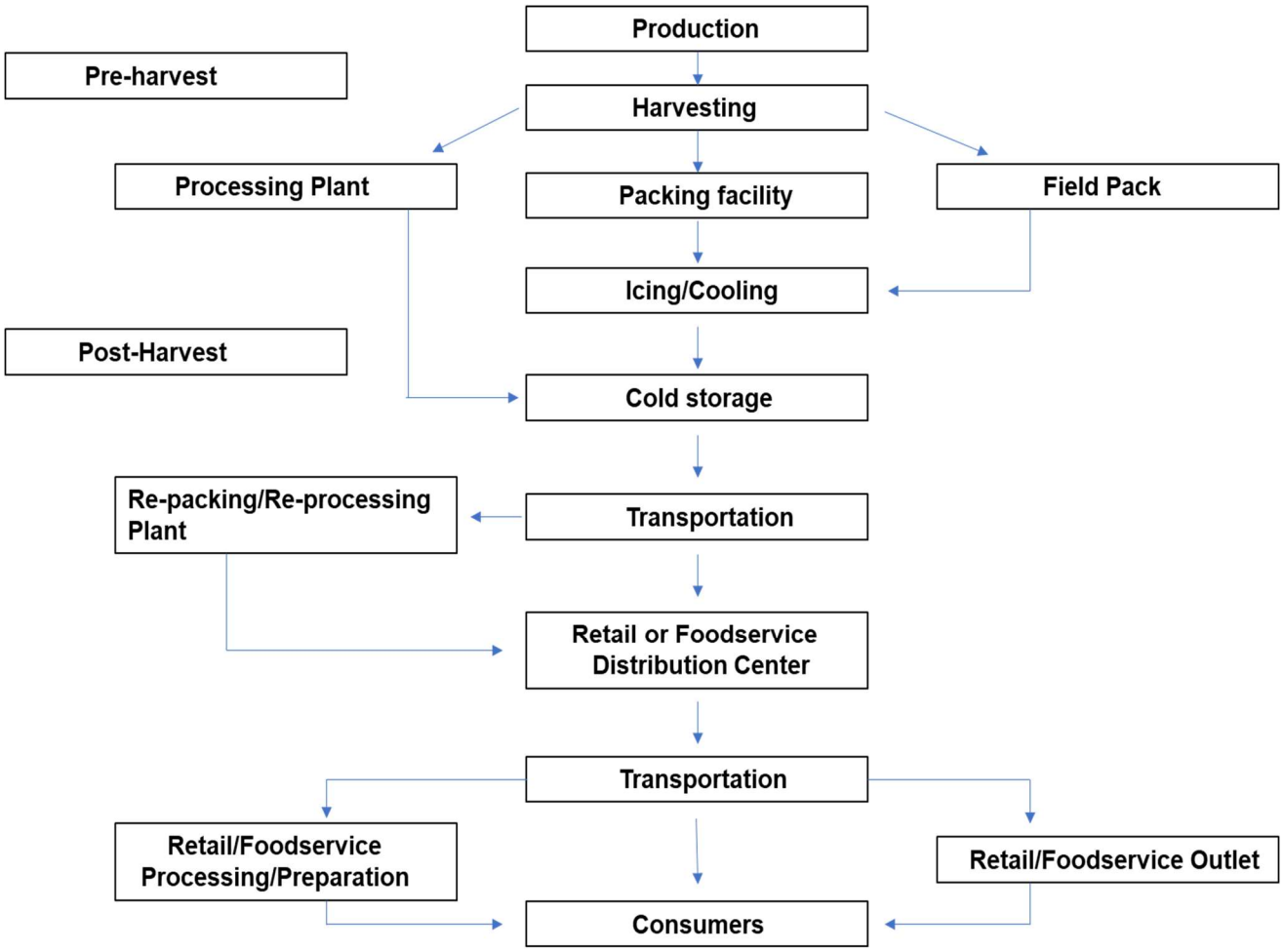

Fig 2.1: General supply chain flow for culinary herbs (Source: FDA, 2013)

Pre-harvest

To address contamination on herbs, it is pertinent to understand the production and supply chain flow to identify critical control points. Contamination of culinary herbs like parsley (*P. crispum*)

and cilantro (*C. sativum*) can occur at pre-harvest. As outlined in the flow chart above, the points of production, harvesting, and packing (processing plants, field pack, packing facility) are arguably the most critical points of contamination in the continuum. Emphasis is placed on the safety of culinary herbs because they are ready to eat food commodities without a 'kill step'; therefore, when contamination occurs, the complete removal or death of the contaminating pathogen is unlikely. By classifying production and harvest into pre-harvest operations, we can broadly group contamination routes resulting from natural factors such as contaminated soil, livestock, irrigation water or compost and those resulting from inadequate hygienic practices (field workers). (FDA, 2013; FDA, 2017).

Cilantro and parsley are typically grown in cool, humid environmental conditions that favor the growth and persistence of human pathogens. High rainfalls which characterize the cooler months in the United States and most parts of the world could potentially expose plants grown close to a contaminated area or on contaminated soils to cross-contamination from rain splashes and run-off (FDA, 2013). In addition, soils can become contaminated by droppings of a wild or domesticated animal or by addition of organic soil amendments such as sewage sludge, slurry, livestock excreta and industrial and municipal waste residue (Alegbeleye, 2018). It is possible to considerably reduce pathogens in soil amendments through proper composting (Day, 1998) but, control of run-off and exclusion of all animals from the field including birds, reptiles, and insects is complex and challenging (FDA, 2013). To overcome these challenges, plants with detectable fecal contaminants or those that might be potentially contaminated from animal damage are not harvested.

Irrigation water is another very important food safety component at pre-harvest as water can be a vehicle for microbial cross-contamination on herbs and leafy greens especially wastewater and

raw sewage (FDA, 2013; Armon et al, 2002; Amoros et al, 2010). The irrigation regime and morphology of the plants are important factors to consider when highlighting potential contamination through an irrigation water source.

Irrigation regime and contamination

Irrigation water regime employed is usually designed to cater for its intended use. For example, the use of canals in the furrow irrigation system might be suitable for irrigating culinary herbs most probably because of their shallow root systems, but not suitable for mixing pesticides that are applied aerially on the edible portions of the plants (FDA, 2013). It has been noted that although several irrigation systems exist, including surface irrigation, drip irrigation, sprinkler irrigation, and sub-irrigation, each is complex and has its own drawbacks. Most designs pose substantial food safety threats as a result of ecological environments with multiple potential sources of pathogenic contamination (Alegbeleye et al, 2018).

In many commercial production fields of cilantro and parsley in the United States, a combination of the overhead sprinkler systems and drip irrigation but mostly overhead sprinklers are used for irrigation (Smith et al, 2011). Both crops possess shallow root systems hence frequent short irrigation causes the plants to thrive and uniformly moist soil is maintained for maximum production. Due to the irrigation needs of cilantro and parsley, their short growth cycle and a need to minimize labor requirements, overhead solid-set sprinklers are preferred. The use of a sprinkler system of irrigation, however, is said to pose more threat of microbial contamination than the use of a furrow system although in both cases, significant microbiological contamination

occurs during transport of water from the source to the field (Alegbeleye et al, 2018; Pachepsky, 2011). The exact role of water in the contamination of leafy greens and herbs is not totally clear but evidence points to irrigation water as a vehicle for microbial contamination (Hanning et al, 2009; Hintz et al, 2010). In addition, it is important that the microbial quality of irrigation water be assessed frequently (Pachepsky, 2011). Existing microbial detection tests on water used to irrigate culinary herbs target common pathogens like *E. coli* but examination for parasites like *Cyclospora* and *Cryptosporidium* is not available.

Water used for the preparation of pesticides and insecticides or the cleaning of on-farm equipment is rarely monitored (Pachepsky, 2011). Generally, pesticides and insecticides are applied prior to emergence (at pre-emergence) of leafy greens on the field or post-emergence of crops depending on the manufacturer's recommendation. For example, Movento (spirotetramat) is applied when enough leaves are present on crops to allow for maximum uptake of active ingredients into the plant. The frequency of application might vary depending on the level of contamination in fields. During mechanical harvest operations, which are mostly employed by commercial producers, plants could be exposed to on-field contamination if the equipment is contaminated with pathogenic microorganisms. The use of well water is also a common practice.

Post-harvest

Harvesting cilantro and parsley by hand-picking, and post-harvest operations, field packing, packing facility or processing plants share one potential route of contamination which is a break down in hygienic practices. Poorly designed equipment with unreachable fresh produce contact surfaces could serve as potential vehicles for pathogenic microbial contamination and the

formation of biofilms. Good equipment design is however only as good as a constant equipment sanitation regiment and maintenance practice.

Poor worker hygiene is perhaps the most important route of contamination on herbs during post-harvest operations. Workers who are asymptomatic for certain diseases, for example, cyclosporiasis, can contaminate fresh produce, water supplies, other workers and can transmit human pathogens (FDA, 2013).

CURRENT MITIGATION STRATEGIES AND CHALLENGES

Discussion on current mitigation strategies against contamination on cilantro and parsley will draw heavily from the Fresh Culinary Herb Guidance document published in 2013 (FDA, 2013). Existing strategies are designed to address all known routes of microbial contamination and are tailored to be of relevance to culinary herb production. As described above, possible routes of contamination exist during production, harvest, and packing of fresh herbs. Given their status as RTE food commodities, pre-harvest contamination is of utmost importance and guidelines do not differ considerably from existing regulations for all fresh produce production outlined in the Good Agricultural Practice (GAP) documents, the Current Good Agricultural Manufacturing Practices (cGMP) (Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetable), or the recent Food Safety Modernization Act (FDA, 2019) and the Leafy Green Marketing Agreement (LGMA). The guidance document addresses safety at all aspects of culinary herb production including traceback and operational records keeping, pre-planting environmental assessment, advisory on animal activity on the field, water use both for foliar and non-foliar application, soil amendments, non-synthetic crop treatment, equipment sanitation, field and harvest personnel, flooding, field packing operations, GAPs and cGMPs for packing

and cooling facilities, post-harvest product containers, packaging materials, finished product materials, construction of facility outlining areas like toilet and handwashing station designs, cold storage, warehousing and necessary employee training.

While the guide document addresses virtually every aspect of safety during culinary herb production, there is no note of contamination by parasites when discussing corrective actions. *E. coli* is highlighted as the target organism during pre and post-harvest water use analysis and other routes like soil amendment do not necessarily address parasitic contamination. This is a challenge with parasitic contamination of fresh produce. A reason for this gap might be the relative length of time it takes to assay for parasites in water and or on fresh produce commodities as compared to assays for bacteria.

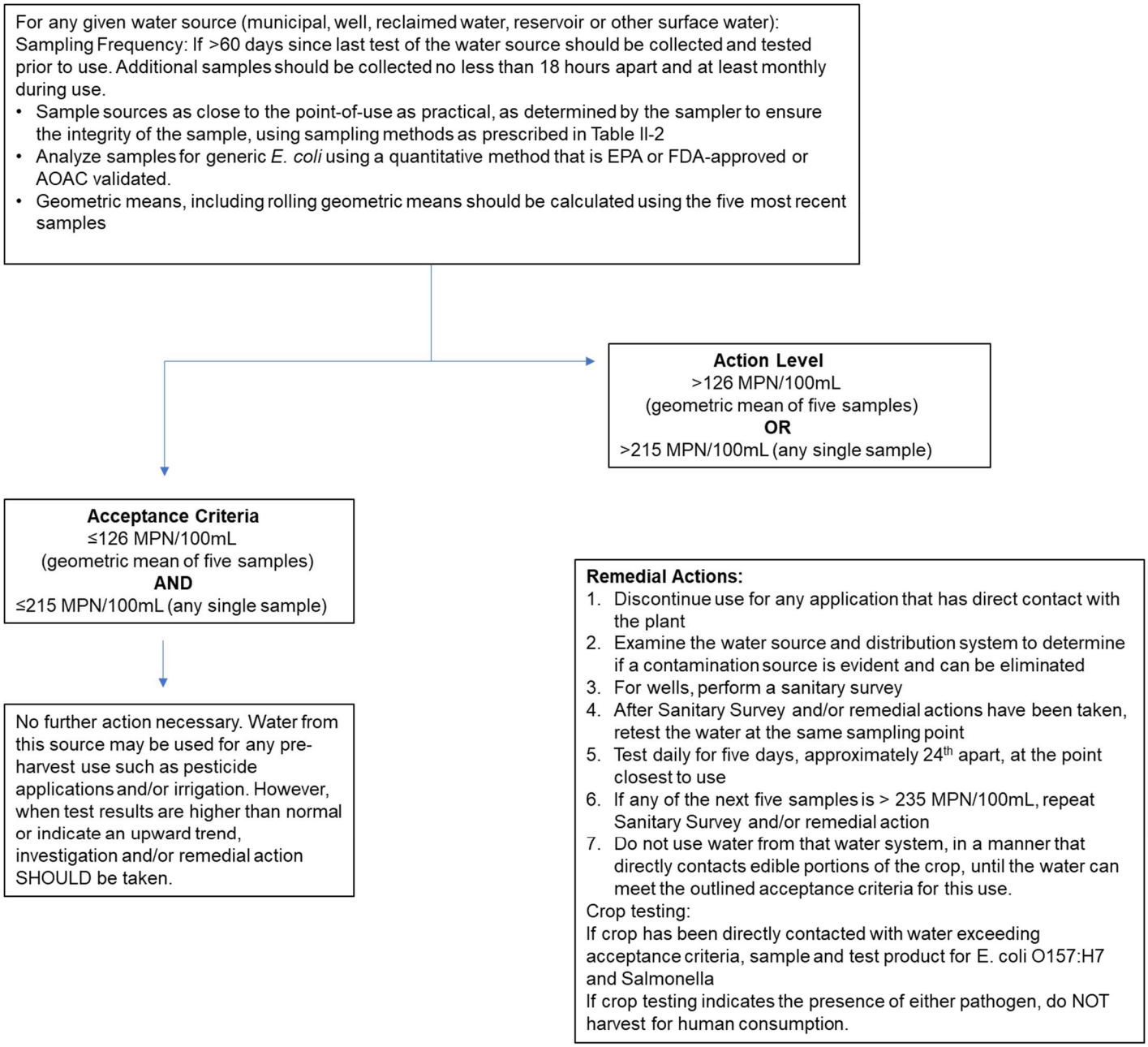

Fig 2.2: Decision tree for pre-harvest water use during the production of culinary herbs (Source: FDA fresh Culinary Herbs, 2013)

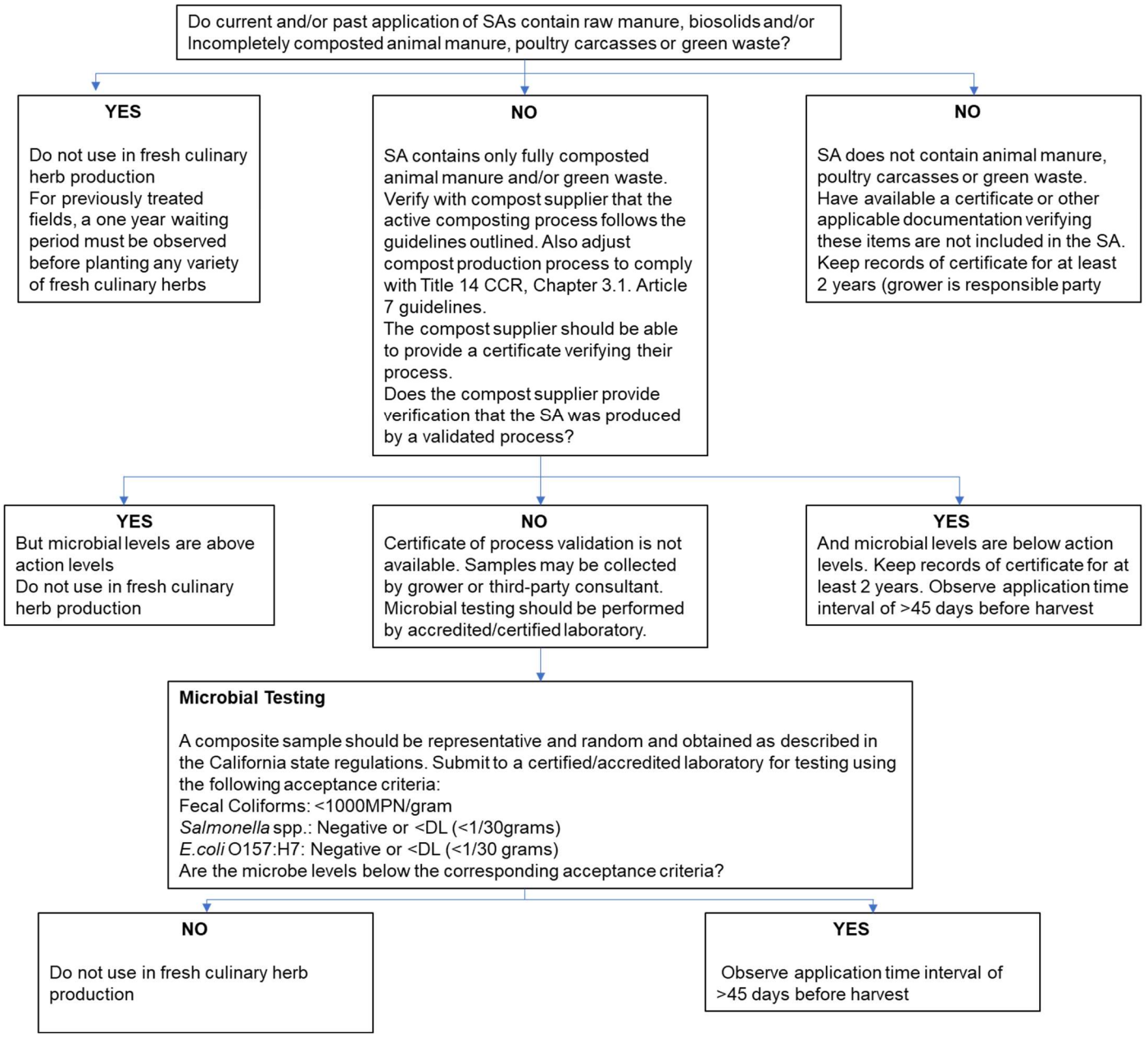

Fig 2.3: Decision tree for composted soil amendments during the production of culinary herbs

(Source: FDA Fresh Culinary Herbs, 2013)

STUDIES ON INACTIVATION OF PATHOGENIC MICROBES

Several studies have reported that pathogenic bacteria, parasites or viruses can be detected on fresh produce sold in different commercial outlets globally. Increased interest in the emergence of pathogenic microbes on food as a public health threat has resulted in numerous studies on the biology, transmission, epidemiology, genetic characterization, for example PCR amplification of specific genes (Zahraei-Salehi et al, 2006), transmission, and treatment of infection from these organisms. In the laboratory, many of the known pathogenic microbes have been challenged to different physical, chemical or biological hurdles on common food matrices in order to evaluate their microbiological and biochemical characteristics (Lapidot and Yaron, 2009; Lapidot et al; 2006). Microorganisms respond differently to different hurdles depending on the type of organism and the lethality of the hurdle (Lang et al, 2004; Macarisin et al, 2010a, Macarisin et al, 2010b).

Arguably, the most studied techniques are the physical and chemical microbial controls. Generally, for different food matrices, some of the well-studied techniques include physical controls: freezing, heating, use of UV light, irradiation, high pressure, and chemical controls: disinfectants and sanitizers (Clancy et al, 2004; Erickson and Ortega, 2006). Each control has been either used alone, in tandem with other controls within the same technique or in concert with other controls from other techniques to eliminate microbial contamination on food. For example, a sequential combination of ozone and chlorine has been used to inactivate protozoan parasites experimentally and a combination of electroporation, a physical technique and chlorine have also been shown to have a synergistic effect (Erickson and Ortega, 2006). The food matrices are, however, an important consideration when conducting research on microbial controls as this could influence the efficacy of techniques used. On leafy greens and herbs, for

example, the use of chemicals (sanitizers) is the most preferred control method because based on researched acceptable levels, they are effective in reducing or eliminating pathogenic organisms of public health concern without adversely affecting the quality of produce or its safety for consumers.

PARASITES ON PRODUCE AND HERBS

Cryptosporidium parvum and *Cyclospora cayetanensis* are coccidian parasites and have caused food and waterborne outbreaks. Both coccidia causes severe diarrhea in the immunocompetent, and more severe and prolonged diarrhea in immunocompromised individuals who are exposed to contaminated water sources even at low doses (DuPont et al, 1995). Recently, however, oocysts have been detected on fresh produce, particularly leafy greens (Mota et al, 2009). Reports indicated that these parasites might have been introduced on leafy green crops through contaminated irrigation water or contaminated fecal matter in the form of manure. Leafy greens harvested in regions of Poland with a high livestock population had more parasite oocysts than in regions with lower livestock activity (Rzezutka et al, 2010). Their results showed that of all 63 farms examined, those that had oocyst contamination were in locations with the highest livestock population, and regions with little or no livestock had no contamination. Direct contact with infected livestock could also lead to infection in humans as seen in an outbreak in Norway in which schoolchildren visiting a farm acquired cryptosporidiosis after exposure to lambs and goats (Lange et al, 2014).

Calves typically acquire cryptosporidiosis the first week after birth and those younger than 15 days old frequently shed high numbers of *Cryptosporidium* oocysts (9 log-10 per day). These parasites are excreted by the infected calves and introduced in the environment when the feces

are used for composting of soils (Davies et al; 2003). Feces could also end up in surface waters such as rivers or lakes/ponds. *Cryptosporidium* oocysts are highly resistant to pesticides (Sathyanarayanan and Ortega, 2004), hence, they could be deposited directly and persist on the surface of the edible portions of crops if contaminated waters are used for the application of pesticides. This is also the likely cause when the parasites are introduced through contaminated irrigation water.

Cryptosporidium and *Cyclospora* have been detected in bagged salads from Spain and the United States, and on vegetables collected from markets in Peru, Mexico, and Nigeria (Maikai, 2013; Dixon et al, 2013; FDA, 2019); the two parasites have also been linked to several foodborne outbreaks (Herwaldt et al, 2000; CDC, 2018). Some studies have sought to understand the life cycle and propagation of these parasites, and also to determine the transmission dynamic of microbial pathogens, their survival or persistence on infected crops (Wagner-Wiening and Kimmig, 1995; Winsen et al, 2001; Wood, 2013) however, most studies on plant inoculations with pathogenic organisms involve spiking crops experimentally in the lab, or growing crops in chambers at controlled environmental conditions. None of these studies model the introduction of foodborne pathogenic parasites naturally while in the field, or the effect of natural climatic conditions and seasonal changes on their persistence or survival. Other studies focus on developing improved detection and or identification techniques for different pathogenic microbial strains (Shi et al, 2015; Slifko et al, 1997). Some field studies have made some effort to have this natural model using *Salmonella* Typhimurium and *E. coli* mostly on soil fertilizer contaminated by these pathogens, few on fresh leafy greens (Islam et.al, 2004; Ongeng et. al, 2015), but none for parasites.

Lack of field studies for the survival of *Cyclospora cayetanensis* and *Cryptosporidium parvum* is coupled with the lack of information on the viability of these parasites especially *Cyclospora cayetanensis*. This gap has been highlighted in some reviews. Many studies on infectivity of *Cyclospora cayetanensis* have reported inconclusive results for different animal models including humans exposed to experimentally sporulated oocysts perhaps attributed to host specificity (Ortega and Sanchez, 2010).

We have no data on the survival of coccidian parasites on fresh produce in the natural environment, and while contaminated irrigation water could transfer these parasites onto fresh produce (Decol et al, 2017), no study has evaluated or substantiated this claim. Researchers have reported a marked seasonality in the epidemiology of these parasites but even the distribution of outbreaks in different countries proves to be quite an enigma. It is apparent that lots of questions are still left unanswered with regard to coccidian parasites and their introduction to fresh produce.

Cyclospora cayetanensis

Cyclospora is an apicomplexan intracellular coccidian protozoan parasite that infects the gastrointestinal tract (Ortega and Sanchez, 2010; Almeria et al, 2017; Mansfield and Gajadhar, 2004). Oocysts of this parasite are spherical and measure between 7.7 to 9.9μm in diameter. Unsporulated oocyst walls are composed of two layers, mostly consisting of proteins and lipids. This wall provides the parasite with protection against physical and chemical treatments and an autofluorescence capability which might confer protection against UV radiation. Autofluorescence also helps in the identification of *Cyclospora cayetanensis* which appears blue by epifluorescence microscopy and green using a 450nm – 490nm dichroic filter (Sterling and

Ortega, 1999). When the cells are differentiated or sporulated, the oocyst has two sporocysts (with sporocyst walls), and each sporocyst has a stieda and substieda body. Within each sporocyst are two sporozoites.

Cyclospora is noninfectious when excreted in the feces of the infected host and requires 7 to 15 days outside the host under the right environmental conditions, 23-27º C, to sporulate and become infectious (Herwaldt, 2000; Ortega and Sanchez, 2010). This characteristic has made it difficult to understand the epidemiology of *Cyclospora*. Clinical symptoms of cyclosporiasis include loose or watery diarrhea, nausea, vomiting, abdominal cramps, loss of appetite, weight loss, fever, chills, joint aches, generalized body aches and fatigue (Cama and Ortega, 2018). Cyclosporiasis has emerged as a global health problem (Bonilla, L. 2013). In 1996, *Cyclospora* was linked to an outbreak involving raspberries imported from Guatemala (Herwaldt et al, 1997). Afterward, there was an increase in reported cases of *Cyclospora* outbreaks involving food sources in both developing and developed countries including the United States. Outbreaks and infection have been reported in Nepal, Canada, Southwest Germany, Mexico, Indonesia, Guatemala, Colombia, and Peru (Ortega and Sanchez, 2010). In 2013, during June – July, 643 cases of cyclosporiasis were reported in 25 states, including the states of Texas (278) and Iowa (153) and Nebraska (86). Clusters in the states of Iowa and Nebraska were linked to consumption of a bagged salad mix imported from Mexico and those in Texas were linked to consumption of imported cilantro. In 2014, 304 cases of cyclosporiasis were reported in 19 states with 64% of cases in Texas. In 2015, cilantro was implicated in a cyclosporiasis outbreak which led to a case count of 546 with 21 hospitalizations in 31 states of the U.S. Again, in 2016, 384 cases of cyclosporiasis were reported with unknown source of infection. Between 2016 and 2019, 2,415 clinically confirmed cases of cyclosporiasis were reported (CDC, 2018).

Generally, U.S. cases of *Cyclospora* infection have been reported to cluster around the summer period from May through August with peaks in June and July (Herwaldt, 2000). The marked seasonality of *Cyclospora* in different countries shows that outbreaks fall in different months of the year. Outbreaks have been recorded in Canada during the months of June-July and in Peru during the months of December to April (Ortega and Sanchez, 2010). In Nepal, infection has been observed to occur predominantly during the warm and monsoon months typically May-August but decreases just before the rains end. Data from Haiti showed a different pattern with outbreaks clustered around the relatively drier and cooler months in the first quarter of the year (Herwaldt, 2000). It has been suggested, however, that more attention be paid to environmental conditions like temperature and relative humidity over seasons when studying *Cyclospora.* Some studies have reported the detection of *Cyclospora* oocysts in market water samples, and wastewater samples suggesting that irrigation water is a plausible route of introduction on fresh produce (Sturbaum et al, 1998).

Cryptosporidium parvum

Cryptosporidium is a coccidian parasite that causes a gastrointestinal illness called cryptosporidiosis. For people with weakened immune systems, symptoms can be severe and could lead to life-threatening illnesses (Caccio and Widmer, 2014). The life cycle of *Cryptosporidium parvum* includes sporulation within a host and the shedding of sporulated and infectious oocysts into the environment. This is one of the major differences between the coccidian parasites *Cryptosporidium* and *Cyclospora.* *Cyclospora* sporulates externally in the environment and is noninfectious when shed, *Cryptosporidium* is highly infectious when shed into the environment hence person to person transmission, as well as the oral-fecal route, are

modes by which *Cryptosporidium* is disseminated. The oocyst stage is of primary importance in the survival, dispersal, detection, and identification of *Cryptosporidium* (Fayer et al, 2000). Under laboratory conditions, *Cryptosporidium* has been shown to survive, with 25% viable cells observed after 14 days of exposure at 4ºC and under ambient temperatures with exposures to light and dark cycles (Utaakar et al, 2017). This means that *Cryptosporidium* can potentially survive field temperatures and conversely will not be affected by refrigeration. This is consistent with studies that have reported the detection of *Cryptosporidium* on salad and precut ready to eat fresh produce, irrigation water and leached through soil (Amoros et al, 2010; Dixon et al, 2012; Boyer et al, 2009; Jenkins et al, 2010). In one study, *Cryptosporidium parvum* was exposed to a range of environmental pressures including freezing and desiccation. Snap freezing at a temperature of -20 ºC was shown to kill the oocysts and slow freezing was less effective. In this study desiccation for >2h resulted in 100% death of oocysts. *Cryptosporidium* has also been shown to be resistant to disinfectants, so treatment of water with chlorine and monochloramine has a negligible influence on its persistence in water (King and Monis, 2007; Zintl et al, 2010). Just like the related coccidian parasite *Cyclospora*, there seems to be a marked seasonality to the incidence of outbreaks involving *Cryptosporidium* on fresh produce. One study reported an increased incidence of *Cryptosporidium* infection when the climatic temperatures and precipitation increased; this is likely to occur during the summer period (Jagai et al, 2009). However, seasonal patterns seem to vary by location. An example is the incidence of cryptosporidiosis among children in the temperate northern part of India which correlated with temperature but no observable correlation among children in the southern parts of India. These examples point to a need to pay close attention to environmental conditions like temperature, relative humidity, and precipitation in outbreak locations and especially for research. In 1993,

409,000 people in Milwaukee were infected with cryptosporidiosis which was linked to the city's Southern municipal water system. As many as 4,400 people were hospitalized during this outbreak and $96.2 million in revenue lost (MacKenzie et al, 1994). In 1998, an outbreak of cryptosporidiosis was reported after several students at a Washington DC campus reported the onset of diarrhea. The affected persons were reported to have eaten in one of two cafeterias on campus in which 4 employees were also reported to have been infected. Investigations which included genotyping of the etiological agent revealed that *Cryptosporidium parvum* genotype 1 was responsible for this outbreak and had been transmitted by an infected food handler on the campus (Quiroz et al, 2000). *Cryptosporidium spp*. has also been detected on salad products in Spain and on raw vegetables in Nigeria (Maikai et al, 2013; Amoros, 2010). Although most of the cases with *Cryptosporidium* have been waterborne, observed outbreaks and detection on produce call for more attention to foodborne transmission of this parasite.

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CHAPTER 3

PERSISTENCE OF *CYCLOSPORA CAYETANENSIS*, *CRYPTOSPORIDIUM PARVUM,* AND *SALMONELLA* TYPHIMURIUM ON CILANTRO AND PARSLEY UNDER CONTROLLED AND NATURAL CHANGING ENVIRONMENTAL CONDITIONS

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Abstract

Fresh produce and common herbs have been often linked to contamination by coccidian protozoan parasites. In this study, the persistence of *Cyclospora cayetanensis* and *Cryptosporidium parvum* and the bacterium *Salmonella enterica* Typhimurium was evaluated on the popular herbs cilantro (*Coriandrum sativum*) and parsley (*Petroselinum crispum*) under natural environmental conditions on the field in Tifton, Georgia and controlled conditions in a growth chamber, with environmental parameters modeling those in agricultural growing areas associated with products linked to outbreaks of cyclosporiasis. Two separate plots with 10 replicate subplots each of cilantro and parsley were used in this study, and pathogens were introduced on the crops by spray irrigation. Cilantro and parsley plant samples were collected within a period of 23 days and evaluated for pathogen persistence. *Cryptosporidium* and *Cyclospora* were analyzed by molecular assays and *Salmonella* Typhimurium by a culture assay. The parasites were detected in 100% of all samples tested up to day 23 in both crops held in a growth chamber. No significant difference ($P > 0.05$) was observed in persistence on both herbs from the field evaluated in the spring of 2018 and in the spring of 2019. *Salmonella* was isolated up to day 15 on both herb types in the field in the two trials with variations in persistence pattern. All the pathogens were detected in the growth chamber until day 23. There was no significant difference between the persistence of the parasites on both herbs; however, the persistence of *Salmonella* Typhimurium differed significantly between cilantro and parsley under field conditions. Our study demonstrates for the first time that *Cyclospora cayetanensis* and *Cryptosporidium parvum* are resilient pathogens and conditions in a controlled environment could be more conducive for the persistence of foodborne pathogens.

Highlights

- Parasites displayed extended persistence in both natural and controlled environmental conditions
- Persistence of pathogens was significantly higher in the controlled conditions of the growth chamber than in the fields under natural changing environmental conditions
- Data suggests that some foodborne protozoan parasites might be more resistant than others when challenged in natural environmental conditions.

Cilantro (*Coriandrum sativum*) and parsley (*Petroselinum crispum*) are common herbs best known for their medicinal properties and culinary savory. Herb consumption in the United States grew to 55.1 million by 2007 according to the United States health interview survey conducted between 2002 and 2007 and generated $7.1 billion in revenue. This rise in consumption has been attributed both to an increase in aging baby boomers who are motivated to identify alternative methods to improve their quality of life and to the mass media that has created an awareness of herbs by providing regular reports on their putative healing properties. However, in recent years the popular herbs cilantro and parsley have been implicated in several foodborne outbreaks in the United States. Between 1996 and 2015, at least five outbreaks resulting in over 2,000 illnesses and 84 hospitalizations were linked to the consumption of cilantro and parsley both alone and in salad mixes. The predominant etiological agents in these outbreaks have been the pathogenic coccidian protozoan parasites *Cyclospora cayetanensis* and *Cryptosporidium parvum*.

In 1996, an outbreak at a wedding ceremony at a Boston restaurant was described. One hundred and one guests attended the wedding ceremony but when several complaints of diarrheal illness among attendees were observed, an investigation was triggered to ascertain the cause of this outbreak. Fifty-seven cases of cyclosporiasis were detected with an attack rate of 61% and traceback investigations identified raspberries as the tainted food source (Fleming et al, 1998). That same year, a report had been published on widespread outbreaks of cyclosporiasis associated with imported raspberries from Guatemala to the United States resulting in 1465 cases in 20 states (Herwaldt et al, 1997). Consequently, the nascent Guatemalan raspberry industry took a big hit, with a depleted number of raspberry growers from 85 prior to 1996 to just three in 2002 (Calvin et al, 2003). A number of children in Peru (63) had been reported to be infected *Cyclospora cayetanensis* and the first reported case of foodborne Cyclosporiasis in Europe

occurred in Germany in 2000 affecting 34 people who attended a luncheon (Madico et al, 1997; Doller et al, 2002). By 2013, reports on *Cyclospora* outbreaks linked to fresh produce and herbs began to emerge. An outbreak linked to cilantro grown at Puebla, Mexico and exported to the United States was reported that year, which resulted in 631 cases of cyclosporiasis in 25 states with 25 hospitalizations but no death (CDC, 2013). In 2014, another outbreak linked to *Cyclospora* on cilantro was reported. This predominantly occurred in Texas and lead to 304 cases. Outbreaks of cyclosporiasis have been subsequently reported annually. Two large outbreaks occurred in 2018: salads sold at McDonald's restaurants were implicated as a source of a *Cyclospora* outbreak that had resulted in 511 cases in 16 states. As a result, 3,000 McDonald's restaurant locations across 14 states stopped the sale of salad mixes. Concurrently, Del-Monte pre-packaged vegetable trays were also linked to *Cyclospora* outbreaks resulting in 250 clinically confirmed cases and a massive recall of 6 oz, 12 oz and 28 oz prepackaged vegetable trays (CDC, 2018a, CDC, 2018b).

Infection from the coccidian protozoan parasites *Cyclospora cayetanensis* and *Cryptosporidium parvum* had been considered a challenge with people who had traveled internationally; however, an increase in the number of clusters occurring within the United States among persons who reported no international travel was observed, indicating that contamination might be occurring in the US (CDC, 2018b). According to the CDC, "domestically acquired" cyclosporiasis or cases of cyclosporiasis that are not associated with travel to a country that is considered endemic for *Cyclospora* is becoming more common and tends to occur mostly around the spring and summer months (CDC, 2019). In a review by Herwaldt et al, in 2000, the marked seasonality of *Cyclospora* infection had been highlighted as an intriguing issue with this pathogen. The review paper suggested that a certain range of temperature, humidity, and other environmental

conditions might influence the sporulation or survival of the coccidian parasites (Herwaldt, 2000). Also, *Cyclospora cayetanensis* has been reported to exhibit host specificity after unsuccessful attempts to create infection in laboratory animals (Eberhard et al, 2000). Another interesting research gap highlighted by Herwaldt et al, 2000 was the possibility of a difference between the persistence of *Cyclospora* and *Cryptosporidium* when exposed to environmental stresses based on differences in their morphologies.

Coccidian protozoan parasites, much like bacteria can contaminate food crops in several ways both at pre and post-harvest. Some of these routes include contaminated irrigation water, contaminated soil, infected wild or domestic animals and infected farmworkers (Islam et al, 2004; Lapidot and Yaron, 2009; Lapidot et al, 2006, Ongeng et al, 2015; Mansfield and Gajadhar, 2004; Ortega and Robertson, 2017; Rzezutka et al; 2010; Rose and Slifko, 1999; Wood, 2013) . Many discussions on produce safety, points to irrigation water as the most probable source of contamination on produce, especially when the pattern of contamination observed with coccidian protozoan parasites on crops in the field is put in perspective. However, no scientific research has established a link between contaminated irrigation water and fresh produce contamination. In addition, most studies with protozoan parasites have involved general detection on commercial produce, optimized detection methods or studies on food matrices under controlled conditions (Cook et al, 2006; Dawson et al, 2004; Dixon et al, 2013; Erickson and Ortega, 2006; King and Monis, 2007; Kniel et al, 2002; Laberge and Griffith, 1996; Lalonde and Gajadhar, 2008; Macarisin et al, 2010; Murphy et al, 2018; Ortega et al, 1997; Sathyanarayanan and Ortega, 2004; Utaaker et al, 2017; Zintl et al, 2010, Palumbo et al, 2013; Ferretti et al, 2001; Wagner and Kimming, 1995) . No studies have challenged these parasites to the natural changing environmental conditions.

The objectives of this study, therefore, are: To evaluate the persistence of *Cyclospora cayetanensis*, *Cryptosporidium parvum,* and *Salmonella* Typhimurium under changing environmental conditions and to assess the impact of changing environmental conditions on the persistence of these pathogens.

Study 1: Persistence on the field under natural changing environmental conditions

Plot design

Two separate plots measuring 13.7 m by 4.6 m for cilantro (*Coriandrum sativum*) and parsley (*Petroselinum crispum*) with four replicate subplots, located at the University of Georgia horticulture research farm Tifton campus, Tifton Georgia was used in this study. Each subplot contained ten replicate plots within, measuring 2 m by 24 cm. The replicate plots within each subplot contained 10 plants of cilantro on one plot and parsley on the other plot. The acreage was cultivated to achieve a split-plot type experimental block design with four different plots for each herb type (one for *Salmonella* Typhimurium, one for *Cyclospora cayetanensis*, one for *Cryptosporidium parvum* Iowa isolate and one for *Cryptosporidium parvum* Maine isolate).

Production of cilantro and parsley

Parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) were grown according to practices outlined in the handbook for vegetable growers (Lorenz and Maynard, 1980). Seedlings of parsley (cultivar; Giant of Italy) and cilantro (cultivar; Santo) were purchased commercially and visually inspected to ascertain seed quality. Seeds were planted manually (broadcasted) in early January in both 2018 and 2019. Irrigation of plants was achieved through a drip system.

Preparation of *Salmonella* inoculum

Frozen stock culture of *Salmonella* Typhimurium 8243 (genotype *Tn10(dKm),* a derivative, defective transposon avirulent strain derived from S. Typhimurium LT2 by Russell Maurer, Case Western Reserve University, Cleveland, OH, and obtained from Dr. Roy Curtiss III, Washington University, St. Louis, MO (Curtiss and Kelly, 1987) was thawed and 100 µL was transferred into a test tube containing 9 mL of Tryptic Soy Broth (TSB; Neogen Laboratories, Lansing, Michigan) followed by incubation at 37 ºC for 24 h. Culture from the TSB was streaked onto Tryptic Soy Agar (TSA; Neogen Laboratories, Lansing, MI) and further incubated at 37 ºC for 24 h to produce fresh *Salmonella* cultures. Colonies from this fresh culture were collected using a wire loop and inoculated into four different test tubes containing 9 mL of TSB and incubated once more at 37ºC for 24 h. Fresh culture in TSB was transferred to 50 mL centrifuge tubes and washed with E-pure water by centrifuging three times and resuspending the pellets in E-pure water after each wash. Pellets were resuspended in 10 mL of E-pure water at the last wash to achieve a final concentration of 10^9 CFU/mL. Cell concentration was confirmed by conducting an optical density (OD) reading on a spectrophotometer and by serial dilutions on XLT-4 agar (XLT 4; Neogen Laboratories, Lansing, Michigan) and TSA plates. The culture used for inoculation was grown 24 h before intended use and prepared on the same day of sample inoculation to ensure it was fresh and fully viable.

Confirmation of *Salmonella* strain

The specific strain of *Salmonella* used was confirmed by both pulsed-field gel electrophoresis (PFGE) as outlined in the FDA standard operating procedure for Pulse Net PFGE of *E. coli*

O157: H7, *E.coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei,* and *Shigella flexineri*, and polymerase chain reaction (PCR) targeting the *avaR* and *invA* genes. Ten additional strains of *Salmonella* (*S.* Enteritidis ATCC BAA-1045, *S. enterica* Tennessee, *S. enterica* Brandenburg, *S. enterica* Baildon, *S. enterica* Agona, *S. enterica* Gaminara, *S. enterica* Enteritidis ME 18, *S. enterica* Enteritidis M4639, *S. enterica* Enteritidis H4717, and *S. enterica* Heidelberg) were analyzed using the PFGE method for increased confidence.

Random checks were conducted using the *Salmonella* presumptive latex agglutination test kit (Thermo Scientific™ Oxoid™ Salmonella test kit) during this study to ensure consistency of enumerated *Salmonella* on samples.

Enrichment for *Salmonella*

The avirulent *Salmonella* Typhimurium strain used in this experiment was a deletion mutant lacking adenylate cyclase and cyclic AMP receptor protein. This property resulted in its avirulence although it remained immunogenic; also, this caused observable slow growth of the bacteria. To account for this slow growth during enumeration, some modifications were made to the enrichment protocol used. After the initial wash of sample bags containing the herbs, 100 mL of TSB was added into the bag and content was properly mixed by shaking vigorously for 10 seconds. Bags were incubated at 37 ºC for 24 h. Vegetables in the bags were discarded and 1mL of wash fluid was transferred into 9 mL of Rappaport-Vassiliadis broth (RV, Neogen Laboratories, Michigan) followed by further incubation at 37 ºC for 24 h. Pellets from the RV broth (100 µL) were transferred into a 1.5 mL microcentrifuge tube containing 0.1 % peptone and plated onto XLT 4 plates. Plates were incubated at 37 ºC for 48h.

Preparation of *Cryptosporidium parvum* and *Cyclospora cayetanensis*

Three strains of *Cryptosporidium parvum* were used in this study: The first was obtained from fresh feces from 10-15-day old calves at Williams Dairy Farm, Madison, Georgia, the second strain *Cryptosporidium parvum* (Iowa genotype II 353152) has been used in multiple studies on cryptosporidiosis and was originally isolated from a calf in Iowa. The third strain *Cryptosporidium parvum* (Maine genotype II) and was responsible for the cryptosporidiosis outbreak on apple cider in 1993 (Millard et al, 1994). Both the Iowa and Maine isolates were propagated in calves and kindly provided by Dr. Michael Arrowood of the Center for Disease Control and Prevention (CDC, Atlanta, GA). *Cyclospora cayetanensis* was obtained from fecal samples from clinical cases of cyclosporiasis. Fecal samples containing oocysts were processed using a modified ethyl acetate method described by Sathyanarayanan and Ortega, 2006. After resuspension, concentrations of *Cryptosporidium parvum* and *Cyclospora cayetanensis* in inocula were 10^8 oocysts/mL and 5.5 x 10^5 oocysts/mL respectively.

Inoculation procedure and sampling of plants

Inocula were prepared in 400 mL of E-pure water for each of the microorganisms evaluated. The microorganisms were prepared independently and introduced to the allotted replicate plot. Each replicate subplot for cilantro and parsley was inoculated with 200 mL of inoculum containing 1mL of *Cyclospora cayetanensis* (10^5 oocysts/mL), 1 mL of *Cryptosporidium parvum* from a local dairy farm (10^8 oocysts/mL), and 500µL of *Salmonella* Typhimurium (10^9 CFU/mL) by spray irrigation when plants were 20 cm long and 4 weeks old. Sampling was conducted by randomly collecting one plant from each plot for each plant type for analysis on days 0, 1, 2, 3, 7, 15, and 23 over the 2018 and 2019 growing seasons (February – May); 100g for *Cyclospora* and

Cryptosporidium testing, and 25g for *Salmonella* testing. Seven replicate samples for each plant was collected per sampling day for each pathogen (Hence, 7 replicate samples per plant were collected from plot inoculated with *Cyclospora* on each collection day; 7 replicate samples per plant from the plot with *Cryptosporidium* and 7 replicate samples from the plot with *Salmonella* Typhimurium). Uninoculated plants were also included as controls. *Cyclospora* and *Cryptosporidium* were analyzed using Nested PCR according to the protocol outlined in the FDA Bacteriological Analytical Manual (2017) and by Zhou et al (2011) and Xiao et al (1999) while *Salmonella* was enumerated on XLT 4 agar.

Sample washing and processing

Herbs sampled each day were weighed into sterile filter sample bags (VMR sterile sampling bags for blender; VWR International), 25 g of each herb for *Salmonella* enumeration and 100 g for the detection of parasites. For *Salmonella*, 100 mL of Phosphate-Buffered Saline (PBS 1X) was added into the sample bags with herbs and the bags were placed in a stomacher for 15 sec at 260 rpm. Serial dilutions were made in 0.1 % peptone from the wash fluid with the first dilution made in Dey-Engley neutralizing broth (DE 2X). Dilutions were plated on XLT4 agar plates, incubated at 37 ºC and colonies read after 48 h. For samples inoculated with the parasites, 200 mL of elution buffer (1X) was added into the bags and the bags were massaged for 15 min (This was achieved by gently compressing the bags between the palms of both hands, ensuring that the herbs were in contact with the wash solution, for 1 min, followed by a 5 mins rest where bags were allowed to sit; further compress for 1 min, another 5 mins rest and a final compress for 3 min). The wash solution was collected in 50 mL centrifuge tubes and centrifuged at 1,308 x g for 20 min. The supernatant was discarded followed by a subsequent wash with E-pure water and the

pellets resuspended and wash repeated three times. At the final wash, pellets were resuspended in 5 mL of E-pure water and refrigerated at 4 ºC ready for DNA extraction and microscopy.

DNA Extraction for parasites

DNA from the samples was extracted following the manufacturer's manual in the Fast DNA spin kit for soil (Fast DNA^{TM} Spin for Soil, MP Biomedicals, LLC, Solon, OH) with some modifications:180 µL of the sample and 20 µL of 10 % milk was added in the lysing matrix E-tubes. All the centrifugation steps outlined in the manual were performed at 11,337 x g and the 15 mL tubes containing the silica binding matrix and samples were left to stand for 12 min in order to allow for proper settling of the matrix. The elution at the final step was done with 65 µL of DNase/pyrogen-free water.

PCR protocol

A nested PCR method described by (Zhou et al., 2011) for *Cyclospora cayetanensis* and (Xiao et al, 1999) for *Cryptosporidium* spp. was used. Visualization of amplified DNA was done on 2 % agarose electrophoretic gels with 3 µL ethidium bromide. For *Cyclospora*, a 501bp fragment of the 18S rRNA gene was amplified while the 819-825 bp fragments of the 18S rRNA gene were amplified for *Cryptosporidium*.

Environmental Monitoring: The temperature, relative humidity, and solar light intensity on the field were measured using the Onset Hobo relative humidity logger (Pro V2 UX 23-001), and the Onset Hobo temperature and light intensity logger Onset®, Bourne, MA. Loggers were set to collect data at time intervals of 30 minutes and placed in the field from the day of inoculation

and first sample collection (day 0) until the last day of sample collection (day 23). Additional field condition data were obtained from the Coastal plain experiment station University of Georgia Weather Network Tifton, Tift Country, Georgia.

Statistical analysis

Data was analyzed using JMP statistical software (SAS Institute, NC USA). A combination of the student's t-test and the Tukey's HSD test was used to compare observed persistence patterns over the two evaluated years on the field and between the field study and study in the controlled growth chamber. Persistence patterns between the two evaluated crops, cilantro and parsley were also compared using the t-test.

Study 2: **Persistence under controlled conditions of an Envirotron growth chamber**

The varieties of cilantro and parsley, and all the procedures used in the Envirotron growth chamber were the same as those used for the study in the field experiments including protocols for inoculum preparation, inoculation of the herbs, sample collection, and processing, enrichment for bacteria, DNA extraction and nested PCR for the parasites, environmental monitoring and statistical analysis. However, some minor modifications were made:

Design and preparation of cilantro and parsley

This study was conducted in the Georgia Envirotron (Model PGW36, Controlled environments limited, Winnipeg, Manitoba, Canada) facility at the University of Georgia Griffin Campus. The chamber space was 6.7 m^2 and equipped with high-intensity lighting, temperature, humidity, and

carbon dioxide (CO_2) controls and an automated drip irrigation system (Conviron model PGW36; Controlled environment Limited Winnipeg, Manitoba Canada). A hundred and fifty pots of cilantro (*Coriandrum sativum,* variety, Santo) and parsley (*Petroselinum crispum* variety, Giant of Italy) plants (three weeks old) were purchased commercially from Bonnie plants (Bonnie Plants Inc. AL). Plants were placed inside the growth chamber with conditions set to mimic those in Puebla, Mexico (Max temp: 21 ºC, Min. temp: 10 ºC, 10 h daylight time) . Plants were allowed for 1 week to adapt to conditions in the chamber before the study commenced.

Inoculation procedure and sampling of plants

The inoculum was prepared in 400 mL of E-pure water for each of the microorganisms evaluated. The microorganisms were prepared independently and introduced to the allotted replicate plot. Each replicate subplot for cilantro and parsley was inoculated with 200 mL of inoculum containing 1mL of *Cyclospora cayetanensis* (10^5 oocysts/mL), 1 mL of *Cryptosporidium parvum* (10^8 oocysts/mL), and 500 µL of *Salmonella* Typhimurium (10^9 CFU/mL) by spray irrigation when plants were 20 cm long and 4 weeks old (*plants were in pots with 3 plants per pot. However, the pots were arranged accordingly to mimic the replicate plot design on the field; Each plot received a uniformly spread number of spray puffs to equate 200 mL per plot*). Sampling was conducted by randomly collecting one plant from different pots on each plot for each plant type and for each pathogen. Uninoculated plants were also collected as controls. Collections for analysis were made on days 0, 1, 2, 3, 7, 15, and 23. Samples of 50 g for *Cyclospora* and *Cryptosporidium* and 25g for *Salmonella* were collected. Five replicate samples for each plant was collected per sampling day for each pathogen (Hence, 5 replicate samples per

plant were collected from the plot inoculated with *Cyclospora* on each collection day; 5 replicate samples per plant from the plot with *Cryptosporidium* and 5 replicate samples from the plot with *Salmonella* Typhimurium; n = 280 samples analyzed). *Cyclospora* and *Cryptosporidium* were analyzed using nested PCR according to the protocol outlined in the FDA Bacteriological Analytical Manual (FDA, 2007) while *Salmonella* was enumerated on XLT 4 agar.

RESULTS AND DISCUSSION

Persistence of the pathogens

In the Field: *Cyclospora cayetanensis* persisted for 23 days, which was the duration of sample collection (n = 70), with 5% detection on day 23 on cilantro and for 7 days on parsley while *Cryptosporidium parvum* could not be detected beyond day 3 for plants sampled (n = 70) between February – March 2018, when viewed after electrophoresis on 2% agarose gels. *Salmonella* Typhimurium persisted until day 7 and 15 on cilantro and parsley respectively sampled between February – March 2018 after enrichment with both TSB and RV broth and enumeration on XLT 4 agar plates. Detection and persistence of *Cyclospora cayetanensis* for plants sampled between February – March 2019 were comparable to those observed the previous year, but two different strains of *Cryptosporidium parvum* (Iowa and Maine) showed slight differences in persistence; although both persisted until day 3 on the two plants (*Cryptosporidium parvum* Iowa persisted until day 3 while *Cryptosporidium parvum* Maine persisted until day 7). *Cryptosporidium parvum* strain isolated from calves in Georgia persisted until day 3 on both plants on the field (40% positive cilantro samples n = 20 on day 3 and 10% positive parsley samples n = 20 on day 3), however, no positive samples were detected on day 7.

In the Growth Chamber: The persistence of the pathogens under the controlled conditions of the growth chamber was completely different from observed persistence on the field under natural environmental conditions. *Salmonella* Typhimurium persisted for 23 days on both herbs and plant samples collected on day 23 for *Cyclospora cayetanensis* and *Cryptosporidium parvum* (from both geographical locations, Iowa and Maine) were positive (60% detection for *Cyclospora* on parsley n = 12/20 and 80% on cilantro n =16/20; 80% for *Cryptosporidium parvum* Iowa on parsley n = 16/20 and 100% on cilantro n =20/20; and 80% for *Cryptosporidium parvum* Maine on parsley n = 16/20 and 100% on cilantro n = 20/20). Coccidian parasites are known to be typically hardy and as postulated by Herwaldt (2000) the need for oocysts to survive long enough in the environment to sporulate and be ingested might suggest that *Cyclospora cayetanensis* and *Cryptosporidium parvum* are resilient and persist for extended periods in the environment. In the 1997 and 1999 outbreaks of cyclosporiasis in Missouri, it was a challenge to ascertain if contamination of basil had occurred from handlers of the food product or from the field considering the short period of time it takes for the consumption of produce commodities after harvest (Lopez et al, 2001). *Cyclospora cayetanensis* takes between 7-15 days at room temperature to sporulate and become infective. Factoring the typical time for produce consumption in the United States after distribution from farmers, contamination from an infected food handler would not really make sense hence contamination might have occurred from the fields. Our study which incorporated a challenge study on the field modeling natural contamination shows that indeed coccidian protozoan parasites are robust and can survive for extended periods based on the detection of parasite DNA. The viability of *Cryptosporidium* and sporulation of *Cyclospora* were, however, difficult to evaluate on the plant

samples; the current methods involve the use of microscopy and cell cultures which are limited by the high levels of contamination and artifacts from environmental samples.

Influence of changing environmental conditions on pathogen persistence

It has been suggested that the marked seasonality of infection from the coccidian parasites (including the growing and harvest seasons of implicated produce) might indicate that a certain range of temperatures, relative humidity, and other environmental factors could facilitate sporulation or persistence of the parasites (Herwaldt, 2000). In our study, there was no significant difference between the average temperature, relative humidity, and light intensity on the field in the spring seasons of 2018 and 2019 ($P > 0.05$). However, there were significant differences when the conditions on the field were compared to the conditions in the growth chamber ($P < 0.05$). The temperatures and light intensity varied significantly between the two conditions but there was no significant difference between the relative humidity under the two conditions tested. In one study evaluating the effect of temperature on the sporulation of *Cyclospora* oocysts, it was observed that sporulation did not occur at -20ºC and 37ºC but was not affected at 4ºC and 23ºC (Sathyanarayanan and Ortega, 2006). The average mean temperatures on the field and in the chamber were 15.1 ºC and 18.1 ºC respectively. From previous studies on persistence at different temperatures, one would expect that the behavior of the pathogens on the field would be comparable to their behavior in the growth chamber. The difference in persistence observed under the two conditions, however, suggests that factors other than temperature, or most probably the combination of environmental factors in said conditions were more important to the persistence of parasites, especially in real-world conditions. It also suggests that conditions

during the growing seasons of produce and herbs especially in outbreak regions could be more conducive to the extended survival of the coccidian protozoan parasites.

Irrigation water as a source of contamination on crops

Persistence of coccidian parasites particularly *Cryptosporidium parvum* has been extensively studied in water. Several studies have reported the detection of parasites in water sources and postulated a possible link between these contaminated water sources and transmission on produce (Cook et al, 2006; Dawson et al, 2004; Dixon et al, 2013) . In a study conducted in Poland, *Cryptosporidium* could be detected more on crops grown in regions with a high livestock population (calves shed a high number of parasites in their feces). The link between high livestock population and parasite detection is however not clear but one hypothesis is that water could be a means of transmission on produce. No study has validated this claim. In our study, plants spray irrigated with water spiked with pathogenic microbes showed contamination which persisted for extended periods of time (23 days under controlled conditions). The FDA fresh culinary herb document and the Food Safety Modernization Act (FSMA) produce safety rule records specifications for testing irrigation water for fecal coliforms and *E. coli.* According to the fresh culinary herb document, the acceptable geometric mean criteria level for five samples should be ≤ 126 MPN/100mL and ≤ 235 MPN/100mL for all single samples while the produce safety rule places this value at ≤ 410MPN/100mL. There are no requirements however for assessment of foodborne parasites in irrigation water.

CONCLUSION

Despite exposure to stringent conditions on the fields in Georgia, *Cyclospora cayetanensis* and *Cryptosporidium parvum* exhibited extended persistence. The host specificity of *Cyclospora cayetanensis* and the observed marked seasonality of infection creates a challenge during outbreak investigations. If contamination of produce by *Cyclospora cayetanensis* was caused by farm workers infected with the parasite, then the timeline of onset of symptoms during active outbreaks as shown by epi curves will be impacted. Earlier onset of symptoms might imply that contamination occurred in the field prior to harvest, however, this assumption is dependent on several factors including growth conditions of implicated produce and the chain of distribution. For example in the 1999 outbreak of cyclosporiasis in Missouri, basil which was the implicated food source was shipped for 3-8 days before final consumption at events A and B. During this period, produce was held in a cold chain with some hours at ambient temperature (Lopez et al, 2001). *Cyclospora cayetanensis* would need to have been already infective before shipping to result in the reported cases of infection. Contamination by infected workers on the basil production field might not have given enough time for the parasite to become infective, hence, contamination event likely occurred before harvest and distribution. With the data obtained from this study on the persistence of the parasite in the natural environment, we can confidently surmise that contamination probably occurred at some point on the field during production. Although infected farmworkers are a possible route of contamination, other routes like water, both for irrigation purposes (Amoros et al; 2010) and in the application of pesticides/insecticides and farm equipment should be put into consideration.

The natural environment might be a better model for scientific research on pathogen survival. Most studies on the survival of *Cryptosporidium parvum* and *Cyclospora cayetanensis* have been

conducted under laboratory conditions or controlled conditions of a growth chamber. As indicated in our study, the parasites exhibited different behaviors when challenged in the natural environment and controlled conditions of a growth chamber. Isolated environmental challenges like temperature, desiccation, or humidity are good measures of pathogen resilience; however, conditions in nature are not isolated and are highly variable. The major setback with this model is the high level of contamination in environmental samples which makes it challenging to determine the viability of the parasites or the developmental stage in which they are. The current method for the detection of parasites in samples involves a nested polymerase chain reaction (nPCR) technique which does not account for oocyst viability; however, these can be improved upon for future studies.

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Table 3.1: Primer sequences for PCR amplification of *Cyclospora cayetanensis* and *Cryptosporidium* spp.

GENE (18SrRNA)	GENE POSITION	PRIMER	PRIMER SEQUENCE 5'- 3'
Cyclospora cayetanensis	519	External - forward	AAT GTA AAA CCC TTC CAG AGT AAC
	1535	External - Reverse	GCA ATA ATC TAT CCC CAT CAC G
	578	Internal - Forward	AAT TCC AGC TCC AAT AGT GTA T
	1074	Internal - Reverse	CAG GAG AAG CCA AGG TAG GCR TTT
Cryptosporidium spp.	156	External - Forward	TTC TAG AGC TAA TAC ATG CG
	1478	External - Reverse	CCC ATT TCC TTC GAA ACA GGA
	192	Internal - Forward	GGA AGG GTT GTA TTT ATT AGA TAA AG
	1029	Internal - Reverse	AAG GAG TAA GGA ACA ACC TCC A

FIGURES

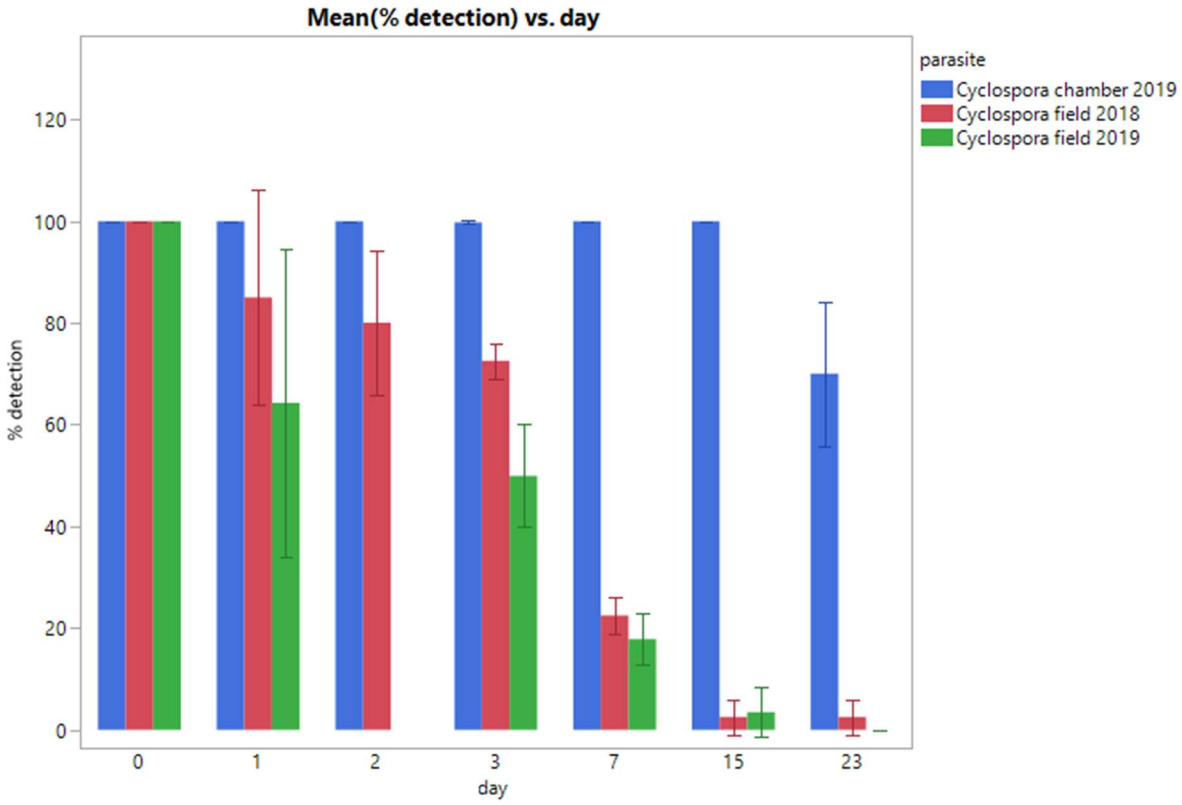

Fig 3.1: Persistence of *Cyclospora cayetanensis* on the field compared to a growth chamber

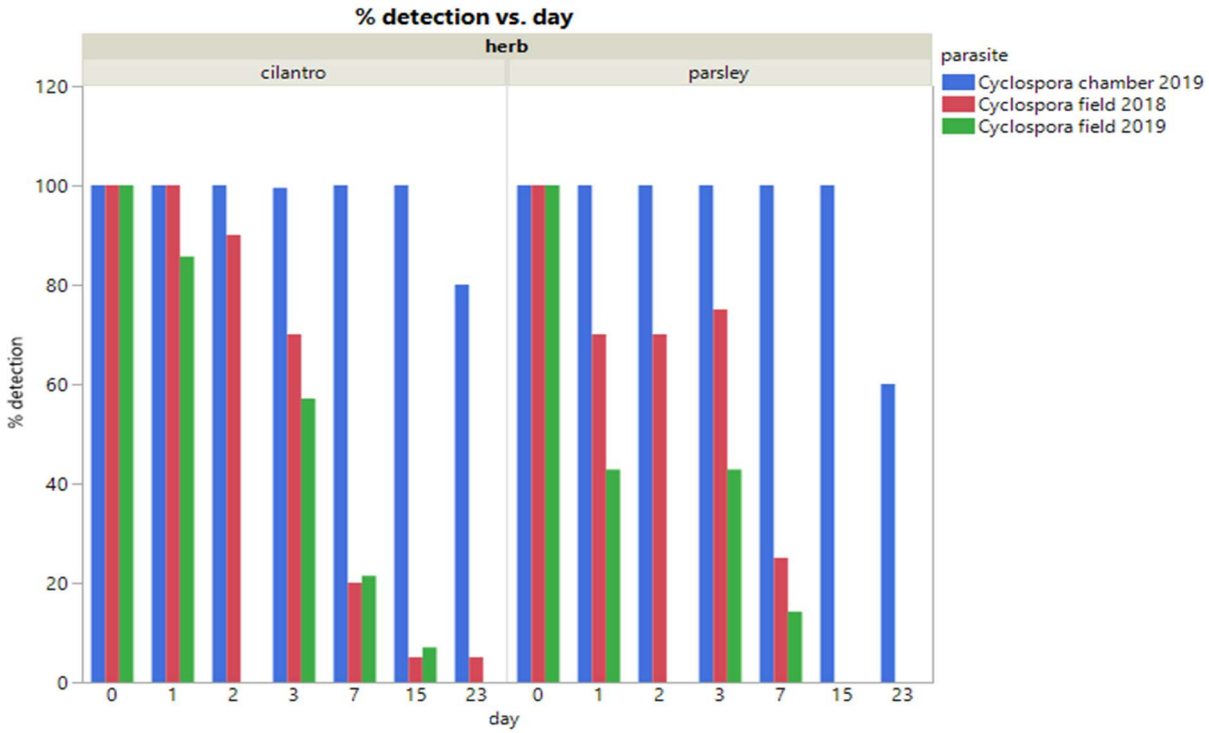

Fig 3.2: Comparison between the persistence of *Cyclospora cayetanensis* on cilantro and parsley on the field compared to a growth chamber

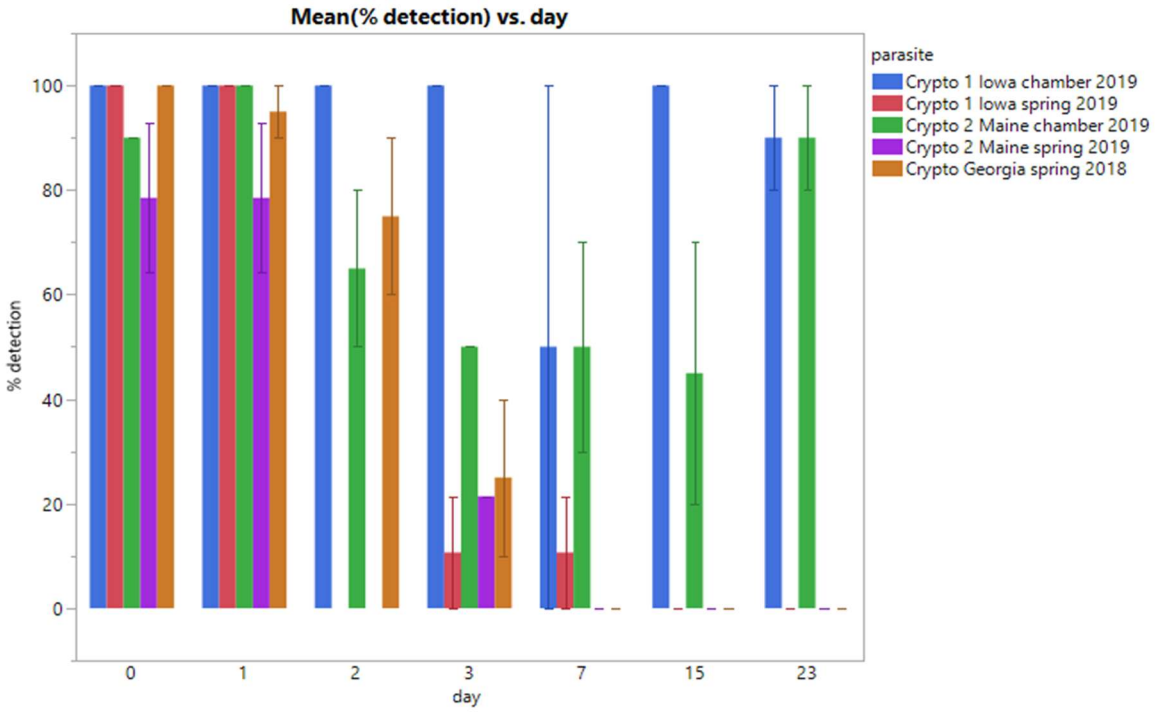

Fig 3.3: Persistence of *Cryptosporidium parvum* in the field compared to a growth chamber

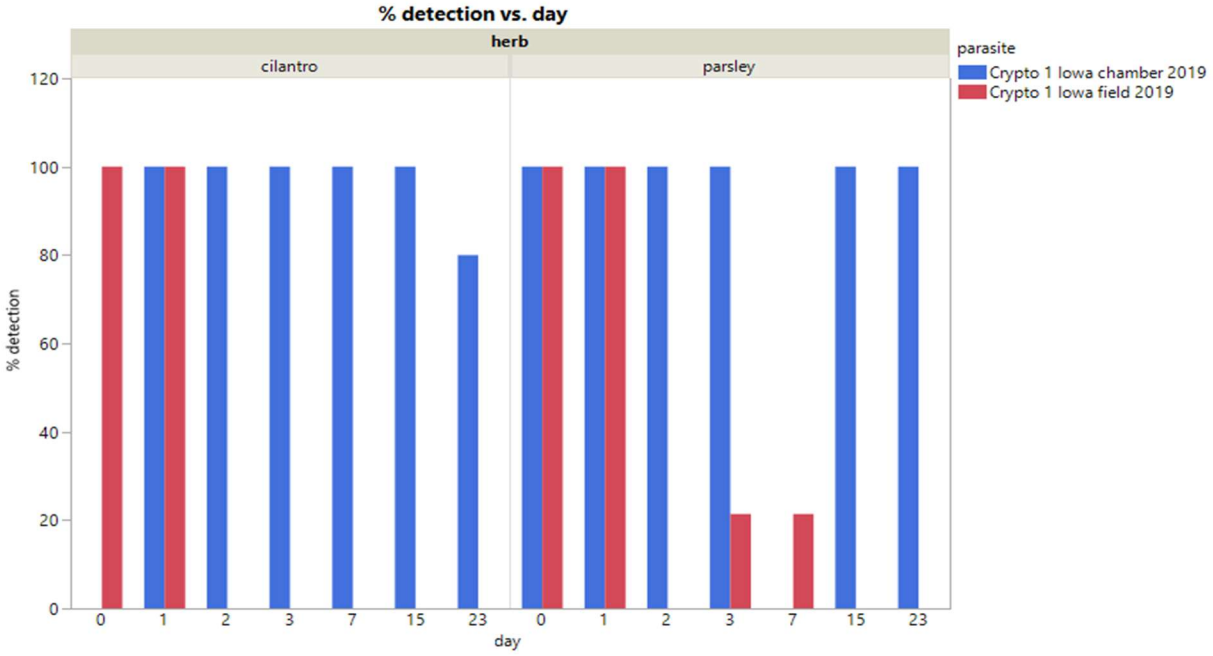

Fig 3.4: Comparison between the persistence of *Cryptosporidium parvum* Iowa on cilantro and parsley on the field and in a growth chamber

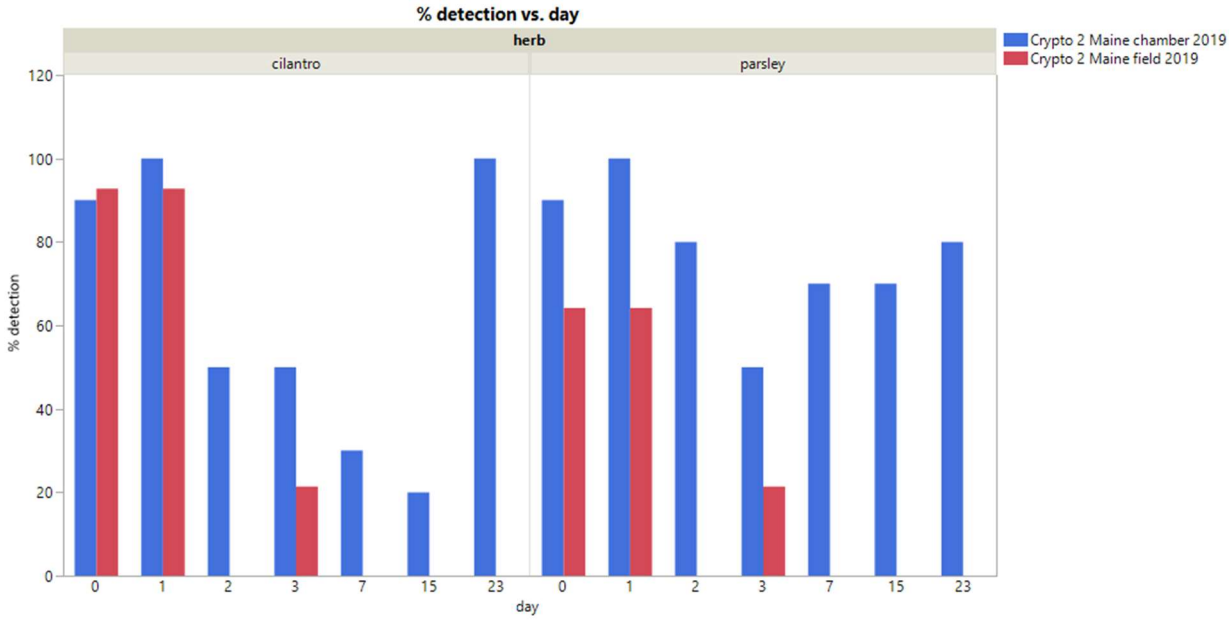

Fig 3.5: Comparison between the persistence of *Cryptosporidium parvum* Maine on cilantro and parsley on the field and in a growth chamber

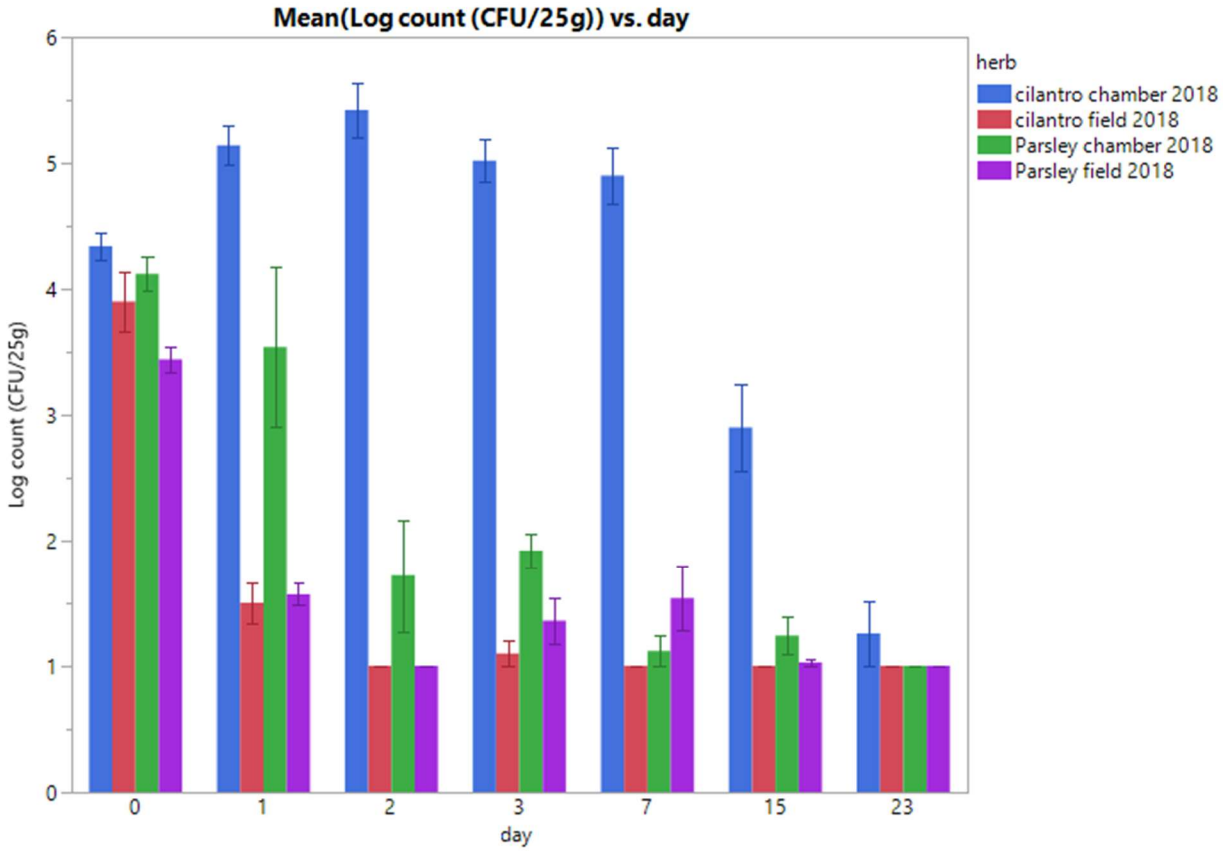

Fig 3.6: Persistence of *Salmonella* Typhimurium in the field compared to a growth chamber in 2018 (Log count 1 represent values below the detection limit < 25 CFU/25 g)

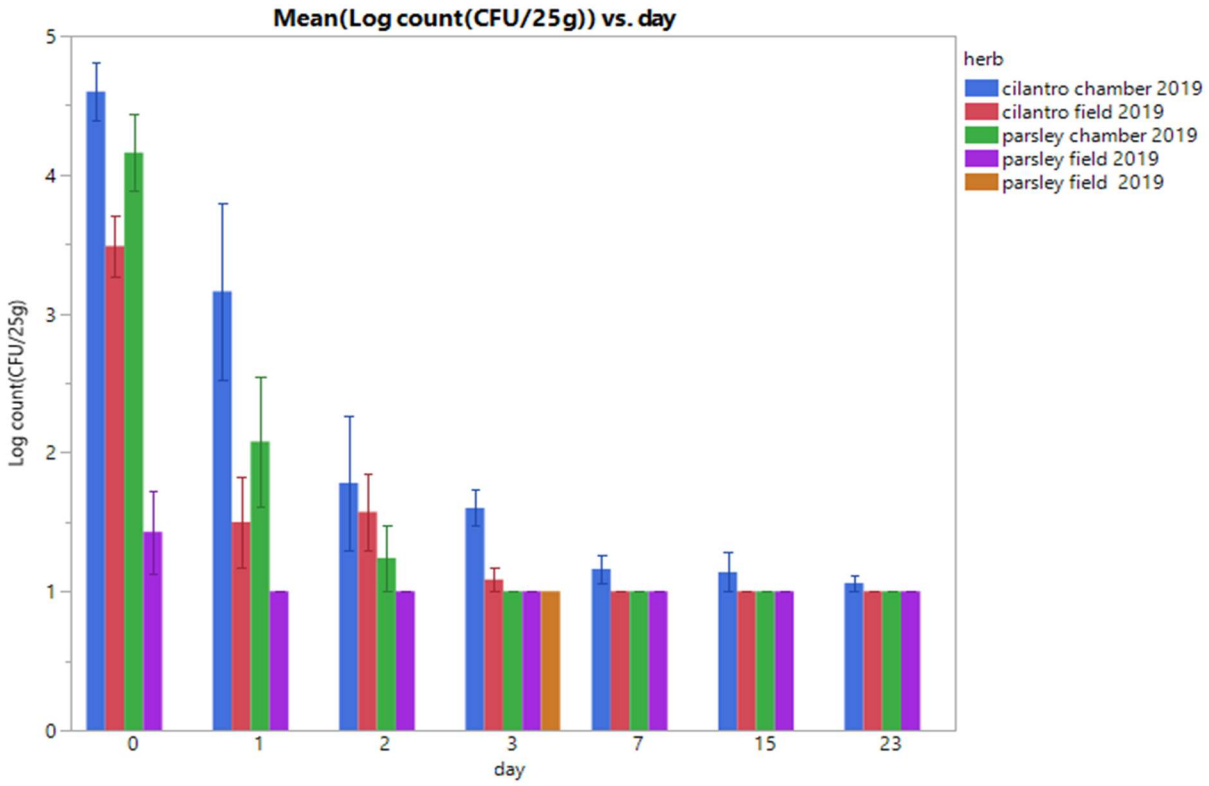

Fig 3.7: Persistence of *Salmonella* Typhimurium in the field compared to a growth chamber in 2019 (Log counts 1 represent values below the detection limit < 25 CFU/25 g)

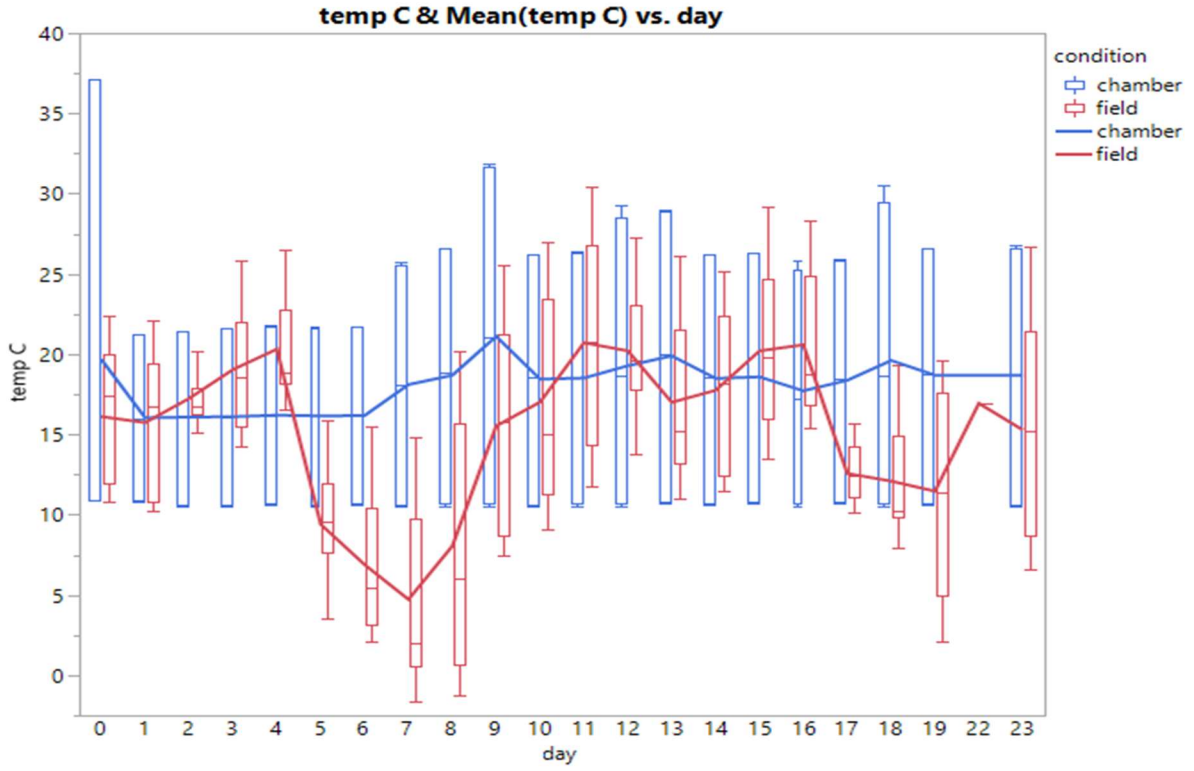

Fig 3.8: Average temperatures on the field and in the chamber

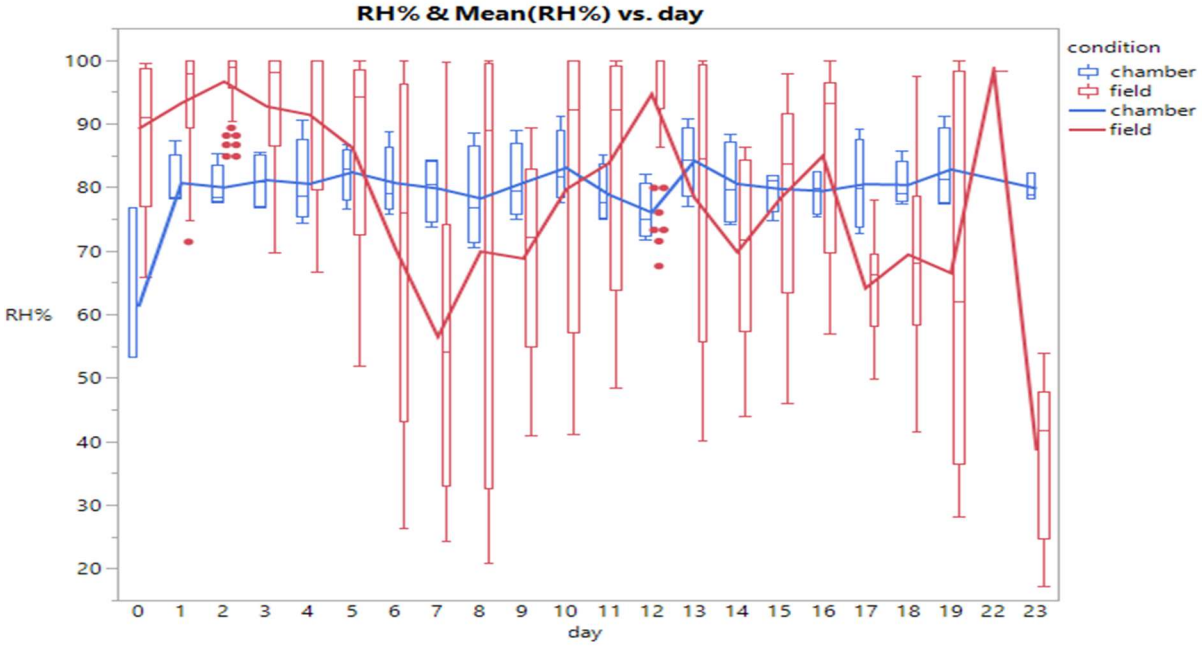

Fig 3.9: Average Relative Humidity on the field and in the chamber

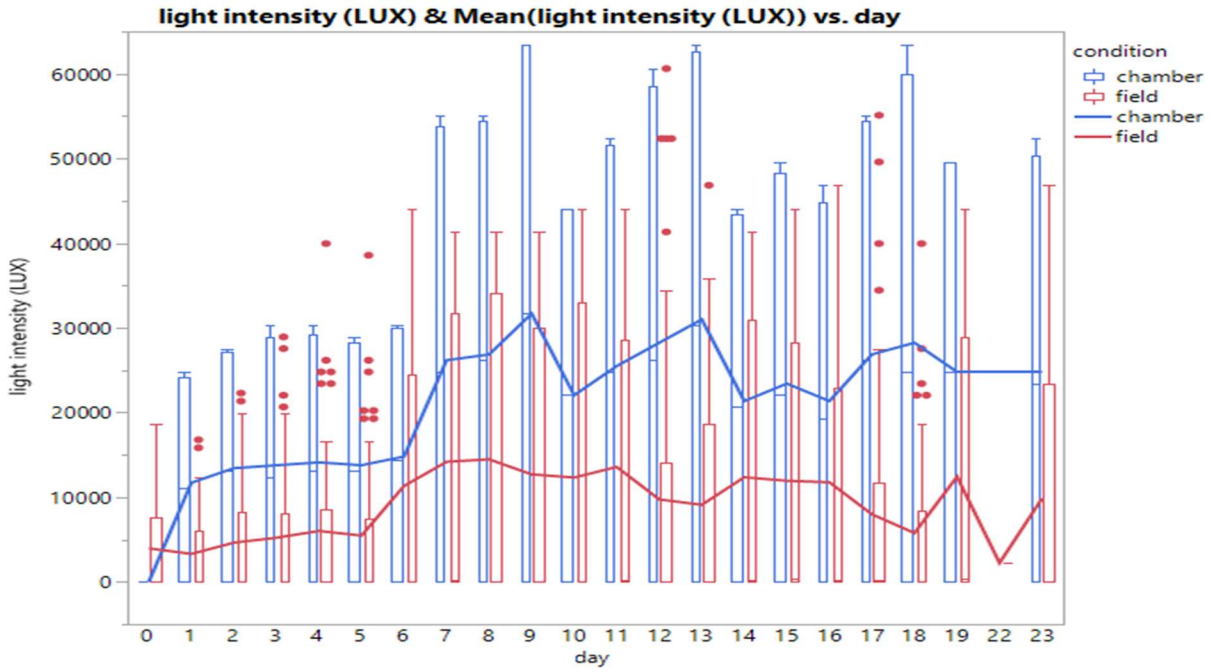

Fig 3.10: Average Light Intensity on the field and in the chamber

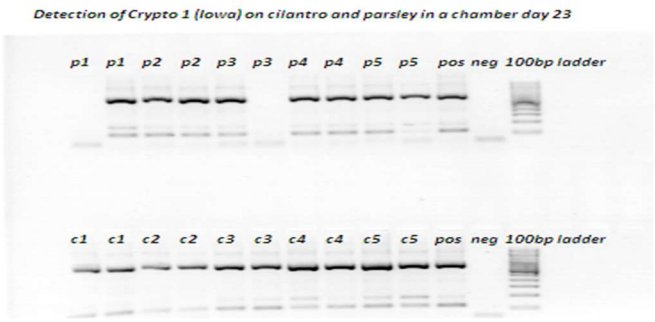

Fig 3.11: Gel detection of *Cryptosporidium parvum* Iowa on cilantro and parsley in a chamber on day

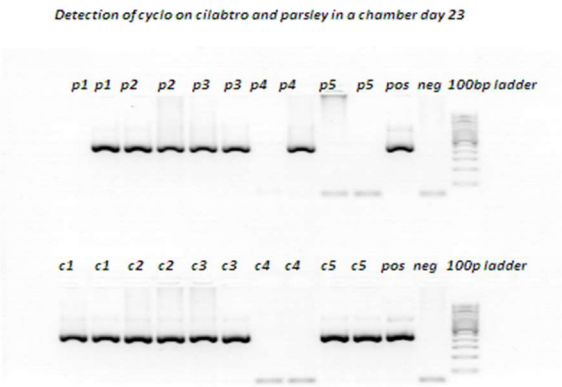

Fig 12: Gel detection of *Cyclospora cayetanensis* on cilantro and parsley in a chamber on day 23