CENTER for PRODUCE SAFETY

CPS 2016 RFP
FINAL PROJECT REPORT

Project Title
Significance of the dormant state in the persistence, interaction with growing plants and virulence of Shiga toxin–producing *Escherichia coli*

Project Period
January 1, 2017 – December 31, 2017 (extended to February 28, 2018)

Principal Investigator
Keith Warriner
University of Guelph
Department of Food Science
Guelph, ON N1G 2W1
Canada
T: 519-824-4120 ext.56072
E: kwarrine@uoguelph.ca

Co-Principal Investigator
Suresh Neethirajan
University of Guelph
School of Engineering
Guelph, ON N1G 2W1
T: 519 8244120 ext.53922
E: sneethir@uoguelph.ca

Objectives
1. *Determine the proportion of dormant cells within populations of the top 7 Shiga toxin–producing Escherichia coli (STEC), including isolates implicated in produce outbreaks.*
2. *Assess the role of different soil types and lettuce root exudates in the induction or breaking of the dormant state.*
3. *Establish the resistance of dormant STEC to free chlorine.*
4. *Evaluate the virulence of dormant STEC with respect to Shiga toxin production and attachment.*

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research

FINAL REPORT

Abstract

The persister state is a form of dormancy whereby bacteria enter a kind of hibernation thereby becoming stress tolerant with extended survival compared to normal cells. To date, persisters have been mainly of interest in clinical microbiology, given that the dormant cells are insensitive to antibiotics and hence can go on to cause infections. Within food systems, the main form of dormancy considered is viable but not culturable (VBNC) and is distinct from persisters with respect to the latter being inducible, in addition to occurring when conditions are conducive for growth. This study investigated the occurrence of the persister state in Shiga toxin–producing *Escherichia coli* (STEC) – a pathogen linked to fresh produce. From screening strains belonging to the Big 7 STEC, it was confirmed that all produced persisters to varying degrees. The highest abundance of persisters was encountered in *E. coli* O157:H7 and *E. coli* O103 compared to the other serotypes tested. A range of agents and extracts of relevance to the fresh produce chain were screened as inducers of the persister state. From the agents tested, indole was found to increase the abundance of persisters within *E. coli* populations. However, seed exudates, bile, iron chelating agent and soil (loam and sandy loam) significantly reduced persisters within STEC. With respect to breakers of the persister state, lettuce extract increased the recovery of persisters although this was found to be serotype dependent. Those responding to breaking the perister state in the presence of lettuce extract were *E. coli* O157, O103, O174, O79 and O91. The breaking of the persister state in other serotypes was less affected by the presence of lettuce extract. The persister state was not alleviated by the presence of indole, soil extracts or mannitol. It was demonstrated that persisters have enhanced resistance to hypochlorite and consequently more likely to survive post-harvest washing. Moreover, persisters retained virulence and could produce Shiga toxin upon recovery. In conclusion, the study demonstrated that STEC can enter the persister state, which enhances stress resistance, in addition to delayed growth during cultivation. Given that STEC are likely to exist in the persister state in the environment, many of the laboratory-based validation and survival studies may over-estimate the die-off rate of pathogens when introduced into soil or post-harvest wash tanks. The persister state could also contribute to the number of false-negative tests when cultured on media. Yet, lettuce extracts were found the break the persister state thereby making cells sensitive to inactivation or growth comparable to non-persister cells. The research opens potential avenues to reduce STEC in the environment by applying breakers of the persister state, in addition to reducing resistance such as passage through post-harvest washes.

Background

The recent foodborne illness outbreak suspected of being linked to romaine lettuce contaminated with *E. coli* O157:H7 underlines the food safety issues facing the fresh produce industry. A trend in recent years is the increased number of non-O157 STEC linked to fresh produce, which greatly outnumbers those of meat (Kase et al. 2015).

There are multiple sources by which STEC can enter the fresh produce chain, with organic amendments and irrigation water representing significant sources (Warriner and Council 2011). In the course of processing, the post-harvest wash process, if not controlled, can result in dissemination of STEC, along with other pathogens, across batches (Murray et al. 2018). To address the food safety issues related to STEC there are guidelines, along with regulations, that aim to prevent the introduction and dissemination of the pathogen within the fresh produce chain. For example, ensuring that sufficient time has elapsed prior to harvest in the case of organic amendments and validating the post-harvest washes prevent cross-contamination between batches, amongst other measures.

Through surveillance studies it has been confirmed that STEC are highly prevalent (>90%) in manure, although those belonging to the Big 6 are sporadically recovered (Arthur et al. 2002; Cernicchiaro et al. 2013; Bai et al. 2015; Bannon et al. 2016). It follows that manure applied to land as part of crop nutrient

management represents a significant source of STEC that can be retained in the soil or leached into water courses used to irrigate crops (Berry and Miller 2005; Fremaux et al. 2008; Franz et al. 2011).

To reduce the risk derived from STEC associated with manure, there is the option of composting or applying manure to land, then waiting for a period of 90 days (for crops such as sweet corn) or 120 days (crops such as cantaloupe) before harvest. The 90–120 day rule was introduced on the assumption that any pathogens present would lose viability over time. To verify or refine the 90–120 day policy, there has been interest in acquiring data on pathogen die-off. To this end, our group and others have reported on the die-off rates of enteric pathogens in soil held within soil microcosms and, more rarely, under natural climatic conditions (Moynihan et al. 2013; Brennan et al. 2014; Erickson et al. 2014, 2015). A common feature in all of the field trials reported to date is the non-linear die-off kinetics that could be separated into three distinct phases: specifically, an initial rapid decrease in pathogen numbers, followed by a slower phase, and then an extended period of persistence. The non-linear decline in pathogen levels has made it problematic to calculate die-off rates. Some researchers calculate a rate based on fitting non-linear models, with others designating the time for numbers to be reduced by a designated log cycle. Regardless of the way to define the die-off, a wide range of *E. coli* O157:H7 persistence has been reported, varying from 16 to 231 days. Through our research, along with that of others, the factors that influence the rate of *E. coli* decline are the fluctuations in moisture, temperature, soil type and resident microflora (Fremaux et al. 2008; Franz et al. 2014; Ibekwe et al. 2014; Naganandhini et al. 2015). Strain related factors for *E. coli* persistence have also been noted. For example, it has been observed that certain strains of *E. coli* (termed naturalized) can grow in the environment thereby facilitating indefinite persistence (Ishii et al. 2006). In one study there was a clear higher persistence of *E. coli* O157:H7 compared to non-O157 STEC. Frenz et al. (2011) compared the relative survival of 18 strains of *E. coli* O157:H7 and reported that those derived from human clinical cases exhibited greater persistence compared to those derived from animals.

In the case of post-harvest washing, some guidelines suggest that maintaining a defined free-chlorine level in water will prevent cross-contamination (Nou and Luo 2010). The suggested free-chlorine levels vary from study to study and are largely based on the inactivation kinetics of pathogens determined under laboratory conditions (Gombas et al. 2017).

The common theme between the pathogen die-off in soil or post-harvest wash tanks is that the data is gathered using laboratory-based studies and defining viability in terms of the ability to grow on plates. However, under natural conditions, enteric bacteria such as STEC likely exist in a dormant state that is resistant to environmental stress, in addition to exhibiting delayed cultivation on standard growth media. This would suggest that die-off rates of STEC in the field are under-estimated and efficacy of sanitizers is over-estimated.

The persistent or dormant state was noted in the pioneering studies in the 1940s to describe the residual population remaining after a sensitive culture is exposed to antibiotics. The resistance could not be explained by mutations conferring antibiotic resistance, as the progeny from survivors were equally as sensitive as the original culture (Putrins et al. 2015). The persistent state can be confused with VBNC although the two can be differentiated with respect to the former being able to eventually grow when presented with the required nutrients and extrinsic/intrinsic conditions. In contrast, VBNC is a transient state from tolerant to non-viable that will not grow on media although can be visualized using direct techniques such as microscopy (Ayrapetyan et al. 2015b). It is likely that previous studies looking at VBNC were actually looking at persister cells although in the absence of single cell interrogation techniques were likely missed (Gerdes and Maisonneuve 2012; Ayrapetyan et al. 2015b).

Type I persisters are defined as those that emerge during the stationary phase of growth or pre-imposed stress (e.g., antibiotics) and make up a small proportion (as low as 1 in a million) of the total population (Amato et al. 2013; Orman and Brynildsen 2015). The Type II persister converts to the dormant state spontaneously regardless of growth phase or imposition of stress (Orman and Brynildsen 2015). Type I

have received the most attention, given the relative ease of analysis, although they are likely to consist of persisters and VBNC cells, as these are formed within the stationary phase (Ayrapetyan et al. 2015a).

The genetic regulation of induction into the persister state is relatively well understood and described by the toxin–antitoxin model (Gefen and Balaban 2009; Amato et al. 2013, 2014; Amato and Brynildsen 2015; Shidore and Triplett 2017). Here, a protein (referred to as toxin) is expressed that reduces protein synthesis and alterations to the membrane structure that collectively place the cell in hibernation. To wake up from dormancy, an anti-toxin is produced that neutralizes the toxin and the cell becomes metabolically active again. A large knowledge gap exists with respect to the external signals that trigger the induction of the persister cells or those that break dormancy. It has been reported that iron, indole and plant hormones induce the persister state (Freestone and Lyte 2008; Vega et al. 2012). Agents that break the persister state are less understood but may occur when the imposed stress is removed or in the presence of sugar alcohols (Fisher et al. 2017). The understanding of factors that break the persister cells will enable novel intervention that can make pathogens such as STEC more stress susceptible and culturable.

The virulence of STEC persister cells is unknown but there has been a suggestion that strains that exhibit high stress tolerance are correlated with low pathogenicity (Ongeng et al. 2015). If this occurs in dormant cells where the main population has high virulence remains unknown.

Importance
To date the most focus on the persister state has been with respect to pathogenesis whereby dormancy enables pathogens to avoid the immune system (Fisher et al. 2017). Much less is known about the significance of the persister state in the environment, plant:pathogen interactions, and resistance to post-harvest interventions (e.g., sanitizers). Clearly, the persister state has a significant impact on all aspects of fresh produce food safety, from manure management in the field and the efficacy of post-harvest interventions through to pathogenicity to the final consumer. By gaining an understanding of the persister state it will be possible to derive novel interventions to enhance the susceptibility of dormant cells that would ordinarily survive.

Research Methods

Bacteria strains
Escherichia coli included in the study were O157:H7 and non-O157 STEC (O26, O45, O103, O111 and O121). A single strain of each serotype was selected for study and included those recovered from meat, fresh produce or clinical sources. Additional STEC serotypes tested were O55, O79, O91, O113, O118 and O174. The strains were donated by the CDC for which the genomes have been sequenced (Lindsey et al. 2014). *E. coli* P36 is a non-pathogenic strain previously isolated from spinach plants.

Growth and recovery of persisters
The media tested included Luria Bertani (LB) broth, tryptic soy broth (TSB), brain heart infusion (BHI) or M9 minimal media. The inoculum was prepared in the test media at 25 or 37°C overnight and inoculated into 20 ml of the test media. The *E. coli* was incubated anaerobically at 37°C, with the optical density being monitored throughout. At the mid-exponential phase, ampicillin (200 µg/ml) was added to the culture and returned to aerobic incubation with 1-ml aliquots withdrawn periodically and separated from the growth medium by centrifugation, followed by resuspension in sterile phosphate buffered saline (PBS). A dilution series was then prepared from the suspension, and survivors were enumerated by plating onto the test agar (LB, TSA or M9) incubated at 37°C, with colonies counted every 24 h for 72 h. Colonies were presumed to be persister (i.e., non-growing) cells within the original *E. coli* population.

Induction of persisters within STEC populations
The test *E. coli* strain was cultivated at 37°C overnight and used to inoculate M9 media containing the agent or extract to a final cell density of 2 log cfu/ml, unless otherwise stated. The agents tested were bile,

L-alanine, mannitol, caffeine and indole, which were added to the media prior to inoculation. The culture was incubated at 37°C until the optical density at 600 nm reached 0.2. An aliquot of the culture was removed and then a dilution series was prepared in saline. Ampicillin (200 µg/ml) was added to the remaining culture and incubated for a further 8 h. At the end of the incubation period a dilution series was prepared in saline, then subsequently plated onto M9 agar and incubated up to 5 days at 37°C and inspected daily for colonies.

The proportion of persisters was calculated as: Persisters = N/Np
where N = persisters cfu/ml, and Np = cell density (cfu/ml) prior to addition of antibiotic.

The effect of soil constituents on induction of the persister state was evaluated in microcosms. About 15 liters of fine sandy loam soil and 10 liters of loam soil were sieved to 0.2 cm (200 mesh screen). After sieving, the two types of soils were mixed separately in two large bins, which were then covered to retain moisture. The soil was transferred to containers (27.9 × 16.8 × 13.7 cm) that had a 3-cm deep layer of gravel as the bottom layer. The microcosm was saturated by adding 1 part water to 2 parts soil and then equilibrated overnight at 20°C. A cover was placed over the microcosm to prevent moisture loss.

The culture to be inoculated was cultivated in M9 medium up to the late exponential phase, then 15 ml was transferred to a dialysis cassette with a molecular weight cutoff of 2 kDa. The dialysis cassettes (4 per microcosm) were placed under the soil surface within the microcosm and left for 48 h. In parallel, controls were performed by placing STEC in dialysis cartridges, in saline solution for 48 h at 20°C. After the incubation period, the contents of the dialysis cassette were transferred to an equal volume of M9, then held at 37°C with the optical density at 600 nm being measured every 30 min. When the optical density of the culture started to increase, ampicillin (200 µg/ml) was added and the incubation continued for a further 8 h. At the end of the incubation period, samples were removed to prepare a dilution series and survivors were enumerated on M9 agar incubated at 37°C for up to 52 h.

To assess the effect of seed exudates on induction of the persister state, a similar methodology to that for the soil microcosms was applied. The test STEC was introduced into the dialysis cassette and placed into a bed of sprouting mung beans. Sprouting was initiated by soaking the beans for 24 h and incubating at 20°C. The water was drained and the sprouting mung beans were irrigated every 24 h by steeping in water. On the fifth day, the dialysis cassette was removed and persisters determined as described above.

Alleviating the persister state

Persisters were obtained by cultivating the test STEC strain in M9 medium containing 0.5 mM indole, adding ampicillin (200 µg/ml) at mid-exponential phase and further incubating for 8 h. The remaining fraction of cells contained persisters that were subsequently used to assess the potential of activators. In one format, the activator was introduced into M9 agar and incubated at 37°C for up to 72 h, with the number of colonies representing activated cells. In a further format, M9 media was supplemented with activator and then dispensed into a microtiter plate. Persisters were inoculated into one row of the microtiter plate, and then diluted down in a 5-tube most-probable-number (MPN) approach. The titer plate was sealed and incubated at 30°C for up to 20 days, with daily inspection for growth. The MPN was calculated based on growth or no growth along with reference to MPN tables. The third method for assessing activation was through viability counts made via flow cytometry or a hemocytometer. Each aliquot (1 ml) of persisters was stained with 3 µl of Live/Dead Baclight (L7007, LIVE/DEAD *Bac*Light™ Bacterial Viability Kits) and incubated at room temperature in the dark for 15 min. After incubation, 15 µl of the sample was transferred to a hemocytometer and the live and dead cells were observed under a fluorescence microscope. The live cells (viable cells) stained with SYTO9 exhibited green fluorescence while dead cells (non-viable) stained with both SYTO9 and propidium iodide (PI) exhibited red fluorescence. To calculate the number of viable cells per milliliter of sample, the following formula was used: total number of live cells in four corners square/4 × dilution factor × 10^4. The survival level of persister cells was calculated as the ratio of persisters within a sample to the original number of total cells, prior to antibiotic treatments.

Microfluidic chip and imaging

Polydimethylsiloxane (PDMS) microfluidic chips were crafted using a 1:10 mic of PDMS to PDMS curing agent. This PDMS mixture was poured into a prepared mold to form the base of the microfluidic chip and left for 1 h in a desiccator to remove any air bubbles. The formed microfluidic chips were then cured for 4 h at 60°C, before cooling for 15 min at room temperature. The chips were removed using an X-Acto knife, and inlet holes were punched using a hole puncher, with the aid of a microscope. The resulting chips were bonded to a 1.22-mm thick glass slide after first placing both parts in plasma cleaner for 5 min and then placing the PDMS chip on the glass slide and baking for 20 min at 60°C.

The bacterial cultures were introduced into the chip with fresh media through an automated syringe system into and out of the inlets at a set rate. The cells were observed using a Nikon Ti-U Eclipse inverted microscope at 100×, with a monochromatic green filter applied to reduce a rainbow effect on the bacteria.

Resistance of persisters to hypochlorite

Persisters of the test strain were produced as previously described, with a sample of cells taken prior to antibiotic addition as a control. Hypochlorite solutions were prepared in phosphate buffered saline (PBS) and addition of hypochlorite. The concentration of free chlorine was measured using a chlorine test kit, and adjusted to a concentration of 5 ppm. The hypochlorite solution was further diluted in PBS to give a range from 0.5–5 ppm free chlorine. The test strain was introduced into a hypochlorite solution of water; after 30 s, 1-ml aliquots were withdrawn and transferred to 1% thiosulphate. A dilution series was prepared and plated onto M9 agar, which was subsequently incubated at 37°C for up to 72 h.

Results

Presence of persisters in different STEC serotypes

Baseline studies determined whether persisters existed with STEC populations and any inter-serotype variation. The definition of persisters is still debated but it is generally accepted that the state refers to those cells remaining after being exposed to antibiotic that the bacterium is ordinarily sensitive to (Fisher et al. 2017). Persisters are selected by cultivating populations of the test bacteria to mid-exponential phase prior to addition of antibiotic that inactivates rapidly growing cells and eventually, tolerant (slow growing) cells (Figure 1 – see Appendices). In the current study, STEC were grown in LB broth to the mid-exponential phase and antibiotic was added to inactivate growing cells. From the survival curves it was found that three distinct phases occurred (Figure 2). Specifically, the majority of the STEC population decreased upon addition of antibiotic, followed by a slower phase that eventually plateaued in some cases (Figure 2). The survival curve results can be explained by the antibiotic-sensitive strains being inactivated first, followed by those with tolerance, and finally leaving the persisters. The differentiation between tolerant and persister strains is problematic; in the current case a contact time of 7–8 h contact with antibiotic was considered sufficient to select for persisters and was applied in subsequent trials.

The survival curves illustrated that persisters formed a low proportion of the population (<1%) but differences did exist with STEC (Figure 2). *E. coli* O103 and O121 formed a higher proportion of persisters relative to the more widely studied O157:H7. Yet, it should be noted that persisters were recovered in all cases, confirming that STEC do enter the dormant state.

To confirm that persister cells were selected by antibiotic treatment, trials were performed using spectrophotometric techniques. Specifically, absorbance spectra were captured of growing cultures vs purified persister cells. Persister cells could be differentiated from non-persister cells by a peak at 295 nm (Figure 3). The peak can be attributed to unique structures (protein and/or lipopolysaccharide) present on the persister cell surface. When the different spectra were compared it was clear that persister populations can be distinguished from non-persister cells (Figure 4). The significance of the result is that persisters have a unique composition compared to other cells within a population.

Induction of the persister state

Upon confirming that the persister state existed in STEC, trials were performed to establish which factors of relevance to the fresh produce chain could influence formation, specifically, the constituents encountered in manure or soil. When developing the assay it was necessary to confirm if the persister state was induced within a culture during the lag phase or simply formed part of the initial inoculating culture. Here, different volumes of inoculum were introduced into media and grown to mid-exponential phase prior to adding antibiotic and then incubating for 8 h to inactivate non-dormant cells. A significantly ($P < 0.05$) higher proportion of persisters were recovered at low inoculation levels than when higher cell densities were applied (Figure 5). The result confirmed that the persister state was induced during culturing as opposed to passively introduced within the inoculum. Therefore, an assay was developed whereby the inoculum was introduced into media containing the inducing agent under study, with non-persister cells being inactivated by the addition of antibiotic.

A further aspect to consider is whether sub-populations within STEC cultures were pre-disposed to be induced into the persister state (i.e. mutants that are more likely to enter the persister state when induced). Therefore, experiments were performed to assess if selection of persisters increased the proportion of dormant cells under a suitable inducing agent. Here, persisters from a culture were sub-cultured into media, then cultivated until mid-exponential phase and then treated with antibiotic. Although there was a high level of variation in persisters recovered from successive sub-culturing (Figure 6), the results suggest that the persister state could not be selected for, with individual cells having an equal probability of entering a dormant state.

With the inducer assay optimized, trials were performed to assess the effect of different compounds or extracts to induce the persister state. Initial trials evaluated previously reported agents that induce the persister state in bacteria. Indole is encountered in soil and acts as a regulator of metabolic activity in a diverse range of bacteria that includes *E. coli*. When indole was supplemented into the growth media the proportion of persister cells in all the STEC strains tested increased but only if cultivated in M9 minimal media, not in TSB (Figure 7). The results suggest that indole acted as an inducer of the persister state in nutrient poor environments but the effect was countered by nutrients/constituents in rich media like TSB.

Iron is an essential nutrient and is considered to regulate microbial metabolism, especially with respect to enteric pathogens. In the presence of an iron chelating agent, such as EDTA (ethylenediaminetetraacetic acid), the levels of persisters recovered decreased in both M9 and TSB media (Figure 8). The result would suggest that iron (or other divalent ions) act as a regulator of the persister state.

Deoxycholate is a byproduct derived from bile metabolism and is encountered in manure. Inclusion of 0.2% deoxycholate, representing physiological concentrations, resulted in a reduction in *E. coli* O157:H7 persister cells recovered relative to controls (Figure 9). In comparison there were no significant differences in the proportion of persisters in *E. coli* O103 when cultivated in M9 media supplemented with deoxycholate (Figure 9). With stationary cultures grown in the presence of deoxycholate the levels of persisters were not significantly different ($P > 0.05$) compared to controls for both serotypes tested (Figure 10). The results suggest that deoxycholate depresses the induction of Type II persisters but less so in Type I in *E. coli* O157:H7.

To test the effect of seed exudates on persister induction, *E. coli* was placed within a bed of sprouting mung beans for a week. The cells were transferred to M9 media and incubated for 2 h to initiate growth of non-persisters. Those cells incubated within the mung bean bed contained a significantly ($P < 0.05$) lower proportion of persisters compared to controls incubated in saline for 7 days (Figure 11). The results would suggest the exudates released from sprouting mung beans suppressed the induction of the persister state.

Trials were performed to assess the effect of soil constituents on induction of the persister state in different STEC. Here, the test strain was introduced into a dialysis cassette, then placed in a microcosm containing loam or sandy loam soil saturated with water. At the end of the 48-h incubation period the cassette content was transferred to M9 media and antibiotic added upon the initial increase in optical

density. At the end of a further 8-h incubation period the persisters were enumerated by microscopy (viability staining) and counting colonies on plates. The results demonstrated that the controls in saline had the highest levels of persisters (Figure 12). In both loam and sandy loam the levels of persisters were significantly lower than the controls (Figure 12). As previously found, serotypes O157:H7 and O103 produced the highest proportion of persisters compared to the other *E. coli* tested. Yet, the persister state appeared to be suppressed when introduced into soil.

A further finding in the soil microcosm study was the higher levels of viable cells identified by viability staining compared to cultivating on agar plates (Figure 12). These results highlighted that depending on colony counts would underestimate the levels of persisters (or dormant cells in general) present.

Effect of recovery and cultivation media on proportion of persisters

The proportion of persisters within a STEC population was found to be independent of the agar and the incubation temperature to recover persisters (Table 1). The exception was with *E. coli* O45, which had an apparent higher proportion of persisters when BHI agar was used to break the persister state. Again, *E. coli* O103 produced a higher proportion of persisters compared to the other serotypes tested (Table 1).

Compounds and extracts that alleviate persister state

Assays were developed to identify agents or extracts that could break the persister state within STEC. The original approach was to introduce persister cells into a microfluidic system and introduce the compound or extract under test, and then directly observe the outgrowth of cells that transitioned from the dormant to active state. Although cells could be viewed within the microfluidic chamber, the test period was limited to 4 h due to evaporation of water. In addition, the field of view was limited to approximately 100 cells, which ultimately resulted in poor sensitivity in assessing the influence of inducing agents. Although it was found that inducers increased the movement/motility of cells there was no evidence of outgrowth within the 4-h timeframe. Therefore, an alternative assay used media supplemented with the agent or extract under test. From a range of base media tested there was no significant difference ($P > 0.05$) in recovery of persisters using LB, TSA or BHI agar (Table 1). For the assay, M9 was used as the base media given it was defined compared to the non-selective media.

Trials were performed to assess if soil extracts or mannitol could alleviate the persister state in *E. coli* O103. Mannitol was selected, given that the sugar alcohol has previously been shown to stimulate growth of generic *E. coli* persisters. In the current study, there was no significant difference in the lag phase of mannitol-supplemented persisters compared to control (persisters with no addition) (Figure 13). Yet, in those cultures supplemented with soil extracts there was a suppression of persister outgrowth. This result provides indirect evidence that constituents within the soil environment are sufficient to maintain the persister state. The presence of indole had the same effect as soil extracts in preventing outgrowth of persister cells. Given the strong effect of indole in suppressing alleviation of the persister state, it was possible that the effect of soil could be attributed to the heterocyclic aromatic. However, when the soil extracts were tested for indole, none could be detected (detection limit <4µM). Therefore, additional metabolites/constituents associated with soil extract were responsible for the suppressing effect.

A further assay developed was a titer plate format based on 5-tube MPN method. Here M9 alone or supplemented with the active agent was dispensed into wells of a titer plate. A series of 5 dilution sets of a persister population were prepared, which were inoculated into the plate that was subsequently incubated at 30°C for up to 20 days, with the optical density at 600 nm being taken periodically. By using the method it was possible to assess the levels of persisters present, in addition to the duration of the lag phase (Figure 14).

Keeping in line with relevance to the fresh produce chain, trials were performed to determine if lettuce extract could enhance the speed and extent of STEC persisters. The lettuce extract was prepared by an initial coarse chopping, followed by cold pressing. The pH and brix were measured along with being

standardized between batches. The resultant lettuce extract was centrifuged to remove large particulates, and then subsequently passed through a sterile filter prior to storage at -20°C. The extract was supplemented into M9 media at a final concentration of 1% v/v. The numbers of persisters recovered were at a significantly ($P < 0.05$) higher level in M9 supplemented with lettuce extract compared to controls, as shown for *E. coli* O157 persisters but less so for *E. coli* O103 (Figure 14). By comparing viability (microscope) counts with those obtained with the MPN method there was no significant difference observed. Although still to be confirmed, there is evidence that constituents within lettuce can break the persister state within *E. coli* O157 and O103.

Further selections of STEC outside the Big 7 were assessed with respect to recovery of persisters in M9 medium with or without lettuce extract. From the results obtained to date, with STEC serotypes O174, O79 and O91 the breaking of the persister state was better supported by inclusion of lettuce extract in M9 medium (Figure 15 A, B, C). However, with STEC serotypes O118 and O55 the recovery of persisters on lettuce extract containing medium was suppressed compared to M9 alone (Figure 15 D and E). *E. coli* O113 appeared neutral to the presence of lettuce extract despite the more rapid initial growth compared to M9 alone (Figure 15 F).

Resistance and virulence of *E. coli* O157:H7 persister cells

Persister cells were prepared by treating *E. coli* O157:H7 grown in M9 containing indole and then subsequently treating with antibiotic. Prior to addition of antibiotic, a portion of the culture was removed to act as the control although it went through the additional 8-h incubation period.

The tolerance to chlorine was assessed by preparing a series of buffered hypochlorite solutions with a dilution series of free-chlorine concentrations. The persister or control group was added to the different solutions and, after 30 s, aliquots (1 ml) were transferred to sterile thiosulphate solutions to sequester residual chlorine. The survivors were enumerated on M9 media supplemented with lettuce extract. The control group was significantly more sensitive to chlorine compared to persisters (Table 2). Yet, levels decreased below the limit of detection (<1 log cfu/ml) when 3 ppm was applied. The results suggest that in the persister state the tolerance of *E. coli* O157:H7 to free chlorine is higher compared to normal cultures used to assess sanitizer resistance.

The virulence of STEC was determined by the ability of cultures derived from persisters to produce toxin. The persisters derived from *E. coli* O157:H7 were inoculated into M9 medium supplemented with lettuce extract, and grown at 37°C for 48 h. Aliquots of supernatant were recovered by centrifugation and tested for toxin using ELISA. The assay confirmed toxin production by the culture and hence retention of virulence of the STEC strain.

Outcomes and Accomplishments

The research team consisted of two graduate research assistants (post-doctoral researcher and laboratory technician), a PhD and two MSc students, in addition to an undergraduate project student. The researchers gained expertise in pathogen handling, imaging, flow cytometry, assay development and undertaking challenge trials. The lead investigators of the project encompassed a food microbiologist, an environmental microbiologist, a soil scientist and microfluidic and imaging engineering. The research group met on a quarterly basis to review progress and establish research plans. An external collaboration with CDC was established, with the agency providing non-O157 STEC isolates for which whole genome sequencing had been performed. The research team focused on different aspects of the project in a coordinated approach. Specifically, the post-doctoral researcher focused on imaging and microfluidics. The PhD student and research assistant evaluated the establishment and survival of persisters in soil. One of the MSc students, in collaboration with the undergraduate project student, determined the role of inducing agents that enhance the formation of persisters. Agents that break the persister state and assessing stress resistance of dormant cells were investigated by the other MSc student.

The outcomes and accomplishments of each of the 4 objectives are as follows:

1. Determine the proportion of dormant cells within populations of the top 7 Shiga toxin–producing *Escherichia coli* (STEC), including isolates implicated in produce outbreaks.

The study demonstrated for the first time that the persister state exists within Shiga toxin–producing *Escherichia coli*. An unexpected finding was the degree to which the levels of persisters differed among the strains tested. *E. coli* O157 and O103 were more predisposed to enter the persister state compared to the other serotypes tested. A broader range of isolates would be required to assess if the persister state is strain or serotype related. It is anticipated that screening the available genome sequence of different strains will identify genes that are associated with a disposition towards inducing the persister state.

2. Assess the role of different soil types and lettuce root exudates in the induction or breaking of the dormant state.

An assay was developed to assess the induction of the persister state within STEC in the presence of different agents or extracts. From the range of agents screened, only indole was found to promote a greater proportion of persisters. Indole is an established regulator within *E. coli* and hence it was not unexpected to find that the compound also played a role in promoting dormancy within the bacterium. It would be interesting to assess if the persistence of STEC in soil could be correlated to indole concentration thereby providing a marker for high risk fields.

The additional activators tested, which included soil, seed exudates and bile byproducts, suppressed the formation of persisters. Yet, it should be noted that this does not relate to the persistence of dormant cells within plants or in soil but more that growing STEC would not have an increased tendency to enter the persister state.

In the original proposal, the intent was to use microfluidic and flow cytometry to study the effect of inducers of the persister state or activators to break dormancy. Despite efforts to optimize the microfluidic system, there were inherent limitations to the approach. Specifically, the field of view was limited to 60 cells, which made identifying persisters within a general population problematic, given their occurrence as 1 in 100,000 cells. Also, the microfluidic chip dried after 4 h, thereby limiting the timeframe to perform image capture, as was most evident with assays to visualize the response of persisters to activator agents. Although changes in motility were observed by the addition of agents, the 4-h timeframe was insufficient for outgrowth.

Flow cytometry was to be applied to differentiate between growing, tolerant and persister cells, thereby enabling selective enumeration. The approach was based on using viability staining whereby viable cells would appear green, tolerant cells would appear orange, and non-viable cells would appear red. In reality, the technique could differentiate between dead and viable cells but not between persister and tolerant cells. However, it was possible to differentiate persister from tolerant cells using hemocytometry (direct microscope count) and hence was applied in the study. With both the imaging and flow cytometry, different methods were adapted thereby enabling the objective to be met.

3. Establish the resistance of dormant STEC to free chlorine.

The most significant aspect of persisters is the ability to have increased tolerance to stresses encountered within the fresh produce chain. This would include survival in soil through to resistance to chlorine in post-harvest washes. In the current study, persister levels remained static within soil, with “normal” cultures transitioning from viable, then losing culturability until reaching the baseline persister levels. Persister cells exposed to free chlorine also exhibited higher tolerance to the sanitizer compared to the bulk population. This result would need to be accounted for when predicting die-off rates or sanitizer susceptibility tests.

4. Evaluate the virulence of dormant STEC with respect to Shiga toxin production and attachment.

Through trials performed it was found that the outgrowth of persisters resulted in progeny that could produce Shiga toxin–producing *Escherichia coli*, thereby a measure of virulence. There was insufficient time within the timeframe of the project to undertake attachment studies. Yet, it should be noted that the ability to attach to host cells is a more stable trait compared to Shiga toxin production, thereby confirming that persister cells retain virulence.

Summary of Findings and Recommendations

The persister state in bacteria was first documented in the 1940s, although interest has been primarily in clinical microbiology with respect to antibiotic resistance and infectivity. Within the food sector, the main interest in dormancy has been with respect to viable but nonculturable (VBNC). Although still debated, the persister state can be differentiated from VBNC by the fact the former is induced in a population of the cells and ultimately the bacteria can be cultured under appropriate conditions. In contrast, VBNC represents a state that cells undertake by a progressive loss of viability to the point of nonculturability. It is likely that text referring to VBNC was actually observing persisters, given that it was possible to eventually culture cells.

The current study was one of the first to explore the significance of the persister state in STEC. Importantly, the study was placed into the context of how the persister state can influence the activity of STEC within the fresh produce chain. Baseline studies evaluated the extent to which the persister state was encountered within STEC, especially those belonging among the Big 7 serotypes. Although persisters could be recovered from all strains tested, it was evident that *E. coli* O157:H7 and O103 produced a higher proportion compared to the other serotypes tested. Given that only a single strain of a serotype was screened, it would be premature to assume that no intra-strain differences in the perister yield exist. Yet, it is clear that different *E. coli* types have a range of abilities to enter the persister state. Through use of spectroscopic techniques, it was illustrated that persister cells could be differentiated from the main population. Moreover, it was illustrated that the induction of the persister state occurs in the lag phase and a more rapid adaption compared to the VBNC state that requires an extended time to enter.

From the range of inducing agents, only indole was found to increase the proportion of persisters within the STEC strains tested. Indole is encountered in soil in varying amounts depending on the presence of producing and utilizing microbes, in addition to exogenous sources in waste. It is possible that the antimicrobial activity of indole causes the induction of the persister state, or effects could be more indirect as a physiological regulator. It was interesting to note that the other agents tested did not lead to increased persisters but the majority decreased the numbers recovered. Bile salts (or more correctly the breakdown products of bile metabolism) are antimicrobial in a similar way to indole, yet inclusion in the growth medium reduced the levels of persisters. Enteric bacteria typically use bile and bile breakdown products as metabolic regulators to sense the status of the gastrointestinal tract. In this respect, the presence of bile could have provided a metabolic signal for *E. coli* to initiate growth as opposed to entering dormancy.

It may have been expected that dormancy would be promoted in the soil environment, given that the environment would increase the need for survival as opposed to growth. However, it is more likely that the soil environment selected for persisters rather than inducing the dormant state. This may reflect that the history of the STEC bacteria could be more relevant, be it manure or irrigation water, which in turn would suggest the induction of the persister state within water or gastrointestinal tract could be more relevant.

Similar to soil, seed exudates from sprouting seeds (mung beans) depressed the formation of persisters relative to controls. This result may have been expected, given that the release of nutrient-rich exudates would be conducive to growth of STEC as opposed to entering a dormant state. The composition of root

exudates changes during plant development, so it is possible that in maturing plants, constituents may result in a greater proportion of persisters. However, this will need to be demonstrated experimentally.

The higher tolerance of persisters to stresses, such as survival in soil or to hypochlorite, was confirmed in the current study. By down-regulating metabolism, cells become tolerant to stressful environments and hence provide long-term survival. Indeed, it is thought that the dormant state evolved to ensure that a proportion of a bacterial population survived in the event of a sudden imposition of stress. The main significance of the findings in the current study is that one could expect STEC to be primarily in the persister state in the natural environment and hence more stress tolerant than laboratory-prepared cultures. This is one likely reason why validation studies (e.g., with post-harvest washes) do not tend to reflect observations made in commercial practice. This is the case for both predictive modelling for pathogens in soil along with the efficacy of post-harvest washing. Although still to be studied, it could also apply to tolerance of pathogens to stresses encountered within the food industry.

With respect to pathogen control, there is interest in identifying agents that could be used to activate dormant cells, thereby making them more susceptible to stress and amenable to culturing. In the current study, it was noteworthy that inclusion of lettuce extract in recovery media enhanced the recovery of persisters. It is unclear at this time if there is a specific constituent within lettuce that activates the cell or is a combination of compounds. Using nutrient rich media (e.g., tryptic soy or brain heart infusion) did not stimulate recovery of persisters, thereby suggesting there are key components that alleviate the persister state. In the current study, the extract from leafy green lettuce was used and it is unclear if different varieties or other produce types would have the same effect. In a commercial context, it can be envisioned that processed lettuce could increase the risk of STEC persisters being activated and then undergoing outgrowth if the product is temperature abused. Yet, the finding could have positive effects when recovering STEC from produce and environmental samples. In this respect, it is noteworthy that culturing STEC from PCR-positive samples still represents a challenge, with a high frequency of false positives recorded. However, all the aforementioned are purely speculative at the current time and will require further research.

Recommendations

It can be envisaged that the perister state is frequently encountered with pathogens associated with the fresh produce chain. Despite the significance, there are many aspects of the persister state in STEC and other foodborne pathogens that remain unknown. Such knowledge gaps need to be addressed, given the impact the persister state has on pathogen survival, resistance and culturability. Based on the results of the study, further research is recommended to:

- Assess the extent to which persisters are generated by a diverse range of STEC strains and serotypes.
- Identify regulatory genes within STEC that control induction and breaking the persister state.
- Establish if indole concentration in soil promotes persister formation and thereby can be used as an additional marker to identify high (food safety) risk fields.
- Evaluate the significance of the persister state on the survival of STEC in the rhizosphere, phyllosphere and internalized populations.
- Evaluate the resistance of STEC persister cells to a diverse range of sanitizers encountered within the fresh-cut chain.
- Establish if amending fields with lettuce extracts would decrease the persistence of STEC within the environment.
- Identify constituents within lettuce that can reactivate persisters thereby potentially leading to lower survival in the environment, increased susceptibility to sanitizers and increasing the culturability of STEC.

Acknowledgements

We wish to thank the Centre for Produce Safety for their financial support, and to Dr. Rebecca Lindsey (CDC), in addition to Dr. Roger Johnson (PHAC) for donation of STEC strains.

APPENDICES

Publications and Presentations

Publications:
None at this time but papers are in preparation.

Oral Presentations:
Warriner, K. (2017). Significance of the dormant state in the persistence, interaction with growing plants and virulence of Shiga toxin–producing *Escherichia coli.* CPS Research Symposium (Denver, CO; June).

Warriner K. (2017). Significance of the persister state in pathogens to public health and infection control. Centre for Public Health and Zoonosis annual meeting (Guelph, ON; May).

Poster Presentations:
Tremblay, C., Neethirajan, S. and Warriner K. (2017). Significance of the persister dormant state in Shiga toxin producing Escherichia coli. OMAFRA Food Safety Symposium (Guelph, ON; May).

Tremblay, C., Neethirajan, S. and Warriner K. (2018). Induction of the persister state in Shiga Toxin producing *Escherichia coli* by indole and inhibition by seed exudates. Ontario Food Protection Association annual meeting (Mississauga, ON; April).

Alzahrani, A., Neethirajan, S. and Warriner K. (2018). The induction and persistence of Shiga Toxin producing Escherichia coli in the dormant state. Ontario Food Protection Association annual meeting (Mississauga, ON; April).

Budget Summary

The initial project budget was $73,040; all funds were expended.

Item	*Cost*
Salaries	57,544.74
Supplies	8,505.95
User fees	3,783.71
Overhead	3,205.00
Total	$73,039.40

Suggestions to CPS

We appreciate the funding from the CPS for this type of project that doesn't have immediate benefits but provides a foundation to make future advances to enhance the microbiological safety of fresh produce.

Tables and Figures

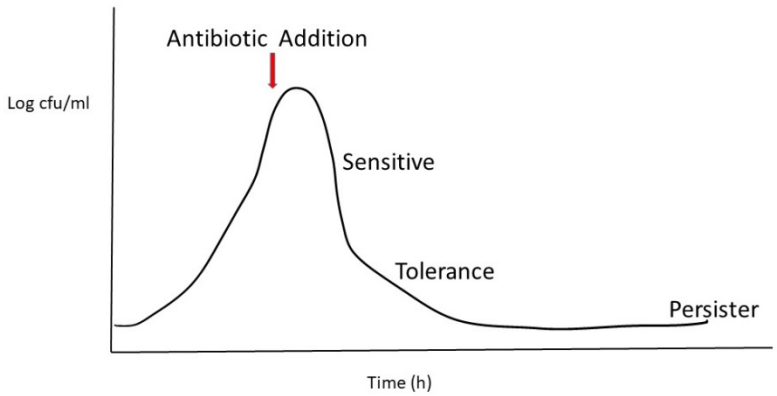


Figure 1: Method for selecting for persisters in bacterial populations. The culture is grown to mid-exponential phase and membrane acting antibiotic added. Growing cells are sensitive to the antibiotic with tolerant (slow growing) being sensitive but persisters resistant. At the end of the incubation period the dormant cells were recovered, then enumerated using culture-based or microscopy techniques.

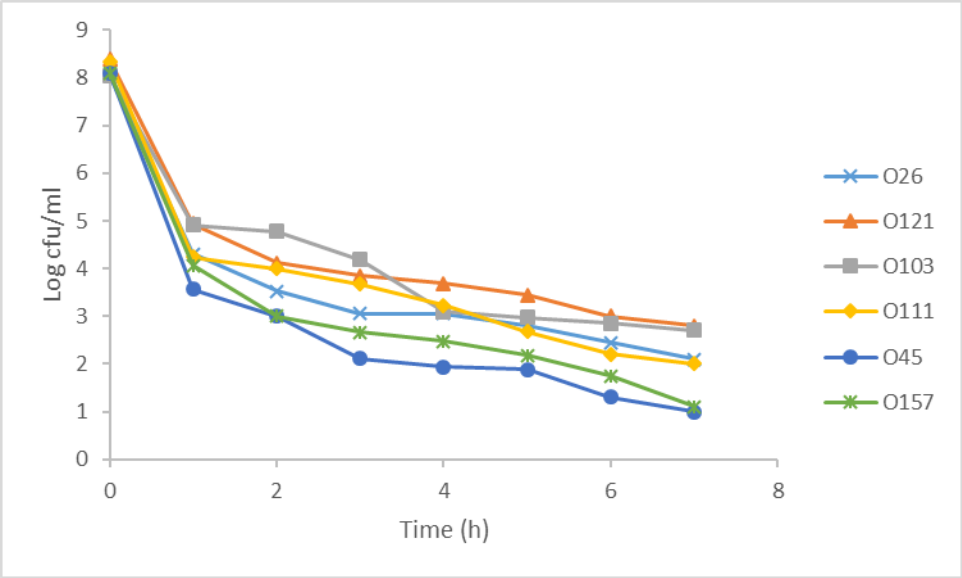


Figure 2: Persisters within different Shiga toxin *Escherichia coli* serotype populations cultivated in LB broth and treated with ampicillin at 200 µg/ml. The test *E. coli* strain was cultivated in LB broth to mid-exponential phase, then ampicillin was added to the culture. Samples were then removed periodically and survivors enumerated on LB agar cultivated at 37°C for up to 72 h.

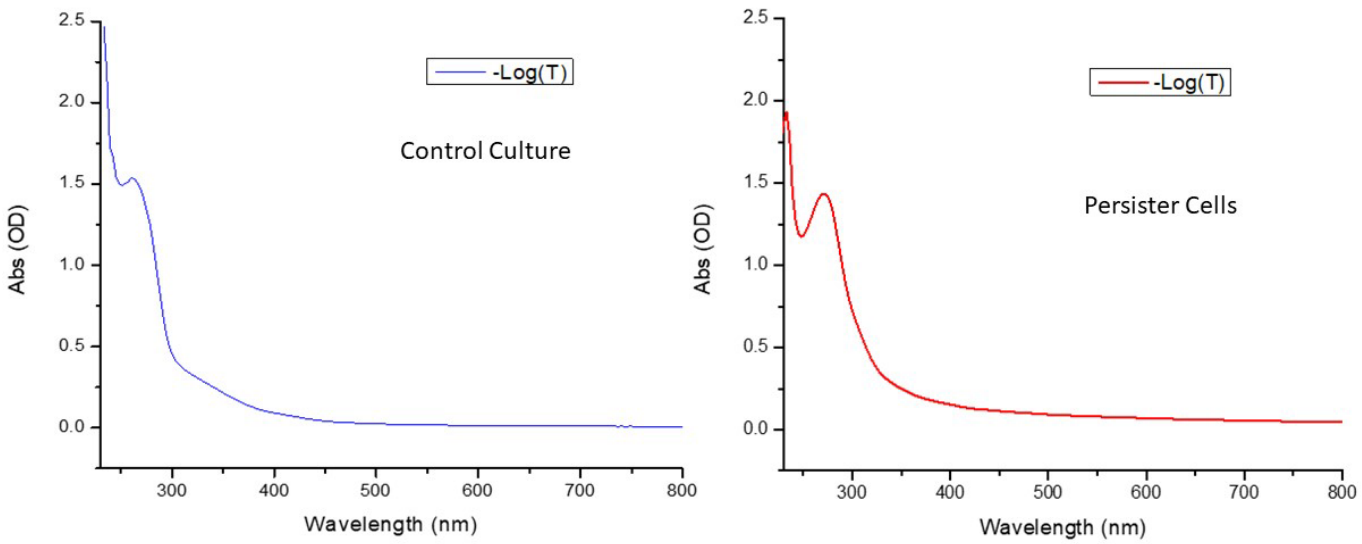


Figure 3: Absorbance spectra of a growing culture and persister cells of *E. coli*. A growing culture and suspension of persister cells were adjusted to the same optical density, and then the absorption spectra captured using Aqualog spectrometer.

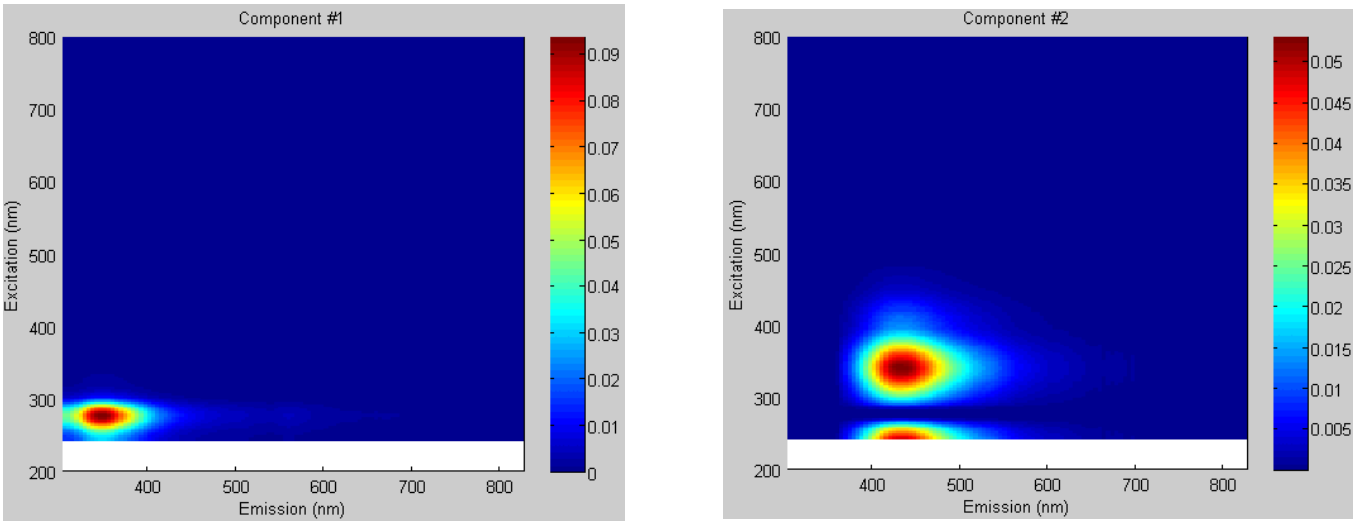


Figure 4: Excitation emission matrices obtained from the persister and resistance cells using the simultaneous fluorescence and absorbance spectrum through PARAFAC model to clearly distinguish the signatures. Percent contribution of surface protein differences can be observed in the figures as shown above. Component 1: General population; Component 2: Persister cells: (n=4).

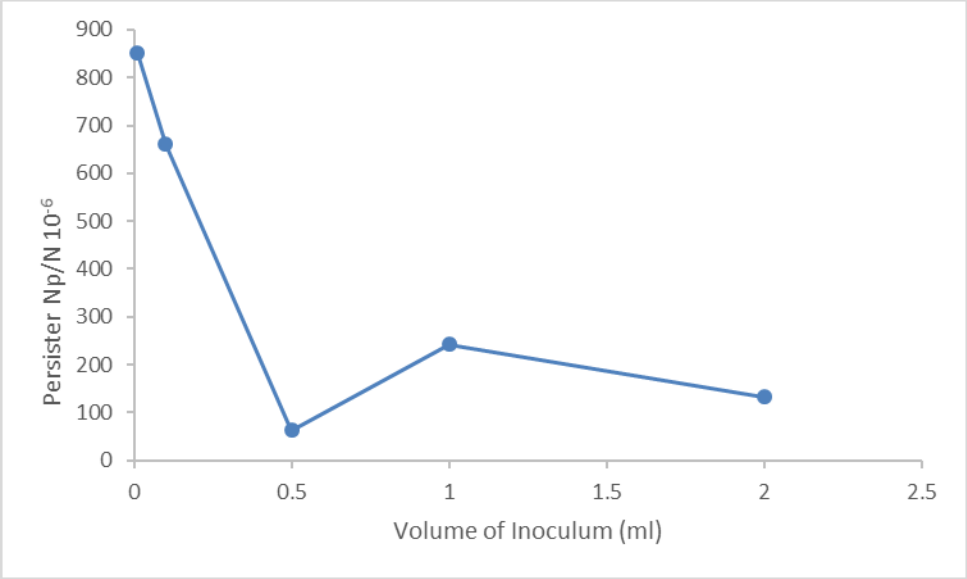


Figure 5: The effect of inoculum cell density of the recovery of persisters. *E. coli* O103 was cultivated overnight in M9 medium, then different volumes (0.01–2 ml) were added to 20 ml of M9 broth. The cultures were grown to mid-exponential phase before addition of ampicillin and further 8-h incubation. The persisters were then recovered by plating onto TSA and incubating at 37°C for up to 72 h.

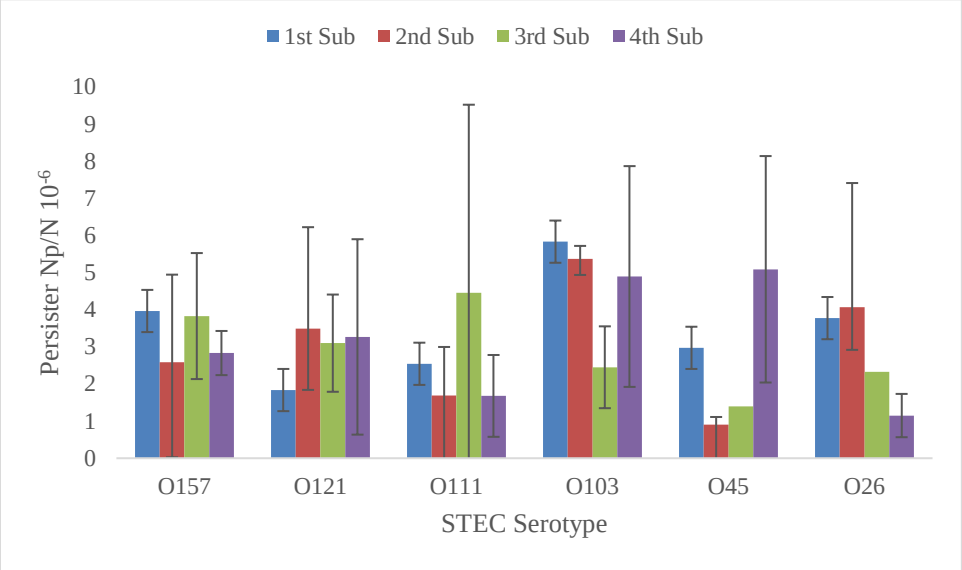


Figure 6: The impact of subculture on persister proportion recovered from STEC strains. Persisters were recovered and used to inoculate a fresh culture that was grown to mid-exponential phase before the addition of ampicillin. The persisters were recovered and transferred to a fresh culture and re-grown prior to the addition of antibiotic. Four subcultures were performed in total.

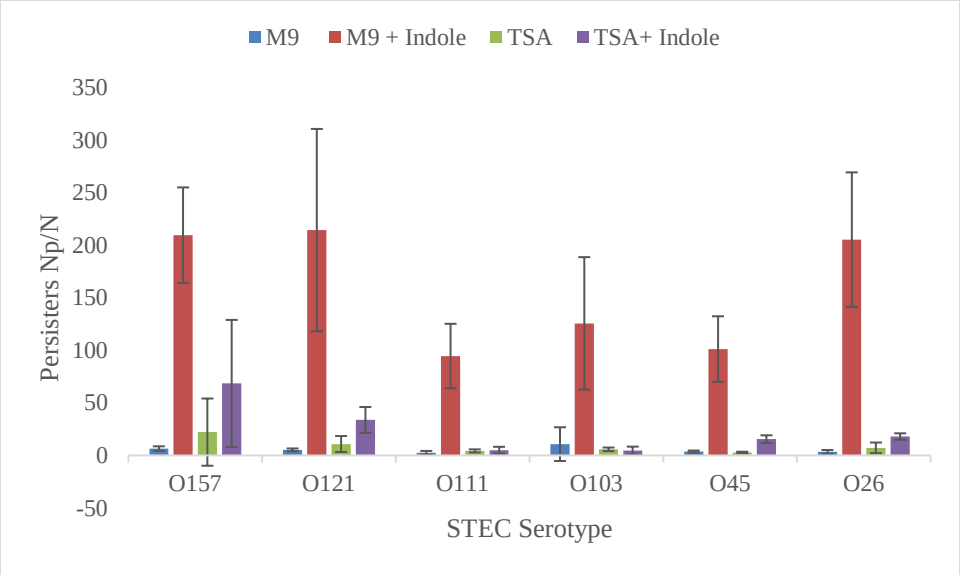


Figure 7. The effect of indole on inducing the proportion of persisters within STEC populations. The test STEC strain was cultivated in M9 media, and then inoculated into M9 or TSB with or without indole (500 µM). The cultures were grown to mid-exponential phase before adding ampicillin and incubating for a further 8 h. The persister cells were recovered by plating onto TSA agar incubated at 37°C for up to 72 h.

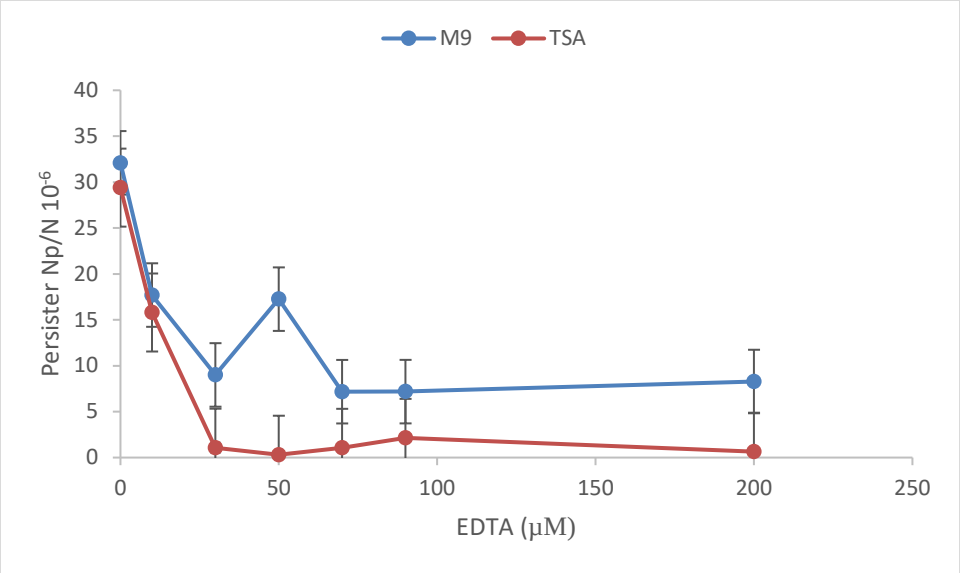


Figure 8: The effect of ethylenediaminetetraacetic acid (EDTA) concentrations in O103 STEC persister cells. E. coli O103 was cultivated in TSB or M9 with different levels of EDTA, then ampicillin added when the culture had reached mid-exponential phase. The culture was incubated for a further 8 h and then persisters recovered on TSA.

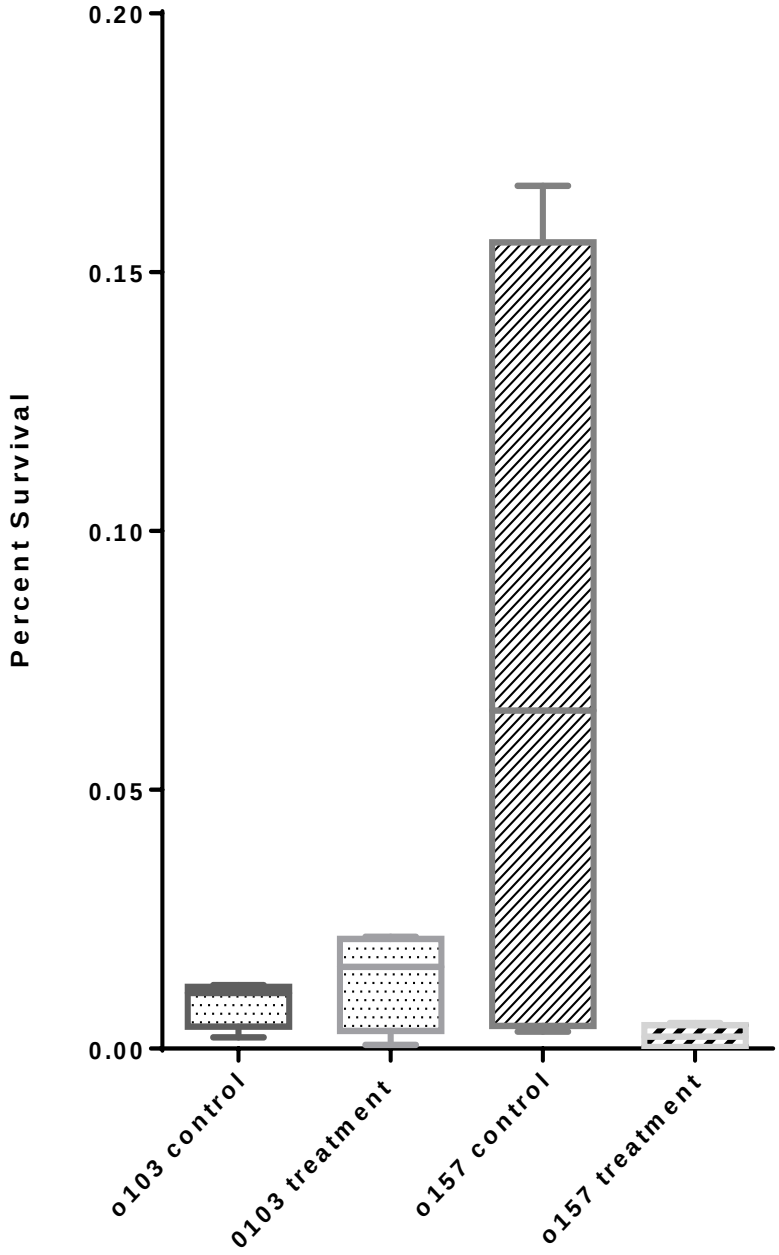


Figure 9: Persister formation in E. coli P36, O103 and O157 grown in M9 medium with or without 0.2% deoxycholate.

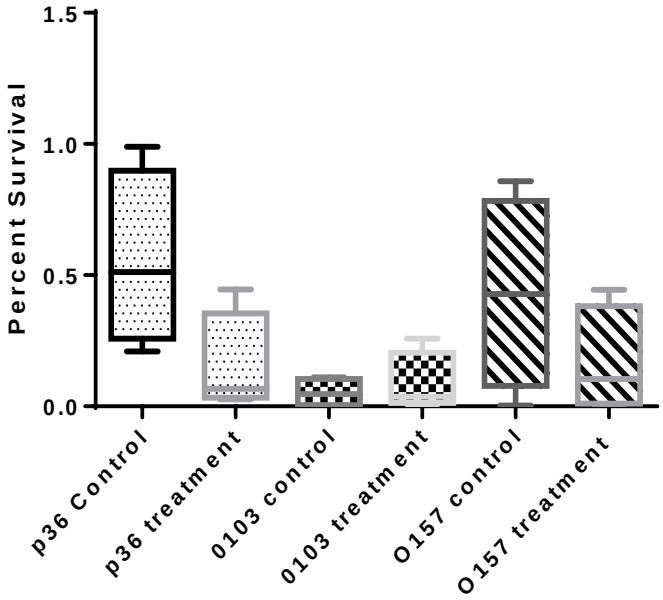


Figure 10: Percent survival of *E. coli* P36, 0103 and O157 grown in M9 media (controls) or M9 media supplemented with 0.2% deoxycholate, and grown to the stationary phase after treatment with ampicillin at 200 μg/ml.

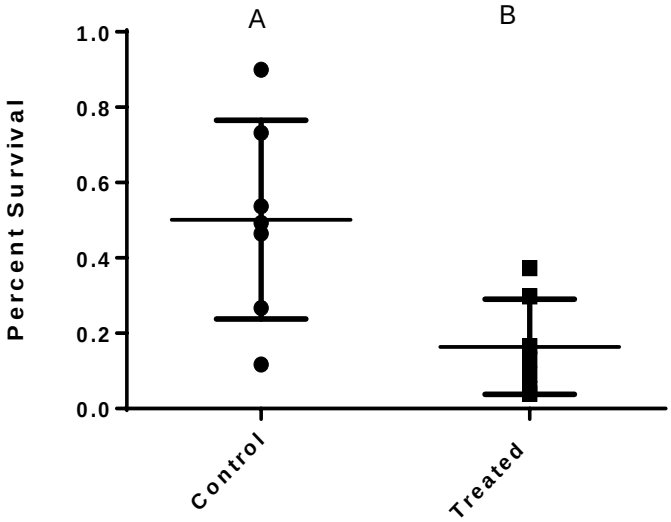


Figure 11: Effect of seed exudates on persister formation in *E. coli* P36. An E. coli suspension was placed in a dialysis cassette that was transferred to a bed of sprouting mung beans. After 7 days sprouting, the contents of the dialysis cassette were transferred to M9 medium and grown to mid-exponential phase, and then ampicillin was added. After a further 8-h incubation the persisters were recovered on TSA. Controls were performed by suspending *E. coli* in saline.

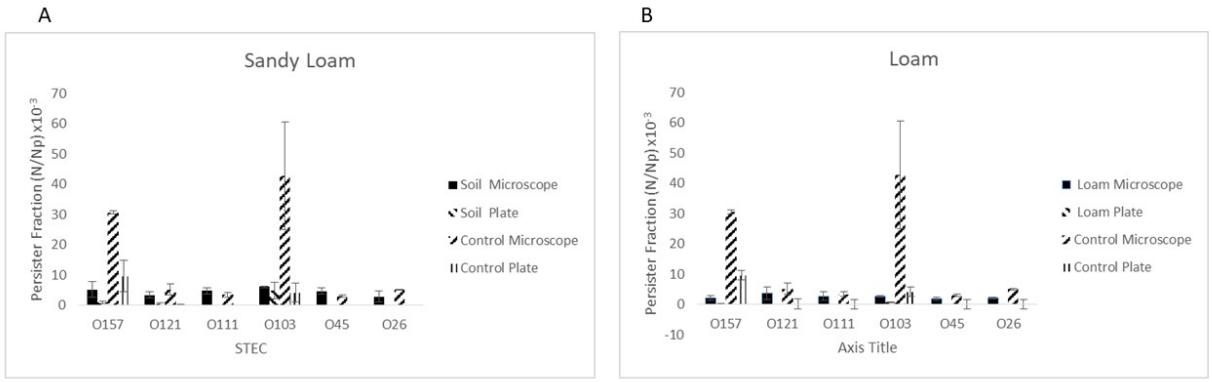


Figure 12: Persister formation in STEC by sandy loam (A) or loam (B) soil constituents. The test *E. coli* strain was introduced into dialysis cassettes, and then placed in saturated soil microcosms. After 48 h the contents of the cassette were transferred to M9 media and incubated at 37°C for 2 h before adding antibiotic. The culture was incubated for a further 8 h, and persisters enumerated by plating, in parallel with microscopy counts. Controls were treated the same except placed in saline rather than the soil microcosm.

Table 1: Persisters recovered from STEC cultures treated with ampicillin, incubated for 8 h at 37°C, then plated onto different agars to enumerate survivors after incubating at 20 or 37°C.

STEC Serotype	Log cfu/ml Recovery Agar and Incubation Temperature					
	BHI		TSA		LB	
	20°C	37°C	20°C	37°C	20°C	37°C
O157	1.26	1.29	1.29	1.31	1.28	1.29
O121	1.62	1.70	1.62	1.89	1.66	1.88
O111	1.42	1.48	1.46	1.34	1.58	1.70
O103	2.39	2.37	2.48	2.48	2.38	2.41
O45	3.15	3.30	1.92	1.96	1.75	1.82
O26	1.68	1.42	1.52	2.16	1.60	1.84

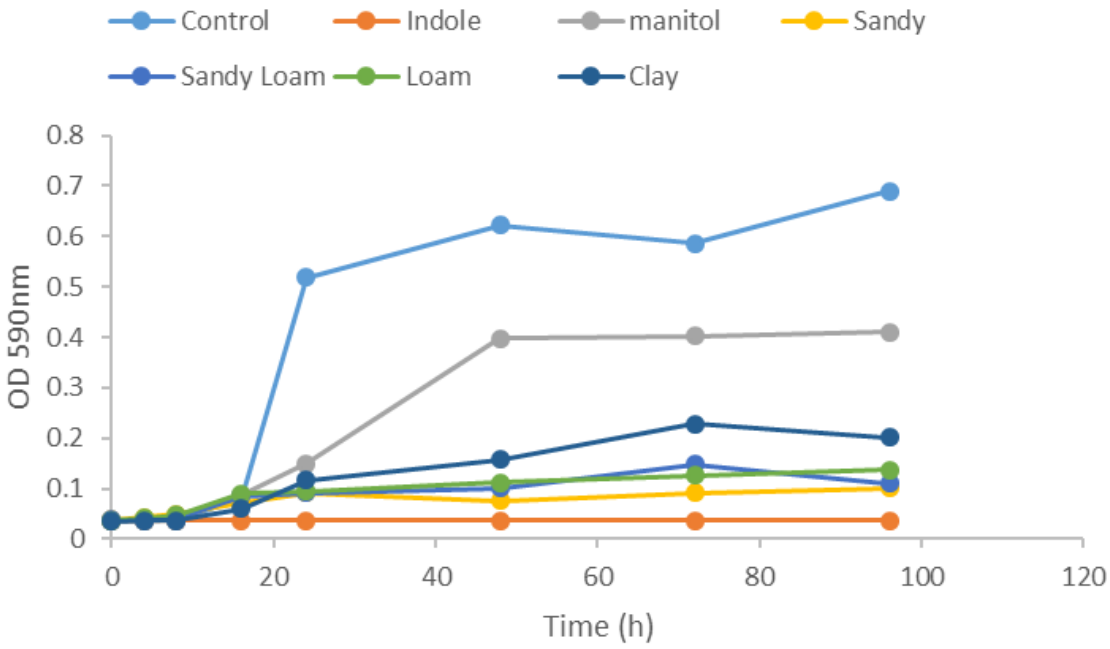


Figure 13: Effect of various agents in alleviating the persister state in *E. coli* O103. Persister cells were generated by treating cultures with antibiotic. The perister cells were diluted to a final cell density of 100 cfu/ml, and then inoculated into M9 media containing the test agent (soil extract, indole or mannitol). The plates were incubated at 37°C, and optical density at 595 nm determined periodically.

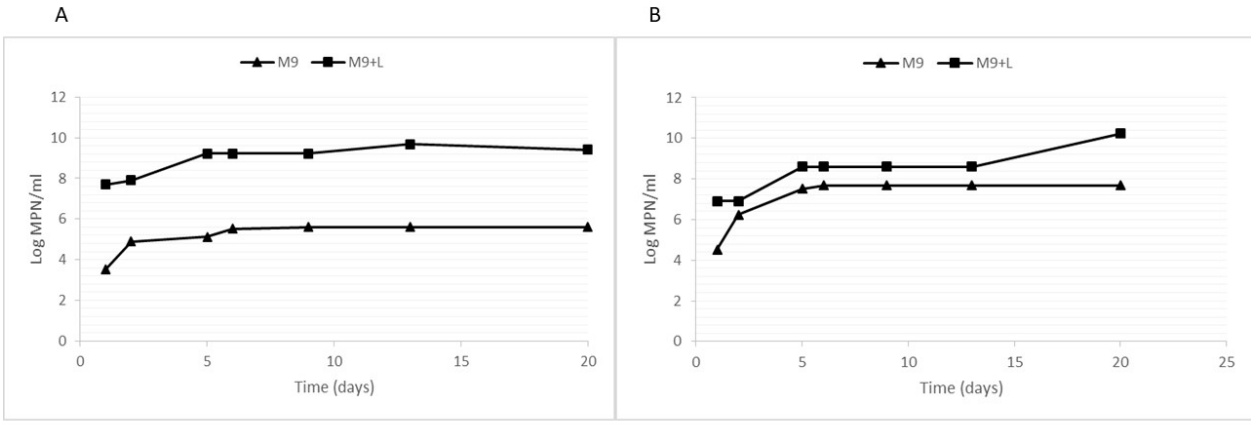
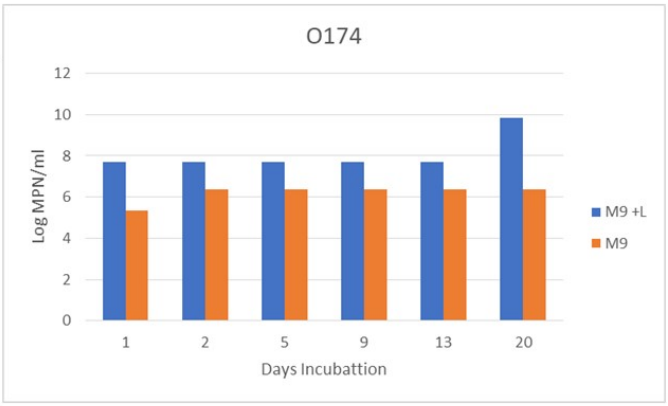
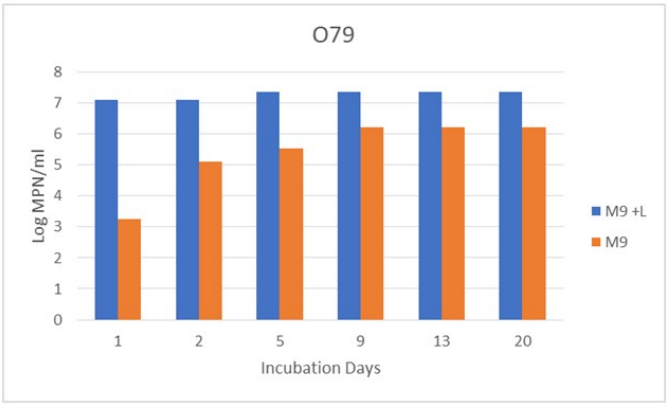


Figure 14: Recovery of *E. coli* O157 (A) and O103 (B) persisters in M9 agar supplemented with or without lettuce extract. *E. coli* were cultivated in M9 medium containing indole and ampicillin added upon reaching mid-exponential phase. The culture was incubated for a further 8 h, and persisters recovered on M9 plates with or without 1% lettuce extract.

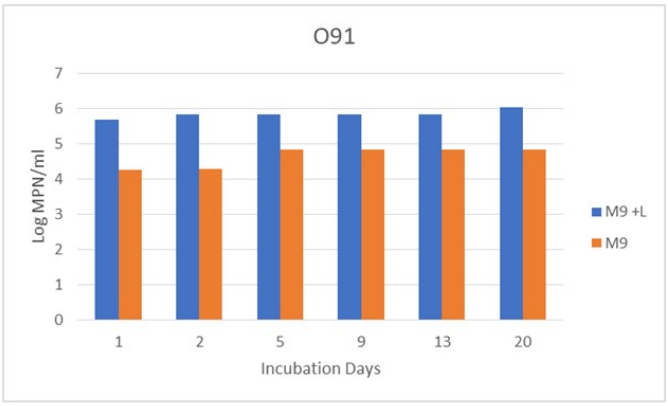
A


O174
12
10
8
6
4
2
0
Log MPN/ml
1
2
5
9
13
20
Days Incubattion
M9 +L
M9

B


O79
8
7
6
5
4
3
2
1
0
Log MPN/ml
1
2
5
9
13
20
Incubation Days
M9 +L
M9

C


O91
7
6
5
4
3
2
1
0
Log MPN/ml
1
2
5
9
13
20
Incubation Days
M9 +L
M9

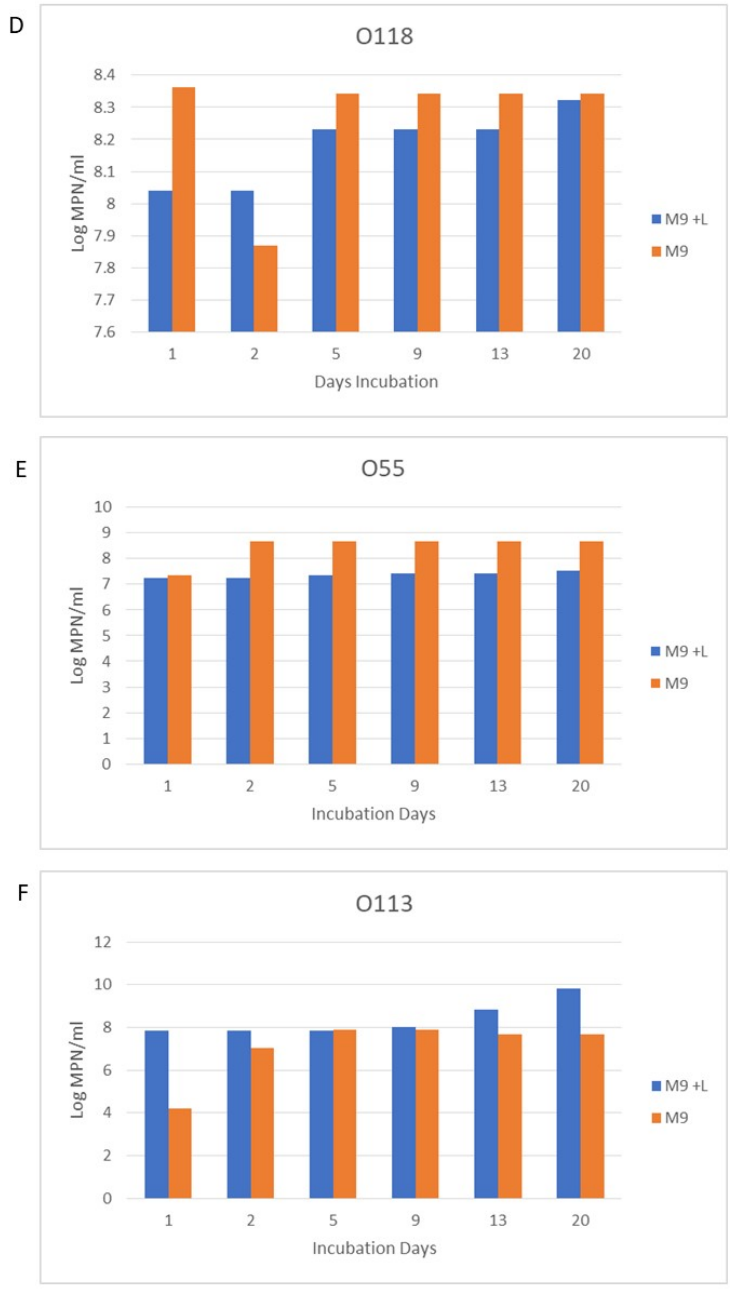


Figure 15: Recovery of persisters derived from different Shiga toxin–producing *Escherichia coli* on M9 agar with or without lettuce extract. Persisters of the test STEC strain were generated by growing the bacterium in M9 media containing indole up to mid-exponential phase, then treating with ampicillin. The culture was incubated for a further 8 h, then used to inoculate a titer plate containing M9 media with or without lettuce extract. The inoculated plate was then incubated for 30 days and inspected for growth periodically. Based on growth or no growth, the MPN was determined.

Table 2: Resistance of *E. coli* O157:H7 persister cells compared to standard stationary phase culture. The *E. coli* O157:H7 strain was cultivated in M9 medium to late exponential phase and then subdivided into two tubes. Ampicillin was added to one tube and further incubated for 8 h at 37°C. The other tube was incubated for the same time but without antibiotic. The cells were then challenged with different concentrations of free chlorine for 30 s and survivors enumerated.

Free-chlorine (ppm)	**Normal Stationary Phase Culture**		**Persister**	
	Log cfu/ml	**Log Count Reduction**	**Log cfu/ml**	**Log Count Reduction**
0	4.14 ± 0.07		2.00 ± 0.32	
0.5	<1.0	>3.14	2.03 ± 0.35	-0.03 ± 0.35
1.0	<1.0	>3.14	1.75 ± 0.64	0.25 ± 0.64
2.0	<1.0	>3.14	1.94 ± 0.48	0.06 ± 0.48
3.0	<1.0	>3.14	<1	>1

References cited

Amato, S.M. and Brynildsen, M.P. (2015) Persister heterogeneity arising from a single metabolic stress. *Current Biology* **25**, 2090-2098.

Amato, S.M., Fazen, C.H., Henry, T.C., Mok, W.W.K., Orman, M.A., Sandvik, E.L., Volzing, K.G. and Brynildsen, M.P. (2014) The role of metabolism in bacterial persistence. *Frontiers in Microbiol.* **5**, 9.

Amato, S.M., Orman, M.A. and Brynildsen, M.P. (2013) Metabolic control of persister formation in Escherichia coli. *Molecular Cell* **50**, 475-487.

Arthur, T.M., Barkocy-Gallagher, G.A., Rivera-Betancourt, M. and Koohmaraie, M. (2002) Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. *Applied and Environmental Microbiology* **68**, 4847-4852.

Ayrapetyan, M., Williams, T.C., Baxter, R. and Oliver, J.D. (2015a) Viable but nonculturable and persister cells coexist stochastically and are induced by human serum. *Infection and Immunity* **83**, 4194-4203.

Ayrapetyan, M., Williams, T.C. and Oliver, J.D. (2015b) Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends in Microbiology* **23**, 7-13.

Bai, X.N., Wang, H., Xin, Y.Q., Wei, R.J., Tang, X.Y., Zhao, A.L., Sun, H., et al. (2015) Prevalence and characteristics of Shiga toxin-producing Escherichia coli isolated from retail raw meats in China. *International Journal of Food Microbiology* **200**, 31-38.

Bannon, J., Melebari, M., Jordao, C., Leon-Velarde, C.G. and Warriner, K. (2016) Incidence of Top 6 shiga toxigenic Escherichia coli within two Ontario beef processing facilities: Challenges in screening and confirmation testing. *Aims Microbiology* **2**, 278-291.

Berry, E.D. and Miller, D.N. (2005) Cattle feedlot soil moisture and manure content: II. Impact on Escherichia coli O157. *Journal of Environmental Quality* **34**, 656-663.

Brennan, F.P., Moynihan, E., Griffiths, B.S., Hillier, S., Owen, J., Pendlowski, H. and Avery, L.M. (2014) Clay mineral type effect on bacterial enteropathogen survival in soil. *Science of the Total Environment* **468**, 302-305.

Cernicchiaro, N., Cull, C.A., Paddock, Z.D., Shi, X.R., Bai, J.F., Nagaraja, T.G. and Renter, D.G. (2013) Prevalence of Shiga Toxin-Producing Escherichia coli and Associated Virulence Genes in Feces of Commercial Feedlot Cattle. *Foodborne Pathogens and Disease* **10**, 835-841.

Erickson, M.C., Habteselassie, M.Y., Liao, J., Webb, C.C., Mantripragada, V., Davey, L.E. and Doyle, M.P. (2014) Examination of factors for use as potential predictors of human enteric pathogen survival in soil. *Journal of Applied Microbiology* **116**, 335-349.

Erickson, M.C., Smith, C., Jiang, X.P., Flitcroft, I.D. and Doyle, M.P. (2015) Survival of Salmonella or Escherichia coli O157:H7 during Holding of Manure-Based Compost Mixtures at Sublethal Temperatures as Influenced by the Carbon Amendment. *Journal of Food Protection* **78**, 248-255.

Fisher, R.A., Gollan, B. and Helaine, S. (2017) Persistent bacterial infections and persister cells. *Nature Reviews Microbiology* **15**, 453-464.

Franz, E., Schijven, J., Husman, A.M.D. and Blaak, H. (2014) Meta-Regression Analysis of Commensal and Pathogenic Escherichia coli Survival in Soil and Water. *Environmental Science & Technology* **48**, 6763-6771.

Franz, E., van Hoek, A.H.A.M., Bouw, E. and Aarts, H.J.M. (2011) Variability of Escherichia coli O157 Strain Survival in Manure-Amended Soil in Relation to Strain Origin, Virulence Profile, and Carbon Nutrition Profile. *Applied and Environmental Microbiology* **77**, 8088-8096.

Freestone, P.P.E. and Lyte, M. (2008) Microbial endocrinology: Experimental design issues in the study of interkingdom signalling in infectious disease. In *Advances in Applied Microbiology, Vol 64* eds. Laskin, A.I., Sariaslani, S. and Gadd, G.M. pp.75-105. San Diego: Elsevier Academic Press Inc.

Fremaux, B., Prigent-Combaret, C., Delignette-Muller, M.L., Mallen, B., Dothal, M., Gleizal, A. and Vernozy-Rozand, C. (2008) Persistence of Shiga toxin-producing Escherichia coli O26 in various manure-amended soil types. *Journal of Applied Microbiology* **104**, 296-304.

Gefen, O. and Balaban, N.Q. (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *FEMS Microbiology Reviews* **33**, 704-717.

Gerdes, K. and Maisonneuve, E. (2012) Bacterial Persistence and Toxin-Antitoxin Loci. In *Annual Review of Microbiology, Vol 66* eds. Gottesman, S., Harwood, C.S. and Schneewind, O. pp.103-123. Palo Alto: Annual Reviews.

Gombas, D., Luo, Y., Brennan, J., Shergill, G., Petran, R., Walsh, R., Hau, H., Khurana, K., Zomorodi, B., Rosen, J., Varley, R. and Deng, K. (2017) Guidelines To Validate Control of Cross-Contamination during Washing of Fresh-Cut Leafy Vegetables. *Journal of Food Protection* **80**, 312-330.

Ibekwe, A.M., Ma, J.C., Crowley, D.E., Yang, C.H., Johnson, A.M., Petrossian, T.C. and Lum, P.Y. (2014) Topological data analysis of Escherichia coli O157:H7 and non-O157 survival in soils. *Frontiers in Cellular and Infection Microbiology* **4**, 11.

Ishii, S., Ksoll, W.B., Hicks, R.E. and Sadowsky, M.J. (2006) Presence and growth of naturalized Escherichia coli in temperate soils from lake superior watersheds. *Applied and Environmental Microbiology* **72**, 612-621.

Kase, J.A., Maounounen-Laasri, A., Son, I., Lin, A. and Hammack, T.S. (2015) Comparison of eight different agars for the recovery of clinically relevant non-O157 Shiga toxin-producing Escherichia coli from baby spinach, cilantro, alfalfa sprouts and raw milk. *Food Microbiology* **46**, 280-287.

Lindsey, R.L., Trees, E., Sammons, S., Loparev, V., Frace, M., Strockbine, N., Sabol, A.L., Sowers, E., Stripling, D., Martin, H., Knipe, K., Rowe, L. and Gerner-Smidt, P. (2014) Draft Whole-Genome Sequences of Nine Non-O157 Shiga Toxin-Producing Escherichia coli Strains. *Genome announcements* **2**.

Moynihan, E.L., Richards, K.G., Ritz, K., Tyrrel, S.F. and Brennan, F.P. (2013) Impact of soil type, biology and temperature on the survival of non-toxigenic Escherichia coli O157. *Biology and Environment-Proceedings of the Royal Irish Academy* **113B**, 41-46.

Murray, K., Aldossari, H., Wu, F. and Warriner, K. (2018) Dynamic changes in free-chlorine levels within a commercial post-harvest wash and prevention of cross-contamination between shredded lettuce batches. *Food Control* **85**, 127-134.

Naganandhini, S., Kennedy, Z.J., Uyttendaele, M. and Balachandar, D. (2015) Persistence of Pathogenic and Non-Pathogenic Escherichia coli Strains in Various Tropical Agricultural Soils of India. *Plos One* **10**, 19.

Nou, X.W. and Luo, Y.G. (2010) Whole-Leaf Wash Improves Chlorine Efficacy for Microbial Reduction and Prevents Pathogen Cross-Contamination during Fresh-Cut Lettuce Processing. *Journal of Food Science* **75**, M283-M290.

Ongeng, D., Geeraerd, A.H., Springael, D., Ryckeboer, J., Muyanja, C. and Mauriello, G. (2015) Fate of Escherichia coli O157:H7 and Salmonella enterica in the manure-amended soil-plant ecosystem of fresh vegetable crops: A review. *Critical Reviews in Microbiology* **41**, 273-294.

Orman, M.A. and Brynildsen, M.P. (2015) Inhibition of stationary phase respiration impairs persister formation in E-coli. *Nature Communications* **6**.

Putrins, M., Kogermann, K., Lukk, E., Lippus, M., Varik, V. and Tenson, T. (2015) Phenotypic Heterogeneity Enables Uropathogenic Escherichia coli To Evade Killing by Antibiotics and Serum Complement. *Infection and Immunity* **83**, 1056-1067.

Shidore, T. and Triplett, L.R. (2017) Toxin-Antitoxin Systems: Implications for Plant Disease. In *Annual Review of Phytopathology, Vol 55* eds. Leach, J.E. and Lindow, S.E. pp.161-179. Palo Alto: Annual Reviews.

Vega, N.M., Allison, K.R., Khalil, A.S. and Collins, J.J. (2012) Signaling-mediated bacterial persister formation. *Nature Chemical Biology* **8**, 431-433.

Warriner, K. and Council, I.U.S. (2011) IUFoST Scientific Information Bulletin. Shiga toxin producing Escherichia coli: Germany 2011 Escherichia coli O104:H4 outbreak linked to sprouted seeds. *IUFoST Scientific Information Bulletin Shiga toxin producing Escherichia coli: Germany 2011 Escherichia coli O104:H4 outbreak linked to sprouted seeds*.