THE PENNSYLVANIA STATE UNIVERSITY
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DRAMATIC INCREASE IN THE FREQUENCY OF *LISTERIA MONOCYTOGENES* PERSISTER CELLS AS THE POPULATION TRANSITIONS FROM EXPONENTIAL PHASE TO LONG-TERM-SURVIVAL PHASE SUGGESTS THEIR TRUE NATURE AND BIOLOGICAL FUNCTION

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ABSTRACT

Persisters are dormant cells that are found in all free-living bacterial species analyzed to date and are defined by their ability to tolerate multiple antibiotics. They play a significant role in chronic infections, biofilm formation, and food safety. Persisters are found not only in bacteria, but also in most archaea, fungi, and mammalian tumor cells. Therefore, it is critical to understand the true nature and biological function of persister cells. Current knowledge concerning the formation of persisters is limited. Certain genes such as toxin-antitoxins, and environmental stresses have been found to play key roles in persister cell formation. However, the effect of life-cycle phase on persister cell formation, their true nature, and biological function still remain unclear. In this study, persister cell formation was examined by treating cells of Listeria monocytogenes in different life-cycle phases with a lethal dose of penicillin. The frequency of persisters increased sharply after they exited log phase at around log 7.0 CFU/ml (OD~0.02). More importantly, different life-cycle phases exhibited different death kinetics. Homogeneous cultures of L. monocytogenes F2365 in the exponential phase and the Long-Term-Survival phase (LTS) had uniform death kinetics, while a heterogeneous culture containing persisters and non-persisters presented biphasic death kinetics. In conclusion, the dramatic increase in persister frequency after the log phase suggests their true nature and biological function is to enhance fitness by enabling long-term survival in nature.
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PROBLEM STATEMENT

Current dogmas concerning persister cells may be incorrect. If true, this would obscure the true nature and biological function of persister cells. Ever since their initial discovery, most authors have defined persisters as a small subpopulation of cells that are tolerant to antibiotics (1, 2, 3, 4). However, whether they are a small subpopulation still remains unclear. The above definition implies that the biological function of persisters is to tolerate antibiotics, which also may be incorrect. It is important to realize that tolerating antibiotics is an operational definition, based on the tolerance of metabolically dormant cells to antibiotics, and is not a functional definition. Second, persister cells in the stationary phase are claimed to reach a maximum frequency of 1% (5, 6, 7, 8, 9). This claim needs to be re-examined because results from this investigation and from other studies (10, 11) demonstrated that persisters could reach much greater than 1% of the population. Lastly, Type II persisters are defined as those that form spontaneously in the logarithmic phase without being induced by stress (12, 13, 14, 15). However, results from Keren et al. (16) and a mathematical model proposed by Lou et al. (17) suggest otherwise.

Understanding the true biological function of persister cells would not only elucidate current unresolved mysteries in microbiology, but it would also help in understanding life itself since persisters are found in all domains of life (18, 19, 20). Therefore, the purpose of this study was to investigate the different death kinetics exhibited by L. monocytogenes F2365 from the exponential phase to the long-term-survival (LTS) phase. The difference in death kinetics observed throughout the life cycle of L. monocytogenes suggests that persister cells only form in the presence of stresses to ensure long-term survival in nature.
INTRODUCTION

History of persister cell research

While studying the mechanism of penicillin, Hobby et al. described in 1942 a small population of *Streptococcus* that normally survived bactericidal concentrations of this antibiotic (21). According to their observations, 99% of the bacterial population was killed at a constant rate by the action of penicillin, and the 1% left could not be destroyed (21). Two years later, Joseph Bigger noticed the same pattern with *Staphylococcus aureus* and named these surviving cells “persisters” (1). Bigger believed that the dormant, non-dividing state of these cells conferred insensitivity towards this antibiotic. This is consistent with the known mechanisms of action of most antibiotics, namely destruction via the corruption of active metabolisms such as protein, RNA, DNA, and cell wall synthesis (22). Bigger also showed that when persisters were re-inoculated into fresh media they would germinate back to a dividing, metabolically active state, and thus these daughter cells would be as susceptible to penicillin as the previously killed population (1). Numerous subsequent authors have continued to define persisters as a small subpopulation of bacterial cells that survive antibiotic treatment (2, 3, 4).

Many authors have tried to describe and distinguish persisters from non-persisters. Persister cells are phenotypically different from non-persister cells (11). They are coccoid-shaped and smaller (5, 11, 23). Also, persister cell levels are higher in the stationary phase than in the exponential phase, which means the frequency of persister cells increases over time (11, 24). Lastly, scientists have recently described two types of persisters, Type I and Type II (13, 23,
Type I persisters are found during stationary phase and are induced by stress, whereas Type II persisters are defined as forming spontaneously in the logarithmic phase in the absence of stress (13, 23, 26).

**Antibiotic tolerance or resistance**

It is important to understand the difference between antibiotic tolerance and antibiotic resistance. Antibiotic tolerance is a phenotype that is not caused by mutations, while antibiotic resistance is a change in genotype that involves mutations (27). Tolerance is the product of dormancy, a state in which bacteria have little to no metabolic activities. On the other hand, resistance is caused by cells that have evolved genetically to resist stresses. For example, resistance mechanisms include destruction, modification, or efflux of antibiotics (28). As a result, resistant strains can grow in the presence of antibiotics, while persisters cannot. In addition, resistance is inheritable while persistence is non-inheritable. Persistence was first observed by Bigger in 1944 (1); he demonstrated that germinated persister cells were equally susceptible to the bactericidal action of penicillin as the sensitive population. There is a simple way to distinguish between these two in a laboratory experiment. As shown in Fig. 1, the addition of a bactericidal dose of antibiotic resulted in a tail of persisters, because the rest of the population was killed, leaving only the tolerant cells alive. Meanwhile, mutant cells were not corrupted by the antibiotic due to their resistance mechanisms, thus they exhibit growth and no death in the presence of antibiotic.
Figure 1. Difference between tolerance and resistance. A bactericidal dose of antibiotic is added to different bacterial cultures at time zero (1). During the antibiotic exposure, resistant mutants (dotted upper line) are not being affected by the action of the antibiotic. If a wild type culture (black solid line) contains persister cells, there would be an exponential killing curve initially (2), and a slower death kinetics later (3). Biphasic killing kinetics is observed in a wild type population because while non-persisters were killed (green line), the persistent population was not (red line). Hip mutants produce a higher frequency of persisters, but was not considered here to illustrate the difference between tolerance and resistance. Adapted from Maisonneuve et al., 2014 (29).

Mechanisms behind the formation of persister cells

Mechanisms behind the formation of persister cells have remained elusive. Initial approaches included screening for knockout mutants in E. coli (30, 31). If a mutant culture shows a decrease in persister cells, it might be possible to identify the gene responsible for persisters formation. However, none of these screenings identified a particular mutant that completely lacked persisters (30, 31). They only identified some genes that could possibly be involved in the process and found out that these genes encode for global regulators. This
suggested a highly redundant mechanism for persister cell formation, which adds a major difficulty for identifying the particular mechanisms responsible for persister cell formation.

Later, researchers found connections between persistence and Toxin-Antitoxin (TA) systems. Moyed and colleagues discovered the first persister gene in *E. coli*, HipA (32). HipA was first discovered by consistently applying high doses of antibiotics to a bacterial population. HipA is a toxin in the HipBA TA module, and acts as a kinase that deactivates Glu-tRNA synthetase (24, 33). This synthetase attaches glutamic acid onto a tRNA, and inhibition of this enzyme leads to accumulation of uncharged Glu-tRNA. When uncharged tRNAs are loaded at the A site of the ribosomes, they activate RelA and increase the levels of (p)ppGpp (Fig. 2). (p)ppGpp is an alarmone that inhibits RNA synthesis, which leads to reduction in translation. Since (p)ppGpp is responsible for the stringent response (34), it was suggested that accumulation of (p)ppGpp promotes cells to enter a persistent state (33). These findings provided a connection between TA systems and the global regulator (p)ppGpp and their potential role in persistence.

![Amino acid starvation](image)

**Figure 2. Role of RelA in response to amino acid starvation.** An uncharged tRNA due to amino acid starvation binds to the ribosomal A site, and is later recognized by RelA, which binds to the 50S ribosomal subunit. RelA catalyzes the production of (p)ppGpp from ATP and GTP/GDP, and is later dissociated from the ribosome. The dissociated RelA will continue to catalyze the production of (p)ppGpp, increasing the level of this alarmone in the cytosol. Increased (p)ppGpp levels will then lead to regulation of certain cellular processes. Adapted from Hauryliuk et al, 2015 (93).
Further study confirmed the connection between TA systems, (p)ppGpp, and persistence. Korch and coworkers knocked out both relA and spoT genes in a hipA7 E. coli mutant (35). HipA7 is a gain-of-function allele that increases persisters levels by a factor of 1000. However, with the knockout of relA and spoT genes, they observed a decrease in the frequency of persister cells. Since relA and spoT regulate production of (p)ppGpp, it provided further support for the link between (p)ppGpp and persister cell formation.

**Toxin-antitoxin systems and their roles in persister cell formation**

Many findings support that TA systems play a significant role in the formation of persister cells. First, they are overexpressed in persister cells (24, 36); second, they are induced by (p)ppGpp (37); and third, the action of TA toxins is known to disrupt various cellular processes that may lead to dormancy and thus persistence (38). TA toxins respond to stresses by interrupting cellular processes, and their targets are very diverse. TA toxins can inhibit DNA gyrase that is essential during DNA replication (39); they can also cleave mRNAs, and thus are called mRNAs endonucleases or interferases (40). There are also other targets, such as proteins involved in cell wall synthesis (41), ribosomes (42), cytoskeletal proteins FtsZ and MreB in E. coli (43), and the bacterial inner membrane (44).

TA systems are comprised of a toxin, which corrupts cellular mechanisms, and an antitoxin that neutralizes the effect of the toxin (45). Toxins are always proteins, while antitoxins can be either protein or RNA (46). In general, TA systems are classified by the nature of the antitoxin and the mechanism by which it neutralizes the toxin (Fig. 3). For instance, in the Type I...
TA system, the antitoxin is an antisense mRNA that binds to the toxin’s mRNA and inhibits the translation of the toxin (47). Type II is when both toxin and antitoxin are produced as proteins, and they form a heterodimer polypeptide that inhibits the action of the toxin (48). Type III TA systems contain a RNA antitoxin that inhibits the action of the toxin protein through direct binding to the toxin (49). Meanwhile, Type IV is when the protein antitoxin indirectly inhibits the action of the toxin by binding to the toxin’s target (50). Lastly, Type V is when the antitoxin degrades the TA toxin mRNA directly (45).

Figure 3. Different types of TA systems based on the nature of the antitoxin and how it neutralizes the toxin. Toxins are shown in red, while antitoxins are shown in purple. TA systems are classified according to the nature of neutralizing the action of toxin by the antitoxin. In Type I TA system, the antitoxin is an antisense mRNA that binds to the toxin mRNA and inhibits the translation of toxin. Type II is when both toxin and antitoxin are produced as proteins, and they form a heterodimer polypeptide that inhibits the action of the toxin. Type III TA systems have a RNA antitoxin that inhibits the action of the toxin protein through direct binding. Meanwhile, Type IV is when the antitoxin indirectly inhibits the action of the toxin by binding to the toxin’s target. Lastly, Type V is when the antitoxin degrades the toxin mRNA directly. Adapted from Markovski & Wickner, 2013 (46).
Moreover, TA systems can be found in both plasmids and chromosomal DNA, and are ubiquitous in both Bacteria and Archaea (49). TA systems found in plasmids are essential for cell survival, since the daughter cells that do not inherit the parental plasmid with TA genes are killed by the stable toxin (52). In contrast, TA systems found in the chromosome have been hypothesized to be involved in a variety of cellular processes that respond to stressful conditions such as nutrient starvation, bacteriophage infections, oxidative stress, high temperature, and the presence of antibiotics (53, 54, 55).

The nature of toxins and antitoxins explains their role in inducing persistence. TA antitoxins are unstable (more easily degraded by cellular enzymes) and thus are short-lived; whereas, TA toxins are more stable and thus are long-lived (56). Since the antitoxin is constantly degraded, it must be continuously replenished by synthesis of the antitoxin in order to neutralize the effect of the toxin (49). If the production of antitoxin is interrupted by stress, the rate of antitoxin synthesis cannot keep up with the rate of its degradation. The free toxin will then proceed with its normal cellular inhibitory function, leading to growth arrest and cellular dormancy.

While many studies have been conducted on the role of TA systems in the formation of persister cells, it is important to keep in mind that TA systems vary from species to species. As mentioned previously, there are numerous TA systems within a single species and therefore it is hard to pinpoint the exact TA system that causes persistence. For instance, Dörr et al. in 2010 observed a redundancy in the TA systems in their studies with persister cell formation (57). A significant reduction in the frequency of persisters was only observed in E. coli when all 10 TA loci were deleted in E. coli (58). Lastly, there is evidence that Type II TA systems in E. coli seem to have a cumulative effect on persister cell formation.
**Mathematical model of persister cell formation**

Lou and colleagues proposed a mathematical model for *E. coli* persister cell formation based on the HipBA TA system (17). They proposed that a simple double negative feedback loop was not enough to cause formation of persister cells. The double negative feedback includes: first, the toxin HipA inhibits cell growth, and second, cell growth inhibits the accumulation of the toxin HipA. Hence, they developed a more sophisticated mathematical model that took into account several biochemical parameters, such as the dimeric forms of both HipA and HipB, and the regulatory actions of both HipA and HipB on their own transcription. Therefore, the outcomes of this model (Fig. 4) depend largely on the type of the TA system, but it is still useful for predicting persister cell formation of *E. coli* under any particular condition within the parameters of this model (17).

The graphical representation of the mathematical model resulted in three fixed points, two are stable and one is unstable (Fig. 4a). The phase space trajectories, which indicate all possible states of a system, also supported the existence of these states (Fig. 4b). This model describes how a bacterial culture growing at its maximum growth rate in the stable homogeneous log phase does not allow the formation of persister cells (Fig 4a, black circle at bottom). This is consistent with the concept of log phase, where by definition all cells are reproducing at a constant rate (59). The model also predicts a stable homogeneous phase of no-growth, which contains 100% persisters (Fig. 4a, black circle at top).
Figure 4. **Graphical results of the proposed mathematical model for the formation of persisters.** This bistable mathematical model was determined by taking into account the dimeric composition of the HipBA TA system in *E. coli*, the double negative feedback loop, and the maximum growth rate of *E. coli* (d=0.02 min⁻¹). Filled black circles represent the stable states, and open circle indicates an unstable transition state (A). Phase space trajectories, which indicate all possible states of the system, also suggested the existence of two stable states (B). Modified and adapted from Lou et al., 2008 (17)

According to their model, when a bacterial culture is growing at its maximum growth rate, the amount of antitoxin (HipB) is high, which neutralizes all HipA toxin, and thus prevents the formation of persisters (Fig 4a, black circle at bottom). In contrast, when growth rate ceases, such as in the LTS phase, antitoxin is not synthesized but rapidly degraded, which releases the stable free toxin and leads to the other stable state composed entirely of persisters (Fig. 4a, black circle at top). To conclude, this model clearly connects growth rate and TA systems in persister cell formation. It also provides a quantitative explanation to understand the heterogeneity in persister cell frequency as bacterial populations transition between the stable exponential and LTS phases.
The action of penicillin

Penicillin was discovered accidentally by Alexander Fleming in 1928, when he was studying *Staphylococcus* (60). Since then, many scientists have tried to discover the antimicrobial mechanism of action of penicillin. In 1965, Wise and Park formulated the hypothesis that the action of penicillin on Penicillin-binding proteins (PBP) will gradually weaken the cell wall leading to cell rupture (61).

Penicillin was chosen for the present study because it can distinguish between growing and dormant cells by killing only the former, and it does not interfere with translation. Penicillin is a beta lactam drug, which differs from other antibiotics, because it interferes with peptidoglycan synthesis (3). This is very important since drugs that completely inhibit protein synthesis could possibly kill persister cells, thus underestimating the true frequency of persister cells.

To better understand how penicillin distinguishes persister cells from non-persister cells, we must understand the role of penicillin and its target. Like all other Gram-positive bacteria, *L. monocytogenes* has a thick layer of peptidoglycan in the cell wall. Peptidoglycan is a macromolecule that contains glycan chains with alternating N-acetyl muramic acid (NAM) and N-acetyl glucosamine (NAG), cross-linked with peptide bridges (62). Synthesis of peptidoglycan involves the catalytic action of penicillin binding proteins (PBPs) in the final steps (63). In short, PBPs are responsible for cross-linking the peptidoglycan side chains, and were found to be the target of penicillin. They are involved in cell division, cell elongation, and cell shape (64). Moreover, the number and the function of these PBPs depend largely on species, some PBPs are not even involved in peptidoglycan synthesis (65).
It is believed that when penicillin binds to PBPs, it inhibits the peptidoglycan cross-linking, which later leads to a weak cell wall and possibly cell rupture (61). However, later experiments suggested that this hypothesis is not entirely true. Scientists have suspected that autolysins also play a role in penicillin-induced cell lysis (65). In 1970, an experiment with autolysin defective pneumococci demonstrated how a pneumococcal culture treated with penicillin showed minimal lytic effect (66). Autolysins are believed to be involved during normal cell wall biosynthesis through inducing “cuts” in the cell wall so that the bacterial cell can introduce the building blocks of the peptidoglycan. The main autolytic enzyme that was isolated from the pneumococcal culture was L-alanine-muramyl-amidase, a family of hydrolases that cleaves the bond between N-acetylmuramoyl residues and L-amino acid. According to Thomas et al., the sole action of penicillin on PBPs was not enough to cause bacterial lysis. Thus, cellular disintegration seems to be caused by the combined action of two processes: the normal function of hydrolases and the inhibitory action of penicillin on PBPs (67).

*L. monocytogenes* as a model organism for studying persister cell formation

*L. monocytogenes* is a Gram-positive, rod-shaped, motile, and facultative anaerobic bacterium that cycles between saprophytic and pathogenic life styles (68). It is responsible for listeriosis, a highly fatal foodborne disease that results in severe invasive systemic infections in immunocompromised people and the elderly (69). Invasive listeriosis can develop into miscarriage or stillbirth, septicemia, and meningitis (70).
As a facultative intracellular bacterium, *L. monocytogenes* is able to enter non-phagocytic host cells and reproduce in the cytoplasm. *L. monocytogenes* propels itself from one host cell to another by hijacking the actin filaments of the host, and also by the bacteria’s own protein, ActA (71). This feature, along with other virulence factors such as internalins and listeriolysin O, allows *L. monocytogenes* to invade host cells, causing severe systemic infections. In addition, *L. monocytogenes* can reproduce in many hostile environments, including but not limited to the human gastrointestinal tract, soil, and food products (72). The bacterium can grow at temperatures as low as -0.4°C (73), low pH (~ 4.3), and high NaCl concentration (>12%) (74). One study found that *L. monocytogenes* is able to survive in cheese for 259 days at 4°C with NaCl concentration up to 23.8% (75). This high salt, low acid, and low temperature tolerance makes *L. monocytogenes* a serious threat to food safety and public health.

So what makes this bacterium a good model for studying persisters? First, persister cell formation has been observed in this species (14), as well as the LTS phase (11); second, some of its TA systems are known (76); third, it has a highly repeatable life cycle in TSBYE (11); and lastly, it is a Gram-positive bacterium that can be targeted effectively by penicillin.

*L. monocytogenes* ATCC 19115 was shown to dramatically change its morphology as it transitions to the LTS phase (11). The cytoplasm is more condensed as time progresses, and the morphology changes from rod to cocci (Fig. 5). These coccoid cells in the LTS phase are dormant and non-dividing persister cells (M. Doan, E. Dudley, S. J. Knabel, presented at the International Association for Food Protection, Boston, MA, 2014). The electron microscopy images provided an excellent insight into how *L. monocytogenes* transitions from a normal rod-shaped cell to a round persister cell (Fig. 5). Shah and coworkers also found the same morphological change in *E. coli* after the cells exited log phase (5).
Figure 5. *L. monocytogenes* ATCC 19115 morphology transition from late exponential phase to LTS phase. *L. monocytogenes* ATCC 19115 transitioned from typical rod-shaped cells into cocci-shaped cells as the late exponential phase culture developed into LTS phase. Adapted from Wen et al., 2009 (11).

In addition to morphological changes, *L. monocytogenes* demonstrated a highly repeatable life cycle in TSBYE (11). Contrary to the current dogma of the four life-cycle phases of the bacterial growth curve, which include lag, exponential, stationary, and death phases; most free-living bacteria may actually undergo a more complex life cycle (11). When *L. monocytogenes* was grown in TSBYE at 35°C for 30 days, the bacterial culture exhibited eight highly repeatable life-cycle phases: lag, accelerating growth, log growth, decelerating growth, accelerating death, exponential death, decelerating death, and long-term-survival (11). When placed in fresh TSBYE, bacteria germinated from the LTS phase, and returned to the initial log phase, completing a life cycle (11). In other words, contrary to the common belief that bacteria
exhibit a final and independent death phase, formation of persister cells following a brief death phase allows bacteria to enter an LTS phase and survive for long periods, and then germinate back to the start of the life cycle and start over (Fig. 6).

Figure 6. Life cycle of *L. monocytogenes* ATCC 19115 in TSBYE at 35°C. *L. monocytogenes* exhibits a more complex life cycle than suggested by the current “growth curve” dogma. The phases of *L. monocytogenes* life cycle include lag, accelerating growth, log growth (A), decelerating growth (B), accelerating death (C), exponential death, decelerating death, long-term survival (D), and germination. Modified and adapted from Wen et al., 2009 (11).

*L. monocytogenes* was grown in Tryptic Soy Broth with 6% of Yeast Extract (TSBYE) throughout the investigation. The ingredients of TSBYE include pancreatic digest of casein (tryptone), papaic digest of soybean (soytone), sodium chloride, dextrose, dipotassium phosphate, and yeast extract (Table 1). Yeast extract is an auto-lysate of yeasts and it provides mainly vitamins to the bacterial culture (77). In addition, both soytone and tryptone provide nitrogen sources that are needed for bacterial growth (78). Lastly, carbon source is provided by
dextrose. Thus, TSBYE is a nutrient rich medium that can support and stimulate the rapid growth of *L. monocytogenes*.

**Table 1. Amount and main purpose of each TSBYE ingredient**

<table>
<thead>
<tr>
<th>TSBYE ingredients</th>
<th>Main purpose</th>
<th>Grams per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of casein</td>
<td>Source of amino acids</td>
<td>17.0 g</td>
</tr>
<tr>
<td>Papaic digest of soybean</td>
<td></td>
<td>3.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Osmotic regulator</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>C source for energy</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>Buffer</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>Source of vitamins</td>
<td>6 g</td>
</tr>
</tbody>
</table>

Lastly, not only the current dogma of bacterial life-cycle phases is questionable, but also the present beliefs concerning persister cells themselves need to be further examined. To date, the biological function of persisters is still a puzzle (2, 5). The current definition of persisters used by many authors may have confounded the true biological function of persister cells. In addition, the exact phase in which persister cells first appear remains unclear. Moreover, persisters have been divided into two categories, Type I and Type II (25). As mentioned previously, Type II persisters are defined as forming spontaneously in the exponential phase. However, if an exponential phase culture means that all cells are dividing at a steady exponential rate, then dormant, non-dividing Type II persisters would not be expected to form in this phase when nutrients are in excess.

Hence, the purpose of this investigation was to closely examine the changes in the frequency of persisters throughout the life cycle of *L. monocytogenes* F2365.
MATERIALS AND METHODS

Bacterial strains and growth medium. *L. monocytogenes* strain F2365 was used because previous studies on the LTS phase and persister cell formation performed in Dr. Knabel’s laboratory by his Ph.D. students, Jia Wen and Matthew Doan, used this strain. Bacterial stocks were kept at -80°C in TSBYE (BBL) and 50% glycerol. Working cultures were transferred every two weeks in TSAYE. All bacterial cultures were plated in TSAYE using the pour-plate method. TSAYE was prepared before each experiment and was maintained at 50°C in a water bath. All cultures and plates were incubated at 35°C, and CFUs were counted within a 24h-48h timeframe.

Persister cell assay. This assay was performed to determine the frequency of persister cells at a particular time. It is based on treating a bacterial population with a lethal dose of penicillin to kill the susceptible population, leaving only persister cells alive.

**Treatment:** 990 uL of bacterial culture was removed at a specific time depending on the experiment, and was mixed with 10 uL of 10,000 ug/mL penicillin, leading to a final bactericidal concentration of 100 ug/mL. This aliquot was then incubated statically (exposed to penicillin) for 12 hours and then centrifuged at 12,000 relative centrifugal force (rcf) for 2.5 minutes. To remove antibiotics, the supernatant was discarded, and the pellet was resuspended in PBS. After vortexing, 100 uL of this suspension was plated in TSAYE, unless otherwise indicated.

**Control:** when indicated, a control was performed by adding 10 uL of dH2O to 990 uL of the bacterial culture. This aliquot was then incubated statically for 12 hours and centrifuged at
12,000 rcf for 2.5 minutes. Cells were harvested and resuspended in PBS as described above. After vortexing, 100 uL of this suspension was plated in TSAYE, unless otherwise indicated.

**Sequential dilution and growth assay.** The logic behind this assay is that stationary phase cultures (in this case, the overnight culture) contain persisters, which would be carried over to fresh medium (treatment cultures). Therefore, if these cells are allowed to grow until exponential phase, persisters are germinated, thus reducing the initial concentration of persisters. Consequently, if these cultures are subjected to a series of growth and dilution, there would be a point at which all persisters are diluted out and/or germinated, and thus eliminated prior to inoculating the treated culture. Hence, a sequential dilution and growth treatment was performed when there was a need to create a homogeneous exponential phase culture with no persister cells present (Refer to Appendix A for more details). Keren et al. in 2003 (16) used such a strategy to eliminate persister cells of *E. coli* in the exponential phase. An overnight culture was inoculated 1:100 into fresh 10 mL of TSBYE medium. After the culture reached OD$_{600}$ ~0.02, cells were inoculated into another 10 mL of TSBYE to reach a final dilution of 1:100. This process was repeated twice, being last diluted 1:1000 into a 100 mL TSBYE “treatment” culture. After it reached exponential phase (before OD$_{600}$ ~0.02), cells were harvested for persister cells assay and plated into TSAYE for total viable population count.
**Determining when *L. monocytogenes* exits the exponential phase in TSBYE at 35°C.**

Determining exactly the end of exponential phase of *L. monocytogenes* F2365 in TSBYE was critical in this study. For instance, it aided in the sequential dilution and growth assay in setting the parameters for harvesting the cells after incubation (Appendix A). 100 uL of overnight culture of *L. monocytogenes* F2365 was diluted 100-fold into 10 ml of TSBYE, and 100 uL of this diluted culture was inoculated into 100 mL of TSBYE. This culture was then incubated statically for a total of 12 hours. During each hour, absorbance of OD$_{600}$ was measured and population counts were determined by plating 100 uL of the culture into TSAYE. The point when cells exited the exponential phase was determined as described by the method used in the study of Sezonov et al. (79). OD$_{600}$ was plotted against time, and the point at which cells deviated from exponential growth was determined from the graph. Exit from log phase was also further confirmed by a break point assay (79), which detected where the mass per cell dropped.

**Monitoring the frequency of persister cells over time.** In order to determine the frequency of persister cells throughout the life cycle of *L. monocytogenes*, persister cell assays were performed on a growing TSBYE culture, while the absorbance and the total viable population count were also measured. 100 uL of overnight culture was diluted 1000-fold into a 250 mL TSBYE and incubated statically for a total of 8 hours. Total viable population counts were measured each hour through plating and absorbance reading of OD$_{600}$. Frequency (%) of persister cells was calculated by dividing surviving CFU/ml by the initial viable CFU/ml, and then multiplying by 100.
Effect of life-cycle phase on cell death kinetics. Death kinetics of various L. monocytogenes cultures from exponential and LTS phases were compared:

Diluted-inoculum treatment cultures: An overnight culture was inoculated 1:100 into a fresh TSBYE medium. This culture was then subjected to sequential dilution and growth assay, and was grown until exponential phase in TSBYE (OD600 ~0.02). After this, a persister cell assay was performed, where a bactericidal dose of penicillin (final concentration = 100 ug/mL) was added to 12 aliquots. At each time point, an aliquot was centrifuged to remove penicillin, resuspended in PBS, plated in TSBYE, and then incubated as described above.

Non-diluted-inoculum treatment cultures: An overnight culture was inoculated 1:1000 into a fresh 100 mL TSBYE media and was grown until OD600~0.02. Then, the culture was subjected to persister cell assay (both treatment and control)

Treatment: Penicillin was added to 12 aliquots to achieve a final concentration of 100 ug/mL. These aliquots were incubated and one aliquot was removed at a particular time point to be centrifuged. The pellet was resuspended in PBS and the solution was plated in TSBYE as described above.

Control: sterile dH2O was added in place of penicillin to 12 aliquots and these aliquots were then incubated. One aliquot was removed at a particular time point to be centrifuged. The pellet was resuspended in PBS and the solution was plated in TSBYE.

Long-Term-Survival Phase: An overnight culture was inoculated 1:1000 into a fresh TSBYE medium and grown until early (1.5 weeks) and late (8 weeks) LTS phases. In both cases (early LTS and late LTS), 12 aliquots were removed from the culture and were subjected to the persister cell assay. Then, one aliquot was removed at a certain time point and centrifuged. The pellet was resuspended in PBS and the solution was plated in TSBYE as described above.
RESULTS

Determining when *L. monocytogenes* exits the exponential phase in TSBYE at 35°C. The exponential phase of *L. monocytogenes* F2365 in TSBYE at 35°C ended at approximately log 7.0 CFU/ml (OD~0.02) in both experiments (using different initial concentrations of inoculum). As shown in Fig. 7A and 7B, there are real breaks in the slope of the OD$_{600}$ curves when optical density was beyond ~OD$_{600}$ 0.02. These breaks were further confirmed by the plot of mass/cell vs OD$_{600}$ (Fig. 7C, 7D), where the amount of mass per cell was high initially and then dropped at OD$_{600}$ 0.02 as well.
Figure 7. Determining when *L. monocytogenes* F2365 exits the exponential phase in TSBYE at 35°C. An overnight culture of *L. monocytogenes* F2365 was diluted 10,000-fold (A and C) and 1,000-fold (B and D) in TSBYE. These cultures were incubated for 12 hours at 35°C. During each hour, OD$_{600}$ was measured and 100 μL of cultures were plated in TSA YE. For (A) and (B), OD$_{600}$ was plotted against time. For (C) and (D), the ratio of mass/cell was determined by OD$_{600}$/CFU per mL multiplied by $10^9$. Diamonds represent the optical density measurements, squares represent CFU/ml, and spheres represent the ratio of mass to cell. *L. monocytogenes* F2365 was found to exit exponential phase in TSBYE at approximately log 7.0 CFU/ml (OD~0.02).
 Monitoring the frequency of persister cells over time. Persister cell frequency varied over time. It seems that there was an initial drop of persister frequency at t = 1h. Later, it rose at around t = 5h when the optical density was OD$_{600}$ 0.028, with a total viable population of log 7.57 CFU/mL (Fig. 8).

**Figure 8.** Monitoring the frequency of persister cells over time. An overnight culture of *L. monocytogenes* F2365 was diluted 1000-fold into a 250 mL TSBYE and incubated for a total of 8 hours at 35°C. OD$_{600}$, CFU/ml, and persister cell frequency were measured each hour. The pink line indicates the growth of total bacterial population in log CFU/ml, while the bars represent the persister cell percentage calculated as follows: CFU/ml of persister cells ÷ CFU/ml of total cell population × 100.
Effect of life-cycle phase on cell death kinetics. When bacterial cells from the overnight culture were inoculated into fresh TSBYE and subjected to sequential dilution and growth until exponential phase, they exhibited uniform death kinetics during penicillin treatment until no persister cells were observed after 84 hours (Fig. 9, blue circles). Uniform death kinetics were also observed with both LTS cultures of 1.5 weeks and 8 weeks that were treated with penicillin (Fig. 9, yellow and green circles, respectively). However, when bacterial cells were inoculated from an overnight culture directly into fresh TSBYE and incubated with penicillin, these cultures displayed biphasic death kinetics (Fig. 9, orange circles). Most cells from the non-diluted-inoculum treatment cultures died logarithmically at the same rate as the diluted-inoculum treatment cultures; however, once the non-diluted-inoculum treatment cultures reached approximately log 2 CFU/ml, death ceased. The control, treated with water and originated from the same culture as the non-diluted-inoculum, showed an oscillating pattern (Fig. 9, orange circles with black border). Frequency of persister cells in the diluted-inoculum treatment culture reached to 0% after 84 hours, and reached to 100% in both LTS phases.
Figure 9. Cell death kinetics at various phases of the life cycle of L. monocytogenes F2365 in TSBYE at 35°C. A lethal dose of penicillin was added to cultures at different phases. Early exponential phase was divided into two cultures, one was subjected to sequential dilution and growth (blue circles), while the other was non-diluted (orange circles). Moreover, LTS phases of different ages were tested, one was 1.5 weeks old (green circles), the other was 8 weeks old (yellow circles). In addition, a non-penicillin-treated control (orange circles with black border) was added for comparison purposes, these were originated from the same culture as the non-diluted-inoculum cultures. Blue dash line indicates no CFUs detected (viable counts were below limit of detection).
DISCUSSION

Determining when *L. monocytogenes* exits the exponential phase in TSBYE at 35°C

Sezonov et al. described in 2007 how the exponential phase of *Escherichia coli* in Luria-Bertani broth ends earlier than previously suspected, at an OD$_{600}$ of around 0.3, log 7.7 CFU/ml (77). In theory, exponential phase is the period in which all cells are reproducing exponentially and all intrinsic parameters of the cells are constant, i.e., mean volume and density (80). Therefore, the number of cells, DNA, RNA, proteins per milliliter, and the optical density of the culture should increase exponentially with the same doubling time. Previously, it was believed that the OD range for the end of exponential phase of *Escherichia coli* was between 0.6 to 1.0. However, Sezonov and coworkers noticed that cells in this range were not consistently in the same physiological state, and that the exponential phase ends much earlier than previously thought.

In the present study, the exponential phase of *L. monocytogenes* F2365 in TSBYE at 35°C ended at around log 7.0 CFU/ml (OD$_{600}$~0.02), which is surprisingly early, however, cell density continued to increase by multiple folds beyond this point (Fig. 7). Similar to what Sezonov et al. found with *E. coli* in LB broth, *L. monocytogenes* in TSBYE also showed a real break in the slope of the OD$_{600}$ curve (Fig. 7A and 7B). The break was more visible when the mass per cell was plotted using OD$_{600}$. The mass per cell ratio was high initially, but when the culture was near OD$_{600}$ ~ 0.02, the mass per cell ratio dropped rapidly (Fig. 7C and 7D). This suggests the presence of waste products or depletion of certain nutrients in TSBYE, which may
have reduced the growth rate of *L. monocytogenes* F2365 at 35°C. The physiological state of *L. monocytogenes* likely slowed after the population exited the exponential phase at OD₆₀₀ of 0.02. Therefore, the cells were no longer reproducing at the same rate. Persisters may form when cells exit exponential phase, due to the presence or absence of any substance that could interfere with its maximum growth rate. Populations at log CFU/ml of around 7.0 (or OD₆₀₀~0.02) should be the limit used in sequential dilution and growth assays to ensure all cells are in the exponential phase.

When a population of bacterial cells exits the exponential phase due to nutrient limitations, the alarmone (p)ppGpp is produced (81). This results in both the release of free TA toxins and the synthesis of alternate sigma factors (82, 83). Alternate sigma factors can induce the synthesis many survival proteins (84). Together, TA toxins and alternate sigma factors may result in the formation of dormant, coccoid, and stress-tolerant persister cells, which enable long-term survival and thus enhanced fitness of bacteria in nature.

In order to test whether TSBYE was missing any essential nutrient for the growth of *L. monocytogenes*, a set of nutrient screenings was performed (Appendix C); however, none of the nutrients tested showed a reduction in persister cell frequency. Lastly, when conditions change, the point of exit from exponential phase likely changes as well. Thus, it is important to note that this early exit from exponential phase was seen in *L. monocytogenes* F2365 under the conditions tested.
Monitoring the frequency of persister cells over time

Persister cell frequency was not constant throughout the bacterial life cycle (Fig. 8). Instead, frequency of persister cells varied over time. Persister cells maintained approximately around the same frequency from t = 0h to t = 4h, with a possible initial drop at t = 1h. After OD\textsubscript{600} = 0.028, frequency of persister cells increased (Fig. 8, t = 5h). These results are consistent with what was found previously with the early exit of \textit{L. monocytogenes} F2365 in TSBYE, which happened to be at an OD\textsubscript{600} of 0.02. Moreover, data collected by Keren et al. in 2004 with \textit{E. coli} persisters showed a constant level initially until the population reached early to mid-exponential phase where persister frequency increased dramatically (24), which is also consistent with other authors (85, 86). I speculate that the possible initial drop of persister cell frequency was due to germination of persisters when these cells entered a nutrient rich TSBYE medium.

The consistency between the early exit of exponential phase cells of \textit{L. monocytogenes} in TSBYE (Fig. 7) and the increase in persisters at around that absorbance (Fig. 8) leads me to speculate that factors involved in exiting exponential phase may also induce the formation of persister cells. In contrast to the current definition of persister cells, these findings suggest that the true biological function of persister cells is to tolerate external stresses for the purpose of long-term survival in nature. It is not clear what these stresses are in TSBYE, since screening different nutrients did not yield any clear result (Appendix C) and pH maintained around the same level (pH~7, data not shown). However, that stress is likely responsible for the early exit of log phase and for the formation of persisters at such an early stage. The stress could be a key nutrient, such as vitamin or amino acid, that became limiting and thus triggered the formation of persister cells. Lastly, twelve hours of penicillin treatment time was performed according to the
protocol given. However, I suspected that this treatment time was not enough to completely kill the non-persisters population because the persister cell frequency was high. Thus, for the next experiment, the penicillin treatment time was extended to 252 hours in various life-cycle phases of *L. monocytogenes* and observed different death kinetics in different cultures.

**Effect of life-cycle phase on cell death kinetics**

There was a dramatic difference in death kinetics between cultures from diluted and non-diluted inoculum. A uniform death rate was observed throughout with the diluted-inoculum culture and LTS cultures of 1.5 weeks and of 8 weeks. Despite the significant difference in death slopes between exponential and LTS cultures, they all showed a steady rate of death, which suggests the presence of a homogeneous population within those cultures (Fig. 9, blue, yellow, and green circles). Meanwhile, the biphasic death kinetics observed in the non-diluted-inoculum treatment cultures suggests the presence of a heterogeneous population. The initial rapid decrease in viable cells indicates the death of a susceptible population within that heterogeneous population, while the tail reveals the presence of persister cells, which likely were carried over from the over-night culture (Fig. 9, orange circles).

Heterogeneity in bacterial populations is well-known (85). Moreover, biphasic death kinetics due to the presence of persisters is also commonly known (1, 29). What is interesting from the present study is the difference in death kinetics exhibited through different phases of *L. monocytogenes* F2365. There are some clear similarities and differences between each death rate. For instance, the slope of the diluted-inoculum treatment culture was the same as the slope of the
initial death rate of the non-diluted-inoculum treatment culture. Although one culture is homogeneous and the other is heterogeneous, they exhibited the same slope because they were harvested in the exponential phase, thus the susceptible population in both cultures exhibited the same death rate.

It is important to note that heterogeneity occurs when the population exits the log phase and stays heterogeneous until it reaches the LTS phase, where it again becomes homogeneous (17). The homogeneity observed in the exponential culture (Fig. 9, blue circles) suggests the presence of exponential cells that are homogeneous in terms of their metabolism and rate of replication. When these cells exit the log phase, they become heterogeneous due to the presence of both persisters and non-persisters. Lastly, when these cells enter the LTS phase, they again become homogeneous due to the presence of 100% persister cells in this phase. The non-diluted-inoculum that was harvested from the “exponential” phase showed heterogeneity because the inoculum from the over-night culture likely contained persister cells, which were carried-over into the culture broth and mistakenly made it look like persister cells formed spontaneously in the exponential phase (Fig. 9, orange circles).

Furthermore, another trend was noticeable: the older the culture, the lower the slope. As cells grow older, changes within the cells may make them less metabolically active and thus less affected by antibiotics like penicillin. This may explain the different slopes between early and late LTS cultures (Fig. 9). Referring back to the electron microscopy images of Wen et al. of L. monocytogenes ATCC 19115 in TSBYE (11), cells were smaller in a 214 h culture (~9 days), but were still rod-shaped. Whereas, when these cells were further incubated until 1 month, they transitioned to cocci and appeared more spore-like (Fig. 5). It is believed that, as cells grow
older; they are less metabolically active, and thus exhibit a slower death rate in the presence of antibiotics.

In order to determine whether the slow death kinetics of LTS cells was due to the natural death of cells in the absence of penicillin, a negative control was added. In the control batch, penicillin was replaced with dH2O. These control cells exhibited oscillating death and growth kinetics and showed little death compared to similar cells treated with penicillin. While control cells rose after a period of death, penicillin-treated cells kept dying. This suggests that control cells had active cell wall synthesis, but it is inconclusive with penicillin-treated cells because these cells were under antibiotic stress. However, since cell lysis occurs under the combined action of penicillin and autolysin; the phenomenon that LTS persister cells were still dying could possibly due to the action of autolysin in the presence of penicillin. There are many functions of autolysins; these include peptidoglycan maturation, cell separation, motility, cell expansion, and cell wall turnover (87).

The death kinetics exhibited in this study contradicts many previously incorrect dogmas concerning persister cells. First, Type II persister cells are thought to form spontaneously in the exponential phase (12, 14, 15, 88, 89). However, the present study supports the opposite view. For instance, persister cells were eliminated in the exponential phase through sequential dilution and growth of the inoculum (Fig. 9), which has also been observed by other authors (16, 23). If persisters can be eliminated through prior sequential dilution and growth, then it contradicts the belief that they form spontaneously in the exponential phase. The key to this non-persisters state is achieved through keeping all of the cells in the exponential growth. The study by Sufya and coworkers in 2003 observed no persister formation when \textit{E. coli} cells were grown at their maximum growth rate in a chemostat (90). Likewise, when Dawes and Mandelstam studied
Bacillus subtilis in a chemostat, they also observed no endospore formation when cells were grown at maximum growth rate in the exponential phase (91). However, both studies reported 100% persisters/endospores when growth rate was reduced to zero. These results are all consistent with the mathematical model proposed by Lou et al. in 2008, which predicts no persister formation in the exponential phase and 100% in LTS phase (17; Fig. 4a).

Pretreating a bacterial culture with rifampin or tetracycline can induce persister cell formation (3). Rifampin inhibits transcription and therefore mimics the effect of some TA toxins, while tetracycline inhibits translation and thus mimics other TA toxins (20). In short, both rifampin and tetracycline reduce protein synthesis, and there seems to be a correlation between reduced protein synthesis and persister cell formation (3). Also, in one study, persister cells were sorted from the rest of the population based on persisters’ low protein synthesis (5). In addition, high frequencies of persister cells can be induced by overexpression of toxins, such as mqsR, tisB, and relE. The above findings and the results of the current study refute the existence of Type II persister cells. Data from the current study, along with those found in others (36, 57, 58) indicate that persisters do not form spontaneously, but are only formed when cells are induced by stress.

Furthermore, many articles have described persisters as a small subpopulation of cells that survive antibiotic treatment (3, 14, 92). However, the biological function of persisters should not be thought of in terms of antibiotic tolerance, but rather tolerance to numerous stresses bacteria encounter in nature. For instance, persisters have been observed to tolerate UV light, dry heat, desiccation (A. Mendonca, personal communication), sanitizers (93), and wet heat and high hydrostatic pressure (11). The next problem with the definition of persisters concerns their maximum frequency. Do they constitute only a small subpopulation of cells? In the present
study, the frequency of persisters depended on the life-cycle phase of the culture, as indicated by Fig. 9. As mentioned, persister cell frequency was found to be 0% in the exponential phase, and it only increased after the culture exited the exponential phase to finally reach 100% in the LTS phase (Fig. 9). Therefore, the current definition of persister cells being a small subpopulation of cells that are tolerant to antibiotics is incorrect and very misleading.

So, what is the true biological function of persister cells? It is known that protein antitoxins are intrinsically disordered and thus rapidly degraded by cellular proteases. In order to have enough antitoxin to counteract the action of toxin, the rate of antitoxin synthesis needs to be greater than the rate of antitoxin degradation. When any stress leads to a halt in protein synthesis, the level of protein antitoxin decreases, which eventually leads to a high level of free toxins. These toxins can then perform their main role, which is to interrupt specific cellular processes.

Eventually, cells enter a persister state in which reproduction has ceased and the cells become dormant and stress tolerant. Such cells then germinate into vegetative cells when environmental conditions become favorable again. Therefore, the biological function of persister formation is not simply tolerance to antibiotics, but tolerance to numerous stresses in nature, which results in long-term survival and thus enhanced fitness.

Therefore, I propose here a new definition of persister cells: **Persisters are dormant cells that are tolerant to multiple stresses, which enable their long-term survival and enhanced fitness in nature.**

Lastly, I speculate that persister cells may be a new type of spore, based on their similarities with endospores (Table 2). Both endospores and persisters are dormant and coccoid-shaped. Both endospores and persister cell formation rely on (p)ppGpp and TA systems. Both are formed in response to nutrient limitation and stress and both are involved in the activation of
alternative sigma factors. Lastly, both are very tolerant to antibiotics and stresses (Table 2).

Thus, I speculate that not all spores are formed through engulfment. Instead, persister cells can form a new type of spore by shrinking into a small, coccoid-shaped, dormant and stress tolerant cell for the purpose of long-term survival in nature.

Table 2. Similarities and differences between persister cells and endospores

<table>
<thead>
<tr>
<th>Similarities</th>
<th>Differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both are dormant (94, 95)</td>
<td>Endospores form by the mother cell engulfing daughter cell (spore), while persister cells form by shrinkage of a mother cell (11, 106)</td>
</tr>
<tr>
<td>Both are coccoid-shaped (11, 96)</td>
<td>The alternate sigma factors are different between persister cells and endospores (99, 104)</td>
</tr>
<tr>
<td>Both are part of a complex life cycle (11, 96)</td>
<td></td>
</tr>
<tr>
<td>Both rely on (p)ppGpp and TA systems for formation (24, 36, 97, 98, 99, 100, 101)</td>
<td>Persisters uptake trehalose, while endospores uptake dipicolinic acid (107, 108)</td>
</tr>
<tr>
<td>Both form in response to nutrient limitation and stress (11, 53, 93, 102)</td>
<td></td>
</tr>
<tr>
<td>Both are involved in the activation of alternative sigma factors that redirect protein synthesis for survival (103, 104)</td>
<td></td>
</tr>
<tr>
<td>Both are tolerant to antibiotics and stresses (11, 105)</td>
<td></td>
</tr>
</tbody>
</table>
Appendix A

Sequential dilution and growth procedure

The original practice was to dilute cells 1:100 after 3 hours of incubation in 35°C. But at the end of nine hours (after sequential growth and dilution), the bacterial cultures were too diluted to be able to determine the amount of persisters present. Typically, the total population at nine hours would reach only ~log 1-3 (data not shown), and after the treatment of penicillin for 12 hours, sometimes the result would yield “no persisters”. But one should be careful here, because although reaching a state with no persisters was the first and critical goal, this did not truly reflect a non-persister state. First, the original culture was too dilute, and second, it was not reproducible because sometimes trials would yield ‘persisters’ and sometimes not. In order to correct this, the exponential phase of *L. monocytogenes* in TSBYE was determined. Once the parameters in which *L. monocytogenes* in TSBYE at 35°C exit log phase are determined, the cultures are diluted based on that parameter (Fig. 10).

**Figure 10. Sequential dilution and growth procedure.** An overnight culture is inoculated 1:100 into fresh 10 mL of TSBYE medium. After the culture reaches OD$_{600}$ ~0.02, cells were inoculated into another 10 mL of TSBYE to reach a final dilution of 1:100. This process was repeated twice, being last dilution 1:1000 into a 100 mL TSBYE. After it reaches exponential phase (before OD$_{600}$ ~0.02), cells were harvested for persister cells assay and were plated into TSAYE for total population count.
Appendix B

Persister cell formation between TSBYE and CAABYE media

Purpose

Knowing the type of media that could reduce persister cells would aid in eliminating persisters. Therefore, persister cell levels were compared between a TSBYE and a CAABYE media in order to find a medium between these two that would reduce persister cells level.

Method

100 uL of an overnight culture was inoculated into tubes containing 10 mL of TSBYE and 10 mL of Casein Amino Acids Broth with 6% Yeast Extract (CAABYE). After three hours of incubation, 100 uL of cultures from TSBYE and CAABYE were diluted 100-fold into another set of tubes containing 10 mL of TSBYE and CAABYE. These cultures were again incubated. This dilution and incubation step was performed twice until 9 hours elapsed. At the end of 9 hours, 100 uL of cultures from TSBYE and CAABYE were diluted 1000-fold into 100 mL of TSBYE and CAABYE, and were incubated. Once the culture reached exponential phase, persister cells assay was performed to both treatment and control, as mentioned above in the Materials and Methods section.
Table 3. Composition of amino acids in Casein Amino Acids with 6% of Yeast Extract (CAABYE)

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.961</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.420</td>
</tr>
<tr>
<td>Asparagine</td>
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<tr>
<td>Aspartic acid</td>
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<tr>
<td>Cysteine</td>
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<td>Glutamine</td>
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<tr>
<td>Glutamic Acid</td>
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</tr>
<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
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</tr>
<tr>
<td>Lysine</td>
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<tr>
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<tr>
<td>Phenylalanine</td>
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<tr>
<td>Proline</td>
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<td>Serine</td>
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<td>Threonine</td>
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</tr>
<tr>
<td>Tryptophan</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>0.700</td>
</tr>
<tr>
<td>Valine</td>
<td>1.241</td>
</tr>
</tbody>
</table>
**Results**

Figure 11. Comparing persister cells percentage between TSBYE and CAABYE. An overnight culture of *L. monocytogenes* F2365 was inoculated into two 10 mL tubes of TSBYE and CAABYE and grown for 3 hours. These cultures were subjected to two more 1:100 dilutions, and were incubated between each dilution for 3 hours. After nine hours, cells were treated with either distilled water (control) or penicillin (100 μg/ml) and were incubated for 12 hours. Cultures were centrifuged at 12000 rcf for 2:30 minutes at 20°C. Pellets were re-suspended with PBS, and plated in TSAYE. After 24 hours of incubation, CFU/ml was determined. In short, persister cells in TSBYE medium were below detection limit (<10 CFU/ml), whereas persister cells in CAABYE were 1000-fold more (log CFU/ml of 3.07). All cultures were incubated at 35°C. Dotted line represents detection limit.

Fig. 11 showed a significant decrease in persister cells after a series of dilution and incubation.

The CAABYE medium contained 1000 times more persister cells than the TSBYE medium. Consistent with this result, many experiments performed in CAABYE showed a lower growth rate of *L. monocytogenes* F2365 than in TSBYE. Therefore, TSBYE is used for the rest of the assays.
**Discussion**

Previous studies performed in Dr. Knabel’s laboratory on the absence of certain TSBYE components showed that *L. monocytogenes* F2365 cultures died completely in the absence of tryptone. Tryptone is the pancreatic digest of casein, which is a group of phosphoproteins found in milk. Tryptone is similar in composition to casamino acids. However, tryptone contains oligopeptides, but casamino acids contain free amino acids. Hence, preliminary experiments were performed to further investigate the effect of tryptone, as either free amino acids or peptides, in the formation of persister cells.

It is surprising how individual casein amino acids did not aid in lowering persister cells frequency, since the most straightforward assumption is that free amino acids can be used more easily by cells because there is not as much energy expense as compared with using peptides. Verheul et al. observed how *L. monocytogenes* Scott A possesses two peptide transport systems that required either proton motive force (PMF) or ATP (109). Upon internalization, these peptides are required to be hydrolyzed to amino acids that can be used for protein synthesis. Thus, there are additional steps in processing peptides compared to free amino acids, which could lead to higher energy costs. This phenomenon seems to deviate from the strive for fitness that is common in all organisms.

Interestingly, some studies dating back to the 1950s observed how peptide utilization by bacteria promoted more growth than free essential amino acids (110, 111). There are many causes that could result in this seemingly strange circumstance. For example, there could be some sort of antagonistic effect between amino acids, since all twenty amino acids were present in CAABYE culture. Also, transport systems of free amino acids could be ineffective in comparison with
peptide transport systems. Lastly, CAABYE medium may have a higher osmotic pressure that stressed the cells.

It is important to note that the control does not reflect the total population count. Both water-treated and penicillin-treated aliquots were plated after 12 hours of incubation, and bacteria were allowed to grow during the 12 hours of incubation, so that at the end of incubation, the counts did not reflect the actual population size. Thus, this type of control only revealed that penicillin had an effect on the treated samples but it did not show useful information such as the population size. Therefore, for the rest of the assays, the total population CFU/ml was measured through plating the culture right at the moment of the persister cells assay. The value obtained would then reflect the true value of the population at a particular phase, which would be helpful for determining the persister cells level in any culture. However, Fig. 11 is still reliable in terms of comparing the effects of different media in the formation of persister cells. However, persisters were not eliminated in any of those trials. Even though TSBYE medium in this experiment showed no persisters, but the detectable limit was 10 CFU/ml in this case, so anything below 10 CFU/ml remained unknown. Therefore, further examination and corrections in persister cells assay were performed.
Appendix C

Screens for limitations in amino acids, vitamins, and iron during early exponential phase in TSBYE using persister cells assay

Purpose

Since *L. monocytogenes* was found to exit log phase in a very early stage, a screen for nutrients is needed in order to know if there was anything lacking in the TSBYE that contributed to the early exit of log phase.

Method

Persister cell formation seems to occur when stress is present. In order to eliminate any stress originating from the medium due to lack of nutrients, several nutrients that were proven to stimulate growth in *L. monocytogenes* were screened. These include iron source from ferric citrate, vitamins such as riboflavin, lipoic acid, and thiamine. In addition, dextrose was also tested.

100 uL of an overnight culture was diluted 1000-fold into a control culture of 100 ml of TSBYE and into a treatment culture of 100 ml TSBYE that contained 8 mg/L of ferric citrate (1xIron), 0.1mg/mL of 20 amino acids (1xAA), 5 ug/ml of riboflavin, 0.5 ng/ml of lipoic acid and 1 ug/ml of thiamine hydrochloride. A second screen was conducted by testing the substances separately: TSBYE+0.1 mg/ml of 20 amino acids (1xAA), TSBYE+0.2 mg/ml of 20 amino acids (2xAA), TSBYE+2.5 g/L of dextrose, TSBYE+8 mg/L of ferric citrate (1xIron), and TSBYE+5ug/ml of riboflavin+0.5 ng/ml f lipoic acid + 1 ug/ml of thiamine hydrochloride. A third screen of amino acids was performed by testing different concentrations of the 20 amino acids in TSBYE: 0.1mg/mL (1xAA), 0.05 mg/mL (0.5xAA), 10 ug/mL (0.1xAA), and 5ug/mL (0.05xAA). Lastly, a screen for different ferric citrate concentrations was also performed in TSBYE: 4 mg/L (0.5xIron), 0.8 mg/L (0.1xIron), and 320 ug/L (0.4xIron). Cultures were incubated statically until
they reached OD~0.02, at which point they were subjected to the persister cells assay (treatment) described above and were also plated to determine the total population count.

Results

None of the nutrients tested showed a reduction of persister cells. Compared to control, they all showed a higher frequency than the controls (Fig. 12). When all nutrients except glucose were added together, the average increase in persister level was 18.4% (Fig. 12A). When nutrients were examined separately, the group of vitamins and iron induced 100% of persisters (Fig. 12B). Lastly, the supply of amino acids in different concentrations showed drastic increase in persisters, while the supply of iron in different concentrations showed a subtle increase in persisters (Fig. 12C and 12D).
Figure 12. The effects of different nutrients on persister formation of *L. monocytogenes* in TSBYE. Several nutrient screenings were performed as follows: 100 uL of an overnight culture was diluted 1000-fold and was inoculated into fresh media of 100 mL TSBYE, either as control or as treatment. First screening contained TSBYE with 8 mg/L of ferric citrate (1xIron), 0.1mg/mL of 20 amino acids (1xAA), 5 ug/ml of riboflavin, 0.5 ng/ml f lipoic acid and 1 ug/ml of thiamine hydrochloride (A). A second screen was done by testing the substances separately: TSBYE+0.1 mg/ml of 20 amino acids (1xAA), TSBYE+0.2 mg/ml of 20 amino acids (2xAA), TSBYE+2.5 g/L of dextrose, TSBYE+8 mg/L of ferric citrate (1xIron), and TSBYE+5ug/ml of riboflavin+0.5 ng/ml f lipoic acid + 1 ug/ml of thiamine hydrochloride (B). A third screen of amino acids was performed by testing different concentrations of the 20 amino acids in TSBYE: 0.1mg/mL (1xAA), 0.05 mg/mL (0.5xAA), 10 ug/mL (0.1xAA), and 5ug/mL (0.05xAA) (C). Lastly, a screen for different ferric citrate concentrations was also done in TSBYE: 4 mg/L (0.5xIron), 0.8 mg/L (0.1xIron), and 320 ug/L (0.4xIron) (D). Cultures were incubated until they reached OD~0.02, at which point they were subjected to the persister cells assay of 12 hours penicillin treatment time and were also plated to determine the total population count. Persister cell percentage was calculated by dividing persister cells over total population CFU/ml, and then multiplied by 100. All screenings resulted in an increase of persister cells.

**Discussion**

The increase of persister cells after adding nutrients could be either first, the nutrients tested were not depleted in TSBYE medium, or second, the concentrations of these nutrients were lower/higher than required. Thus, these screenings were inconclusive and further screenings are needed.
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SKILLS AND ABILITIES

Laboratory
- Conducted independent research in a Foodborne Pathogen Laboratory with L. monocytogenes F2365 to examine the effects of media and phase in persister cell formation, 2015-2017
- Received training in parasites and mammalian cells for growth, maintenance, and research in a summer internship at INDICASAT-AIP, 2016
- Performed standardizations, antibiotic treatments, bioactive products extractions and isolations, chemical compounds analysis with H-NMR, GC, IR and gas chromatography
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- Contributor of the Code of Conduct for a school supplies company
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- Teacher of Chinese as a Secondary Language to elementary school students for a year. Planned lectures and assignments.
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Management
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- Medical tours at La Salle 2009-2013
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