Impact of Probiotics on Black Soldier Fly Larvae Transmission of Stenotrophomonas Maltophilia

Emily Marie McLaughlin

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IMPACT OF PROBIOTICS ON BLACK SOLDIER FLY LARVAE TRANSMISSION
OF *STENOTROPHOMONAS MALTOPHILIA*

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Microbiology)

The Honors College
University of Maine
December 2021

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ABSTRACT

*Stenotrophomonas maltophilia* is an opportunistic pathogen which can cause a variety of infections, especially in immunocompromised individuals. It has recently been found that black soldier flies can act as carriers for *S. maltophilia* (Callegari et al., 2020). I have investigated how probiotics impact their vector capabilities. Black soldier fly larvae have been fed substrate spiked with *S. maltophilia*. After several days of feeding, the larvae were investigated by PCR and plating (Sveensson-Stadler et al., 2011). Two groups of larvae were fed with potatoes infected with *S. maltophilia*. Persistence of *S. maltophilia* was determined using PCR by taking samples from days 0, 3 and 6. This research could clarify the role that black soldier flies play in potentially reducing this public health threat.
ACKNOWLEDGMENTS

I would like to thank Dr. Edward Bernard for his support and insight into my thesis, undergraduate education, and my future throughout my thesis process and before. Thank you for teaching me that personality and humor not only has a place within science, but in fact, makes science better. I would also like to thank Dr. Andrei Alyokin and Matt Moyet for their support throughout my thesis and their initiative to assist with me completing my thesis. I would like to thank my advisory committee: Dr. Sally Molloy, Ms. Nilda Cravens MSN RN, and Mr. Chris Mares, who also assisted me through this process.

This thesis project would not be possible without the generous contribution from a Radke and CUGR fellowship. The former and current undergraduate members of the BSFL laboratory were essential in this project as well, Haley Morrill, Marissa Kinney, Grace Harmen and Audrie French. Thank you to Audrie French for teaching me the hard work and poise necessary to be a scientist.

Lastly, I would like to thank my parents, Roberta and Robert McLaughlin for supporting me through my undergraduate journey. Stephanie Nichols, who once thought the longest journey was through Oxford and Somerset Hall, but we will now continue to make the journey through Boston and Portland, you make me who I am. The most wonderful friends, Alec Barranco, Izzy Topper, Annie Stevens, and Sam Nichols who made my days so much brighter. The Moulton family: for their kindness and generosity and for allowing me to be in the life of their amazing daughter Josie, who has changed
my life for the better. Theodore the cat, who kept me company surveying the birds
outside the window during my many hours of thesis work.

Finally, I would like to dedicate this thesis to the Emily McLaughlin who cried in
AP Chemistry every day her junior year and was told that maybe she just wasn’t cut out
for science. She was then and she is now. This thesis demonstrates that humor and
kindness belong in the lab as much as lab goggles and gloves do.
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**INTRODUCTION**

*Stenotrophomonas maltophilia* is an emerging opportunistic pathogen. Although it is not highly virulent, it has emerged as a nosocomial pathogen. *S. maltophilia* is a significant pathogen in immunocompromised patients, with a considerable mortality rate of up to 35.7% attributed to *S. maltophilia* infections (Falagas, et al., 2009). *S. maltophilia* can cause a variety of conditions from pneumonia to meningitis to cellulitis and has been found to be resistant to many antibiotics. Furthermore, it is abundant in many environments. It has been recovered from natural sources such as soil, roots, water, and animals. It has also been recovered from households in sources such as ice machines, contact lens solution, washed salads, faucets and tap water. Part of *S. maltophilia*’s tendency to colonize in a myriad of places is its ability to form biofilms. This is especially important for their ability to infect hospital patients, as biofilm formation has also been associated with 65% of hospital acquired infections (Brooke, 2012). This creates a strong colony of bacteria, which is difficult to treat with antibiotics and is readily able to grow on various surfaces, including human tissues. Recent research has demonstrated that *Hermetia illucens* (black soldier flies) can become colonized with *S. maltophilia* (Callegari et al., 2020). This may allow black soldier flies to act as a vector for transmission of *S. maltophilia* to humans.

This transmission could come in several forms. Larvae containing *S. maltophilia* may be ingested by humans either accidentally or intentionally. It may also be passed through contaminated animal feed made from black soldier fly larvae. Black soldier fly larvae are 42% crude protein and could potentially be used as a dietary source of protein for humans (Wang, et al., 2017). Black soldier flies living in close proximity with
humans could produce feces which may accidentally be ingested by humans. For example, flies may produce waste on food or humans may contact black soldier fly waste accidentally. *S. maltophilia* also readily grows on medical equipment, and poor hygiene from medical staff after touching black soldier flies or their larvae could promote bacterial growth on medical equipment (Brooke, 2012). In addition, black soldier flies and larvae are found widely around the world. Hospitals in developing countries have a larger chance of an accidental interaction of black soldier flies, their larvae and medical equipment or the hands of medical personnel.

Probiotics have become a major area of interest for microbiologists in recent years because they are able to mitigate some types of infections. Lactobacillili have been widely studied for their probiotic abilities. In general, their probiotic potential can be attributed to four main properties: their ability to adhere to cells reducing pathogen adherence, their ability to persist and multiply within the human body, their ability to produce byproducts which can inhibit pathogen growth, and their ability to resist microbicides (Reid, 1999). *Lactobacilli* spp. have already been found to be abundant within the gut of black soldier flies, signifying that the gut has the necessary components to support their growth and suggesting that larvae may also be able to harbor these bacteria as well (Klammsteiner et al., 2020). *Lactobacilli* are resilient bacteria, and their resilience can induce competition which may prevent the growth of *S. maltophilia*. Furthermore, *Lactobacilli* species, including *Lactobacillus acidophilus*, produce bacteriocins which have been found to produce a strong inhibitory effect on *S. maltophilia* (Sahran, et al., 2018). Their ability to colonize black soldier fly larvae and combined probiotic potential indicates that Lactobacilli may be able to outcompete *S. maltophilia*. 
My objective was to answer two questions. Does feeding black soldier fly larvae substrate spiked with probiotics impede the ability of *S. maltophilia* to colonize? Second, how does this impact their capabilities as a vector? My hypothesis was that larvae fed on probiotics will harbor less *S. maltophilia* because the lactobacilli will induce suppression.
LITERATURE REVIEW

Bacteriology of Stenotrophomonas maltophilia

*Stenotrophomonas maltophilia* is a gram-negative bacteria that has become an emerging focus of research for microbiologists and medical professionals alike due to its continuing persistence within clinical environments and potential risk of infection (Sarhan & Ibrahim, 2018). It is a rod-shaped obligate aerobe, which has motility due to polar flagellum. It has been found to be oxidase negative and can survive well in aqueous environments (Brooke, 2012). This bacterium was originally classified as a member of the *Pseudomaonas* genus in 1961, then more research prompted it to be moved to the *Xanthomonas* genus in 1963 until it was finally placed within *Stenotrophomonas* in 1993. Among the ten species that comprise the *Stentrophomonas* genus, *S. maltophilia* stands out because of its ability to cause infection in humans who are immune compromised, specifically those with cystic fibrosis or cancer (Pinot et al., 2011).

*Stenotrophomonas maltophilia* was originally recognized for its occurrences in plants, including a diverse array of crops such as oilseed rape, corn, potatoes, cabbage, mustard, and beets. Some isolates were even found on plants growing in extreme environments, including sand dunes. Many isolates demonstrated an endophytic life style due to their intimate interactions with their hosts (Berg & Martinez, 2015).

*Stenotrophomonas maltophilia* also has an ability to form biofilms which allows it to be highly resilient within a host. Furthermore, *Stenotrophamons maltophilia* can coexist with other bacteria, allowing it to tolerate the immense variety of bacteria which could be living within a host (Brooke, 2012).
The lipopolysaccharide layer (LPS) of the cell wall of *Stenotrophomonas maltophilia* has important clinical and microbiological significance. The LPS layer contains lipid, A, a core oligosaccharide, and the O-antigen. Important to note is that this lipopolysaccharide is charged, which can influence bacterial cell adhesion to surfaces as the positively charged cell wall covers negative charges which are present in the lipopolysaccharide layer. This has clinical significance, as the positively charged cell surface of *Stenotrophomonas maltophilia* strain 70401 was imperative in its ability to adhere to glass and Teflon, allowing it to persist in a variety of household and clinical environment and increase its ability to be transmitted to humans. Temperature is an important factor that can alter the chemical composition of LPS, which could increase *S. maltophilia*’s susceptibility to aminoglycosides, which its resistance to this class of antibiotic. It has been demonstrated that strains grown at 30°C had a >4-fold difference in MICs of aminoglycosides, including gentamicin, than strains grown at 37°C. Analysis of the LPS chemical composition showed that there was a significant increase in the phosphate content at 37°C (Brooke, 2012). Therefore, it seems that the LPS layer is most primed to assist in infection at 37°C- body temperature, potentially promoting *S. maltophilia* infections in a human host.

Much of the pathogenesis of *S. maltophilia* can be attributed to its ability to form biofilms. *Stenotrophomonas maltophilia* has been found to form biofilms on Teflon, glass, plastics, and host tissues. Biofilms have also been estimated to be associated with 65% of hospital acquired infections of *S. maltophilia* (Brooke, 2012). *Stenotrophomonas maltophilia* fimbriae 1 protein (SMF-1) has been found to be essential in biofilm formation *in vitro* tissue culture assays because this bacterium was unable to adhere to
cultured Hep-2 mono layers with anti-SMF-1-antibodies, and this inhibition was most successful in the early stages of infection- within the first half hour (Brooke, 2012).

*Stenotrophomonas maltophilia* has been found to form biofilms on moist surfaces, which may make direct or indirect contact with patients. These surfaces range from hospital plumbing systems to catheters or even household faucets. *Stenotrophomonas maltophilia* is particularly good at forming biofilms on lung cells, which is an important issue for cystic fibrosis patients. In fact, confocal microscopy of biofilms from *S. maltophilia* infected cystic fibrosis patient sputum found that *S. maltophilia* formed microcolonies embedded within the matrix.

There are several environmental factors influencing *S. maltophilia* biofilm formation that are of significant research interest. These factors include phosphate and chloride concentrations, pH, temperature, oxygen supply, and presence of silver or copper ions. Furthermore, clinical isolates of *S. maltophilia* have been found to produce more biofilms at 32°C than at 37°C and 18°C. Also, *S. maltophilia* were better equipped to form biofilms under aerobic conditions than anaerobic conditions; the optimum was observed in a 6% CO₂ atmosphere (Brooke, 2012).

*Stenotrophomonas maltophilia*’s ability to adhere and invade host cells is one of its most important attributes relatively to induction of infection within a host. *Stenotrophomonas maltophilia* has been found to adhere to human bronchial epithelial cells and then invade them. Flagella are important in this process, as they have been found to mediate the adherence of *S. maltophilia* isolates to tracheal mucus in mouse studies. Additionally, it’s important to note that flagella are highly immunogenic structures which are conserved among *S. maltophilia* clinical isolates (Brooke, 2012).
Stenotrophomonas maltophilia has been isolated from a variety of sources, including natural sources like soil, household sources like tap water, clinical surfaces such as in hemodialysis water, and even has been found to be able to grow within amoebae (Denet et al., 2018). Stenotrophomonas maltophilia’s ability to survive well on plants can be a particular issue, resulting in S. maltophilia occasionally being isolated from bagged lettuce. Frighteningly, there is research to prove that S. maltophilia taken from clinical sources and from environmental sources have virulence genes, suggesting that it could have some intrinsic virulence properties which can impact both those who are immune compromised, as well as otherwise healthy individuals (Hall et al., 2020).

Stenotrophomonas maltophilia prevalence in clinical sources is of particular interest as it continues to become an opportunistic pathogen able to cause dangerous infections in immune compromised populations (Table 1). The alarming data showing the prevalence of S. maltophilia infections in cystic fibrosis and cancer patients can be attributed not only to its virulence factors, but also to the sheer abundance of these bacteria in clinical sources, considering cystic fibrosis and cancer patients may spend extended periods of time in a hospital.
Table 1. Clinical Sources of *Stenotrophomonas maltophilia* (Brooke, 2012)

<table>
<thead>
<tr>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital suction tubing</td>
</tr>
<tr>
<td>Electronic ventilator temp sensors, ventilator inspiratory/expiration</td>
</tr>
<tr>
<td>Central venous catheter</td>
</tr>
<tr>
<td>Nebulizers</td>
</tr>
<tr>
<td>Endoscopes</td>
</tr>
<tr>
<td>Dental suction system hoses</td>
</tr>
<tr>
<td>Dental solid waste</td>
</tr>
<tr>
<td>Hemodialysis water and dialysate of renal units</td>
</tr>
<tr>
<td>Contaminated chlorhexidine-cetrimide disinfectant</td>
</tr>
<tr>
<td>Hand washing soap</td>
</tr>
<tr>
<td>Irrigating solutions</td>
</tr>
<tr>
<td>Sink drains</td>
</tr>
<tr>
<td>Faucets/faucet aerators, showerheads</td>
</tr>
<tr>
<td>Water fountain drains</td>
</tr>
<tr>
<td>Patient’s medical charts</td>
</tr>
<tr>
<td>Cystic fibrosis patient cough-generated arenols</td>
</tr>
<tr>
<td>Ice machines</td>
</tr>
<tr>
<td>Tap water</td>
</tr>
<tr>
<td>Water treated by filtration, reverse osmosis, UV exposure or deionization</td>
</tr>
<tr>
<td>Tap water</td>
</tr>
</tbody>
</table>
Clinical Significance of Stenotrophomonas maltophilia

*Stenotrophomonas maltophilia* is a potentially deadly pathogen for those who are immune compromised, mainly cancer patients and cystic fibrosis patients. A U.S multiple hospital study of ICU patients from 1993 to 2004 found that *S. maltophilia* was one of the 11 most frequently recovered organisms, as it made up 4.3% of all gram negative *Bacillus* isolates (Chang et al., 2015). Those who are immune compromised are most likely to suffer from a *S. maltophilia* infection. *Stenotrophomonas maltophilia* can cause a variety of conditions. The most common infections associated with *S. maltophilia* are pneumonia, bloodstream infection, urinary tract infections or infected wounds. Eye, gastrointestinal and neural infections have also been noted. The condition of the patient is the determining factor in their ability to recover from Stenotrophomonas maltophilia (Hall et al., 2020). In addition, hospital acquired bacteremia of this species is often associated with high mortality rates, ranging from 14 to 69% (Brooke, 2012).

The rate of *S. maltophilia* infections in hospitals has been increasing, and so far, the data demonstrate that it can infect patients of all ages. There are a variety of pathways *S. maltophilia* could take to infect a host in a hospital setting. One source of transmission is through direct contact with the source. Another source is transmission through the hands of healthcare workers. Additionally, because cystic fibrosis patients are so susceptible to harboring a *S. maltophilia* infection, the aerosol from their coughs can transmit this bacterium. Furthermore, *S. maltophilia* has evolved some helpful tools to persist within cystic fibrosis patients. Firstly, it can coexist with other bacteria, mainly, it has been found to contently coexist with *Pseudomonas aeruginosa*, a common pathogen for cystic fibrosis patients. Secondly, clinical isolates have been found to have a higher
mutation rate than environmental isolates (Brooke, 2012). So, it can easily adapt to different environments, whether that be an outdoor freshwater source or the surface of lungs in a cystic fibrosis patient.

The current preferred treatment for *S. maltophilia* infection is the use of TMP-SMX, a bacteriostatic compound. However, this is likely not a permanent solution as a 2004 Antimicrobial Surveillance Program found a 3.8% resistance of TMP-SMX for *S. maltophilia* (Chang et al., 2015). Although *S. maltophilia*’s susceptibility to TMP-SMX is still quite good, those with cancer and cystic fibrosis experience a less effective benefit from TMP-SMX than other patients due to the increased severity of infection that *S. maltophilia* causes in this type of patients. This poses a major threat as those are two groups of individuals who overwhelmingly make up most people with risk of *S. maltophilia* infection.

**Virulence of Stenotrophomonas maltophilia**

*Stenotrophomonas maltophilia* has a variety of virulence factors it implores to persist within a host. An increasingly alarming area of interest is *S. maltophilia*’s ability to resist many antibiotics. One virulence tool which *S. maltophilia* implores are encoded β-lactamases (L1 and L2), with both genes found on 200-kb plasmids. These lactamases are quite effective, as they demonstrated heterogeneity for β-lactamase induction when exposed to three antibiotics- imipenem, cefoxitin, and ampicillin. This observation is important as it suggests that β-lactamase activity does not just result from the gene being present in the isolate. It also indicates that β-lactamases may not be the sole reason for increased antibiotic resistance, as they only provide to resistance to three antibiotics and not the wide variety of antibiotics. This idea was further tested when DNA analysis of *S.*
*malophilia* mutants with L1 and L2 knockout genes showed that β-lactamases L1 and L2 expressions are regulated differently. Researchers found that AmpR is needed for basal-level expression of L1 but not for L2. However, AmpR was found to be necessary for induction of L1 and L2. It was demonstrated that AmpR binding to the intergenic sequence between *ampR* and the L2 gene induced the expression of lactamase. More research must be done to fully understand the effects of lactamases in *S. malophilia*, as in addition to *ampR* genes, there are also *ampC, ampN, ampD* and an *ampN-ampG* operon which is required for the L1 and L2 expression (Brooke, 2012).

Lactamases, however, are just one of many virulence factors implored by *S. malophilia* to create harmful infections within a host. *Stenotrophomonas malophilia* has also demonstrated multidrug efflux pumps. These pumps are made up of a membrane fusion protein, a transporter, and an outer membrane protein. The *smeDEF* operon is responsible for the cloning and sequencing of this efflux pump. This efflux pump contributes to *S. malophilia*’s resistance to β-lactams, tetracyclines, erythromycin, quinolones, aminoglycosides, and chloramphenicol. This efflux pump is of particular importance, because when SmeC of *S. malophilia* was introduced into *P. aeruginosa*, it gave *P. aeruginosa* antibiotic resistance demonstrating that *P. aeruginosa* was able to utilize a MexAB-SmeC multidrug efflux pump. This demonstrates an important risk, particularly in the instance of horizontal gene transfer and because *P. aeruginosa* and *S. malophilia* have been known to often cohabitate together (Brooke, 2012).

Integrons are another important virulence factor within *S. malophilia*. Integron-like elements have been found in *S. malophilia* isolated found from a variety of sources worldwide. Integrons contain an integrase-encoding gene which allows antibiotic
resistance gene cassettes to be inserted in between highly conserved nucleotide sequences. Class 1 integrons have been found in transposons in *S. maltophilia*, which can be transferred to plasmids or chromosomal DNA with transposition events. This is important, as it allows *S. maltophilia* a method to disseminate drug resistance throughout a group of bacteria. Class 1 and 2 integrons have been associated with the presence of SmeABC and SmeDEF pumps in 93 *S. maltophilia* clinical isolates from a hospital in Taiwan in 2002. This is of clinical significance because Class 1 integrons have been hypothesized to contribute to increased TMP-SMX minimum inhibitory concentration values for *S. maltophilia* isolates. Namely, *sul* genes have been reported to be the insertion element common region in class 1 integrons. The *sul1* gene has been found in the class 1 integron gene in TMP-SMX resistant *S. maltophilia* isolates, while the *sul2* gene has been found on plasmid DNA in other TMP-SMX resistant *S. maltophilia* isolates (Brooke, 2012).

*Stenotrophomonas maltophilia* has also demonstrated intrinsic resistance to quinolones due to the *qnr* gene. This is due to the induced mutations of topoisomerases and gyrase genes, it has also been hypothesized to arise due to overexpression of the SmeDEF efflux pump (Brooke, 2012).

*Stenotrophomonas maltophilia* implores hydrolytic enzymes which have been reported to demonstrate cytotoxic activity. The *S. maltophilia* K279a genome encodes for extracellular enzymes: proteases, lipases, esterases, DNase, RNase and fibrolysin. Supernatants of *S. maltophilia* clinical isolates which were recovered from liver and trachea demonstrated cytotoxic activity when exposed to African green monkey and human cervical cells. The cytotoxic effects including rounding, membrane blebbing, and
a loss of intercellular junctions, ultimately resulting in eukaryotic cell death after 24 hours (Brooke, 2012).

Finally, proteases are of particular importance because they have been found to exacerbate influenza. Of 14 samples confirmed to contain influenza A virus from animals (pigs, horses and humans who worked closely with animals) 21.11% of them were coinfectected with *S. maltophilia*. It was hypothesized that *S. maltophilia* produced elastase which could cleave and activate the hemagglutinin glycoprotein spike of influenza A which allowed the virus to enter host cells (Brooke, 2012).

**Black Solider Fly Larvae**

The black solider fly, *Hermetia illucens* is of the family Stratiomyidae. This fly was originally thought to be native to the Americas; however, it has now been found worldwide in both tropical and temperate regions. It is not found in colder regions, such as Northern Europe, due to its limited ability to withstand cold temperatures (Moretta et al., 2020). Black solider fly adults can live around 47 to 73 days when provided with enough water, but they also benefit from feeding on carbohydrate solutions. Adult flies live in relative harmony with humans as they generally do not approach humans, nor do they bite or sting. Adult flies, so far, have not been known to disseminate any disease. Black solider fly larvae (BSFL) feed on a variety of organic materials. These substrate sources include but are not limited to, manure, food waster, distiller’s grains, kitchen waste and fecal sludge. Some entomologists propose that the variety of substrate BSFL can feed on and the efficiency in which they feed on this substrate might be the highest among all fly species (Y.-S. Wang & Shelomi, 2017). Larvae can reach about 27 mm in
length and 5 mm in width and require about 3 weeks to complete larval development (Newton et al., 2005).

**Practical Uses of Black Soldier Fly Larvae**

BSFL have been explored for a variety of uses. BSFL are edible and can be used as animal feed. Their feed conversion ratios are superior to crickets and mealworms. Furthermore, they are not thought to be commonly become toxic, but it should be noted that when left to fed on contaminated substrate for too long, BSFL can become contaminated as well. Their long larval development period of 3 weeks is more than that of other flies, such as house flies and carrion flies. This means that larvae consume a larger amount of substrate and attain larger sizes (Y.-S. Wang & Shelomi, 2017). In fact, BSFL have even been explored as a food source fit for human consumption. Interestingly, BSFL can be used as biodiesel as well because they accumulate lipids from their diet to use as an energy source, but those lipids can also be extracted and processed into fuel. BSFL produce great fertilizers as well, as the waste they do not consume combined with their frass is an excellent fertilizer (Y.-S. Wang & Shelomi, 2017).

An important practical application of BSFL is their potential as a waste management agent, particularly in low and middle-income countries where appropriate waste management systems may be unsafe or lacking. Management of municipal solid waste in these countries is oftentimes neglected, which is a key public health issue. As a result, household waste will often remain uncollected in streets and drains, which attracts disease vectors and causes water blockages. In fact, this is such a major issue that around 6.8% of Africa’s greenhouse gas emissions are generated from municipal waste, primarily methane which is released from open dumps (Diener et al., 2011). A 2011
study explored this idea by using BSFL in a medium-scale field experiment in Costa Rica to degrade mixed municipal organic waste. The researchers found that waste reduction ranged from 65.5 to 78.9% depending on the waste added and the conditions. Overall, that study showed that BSFL can be incorporated into an excellent waste management system, particularly in countries which may be lacking resources to create an appropriate waste management system (Diener et al., 2011).

This is one of several studies which explores BSFL’s potential as a waste management system. However, it is known that BSFL can become contaminated if left to feed on contaminated substrate. Before we entertain the idea of welcoming BSFL by the millions into communities, who may already be facing limited medical capabilities, it is important to consider the risks associated with BSFL and their ability to spread pathogens, namely, *S. maltophilia*.

**Microbiome of Black Soldier Fly Larvae**

Like all creatures, BSFL is known is have a robust microbiome. A 2020 study sought to analyze the microbiome of BSFL fed on a variety of low burden diets: chickenfeed, fruits and vegetables and grass cuttings. They found that *Actinomyces* spp., *Dysgonomonas* spp., and *Enterococcus* spp. were the main members of that microbial community (Klammsteiner et al., 2020). Furthermore, those species provided functional and metabolic services which allow BSFL to survive in a variety of environments. The researchers were able to isolate *S. maltophilia* from BSFL gut after several days of feeding (Klammsteiner et al., 2020). An additional study from 2021 had a similar goal but fed BSFL on chicken feed or fiber-rich substrate; they found *S. maltophilia* in 0.6% of samples (Gorrens et al., 2021). These studies were performed using controlled
substrates. However, if BSFL was able to feed on substrate which might already be contaminated with *S. maltophilia*, this would increase the levels of this bacterium already present inside the BSFL.

BSFL are also remarkable for their ability to produce antibiotic chemicals which modify the bacterial communities in the substrate they inhabit. These come in the form of antimicrobial peptides, they act as the first line of immune defense inside BSFL; however they can also go into the substrate as well (Moretta et al., 2020). Morietta et al. (2020) have identified 57 putatively active peptides within BSFL along, many of which will undergo further examination in the coming years to investigate their potential impact on BSFL and humans.

The intersection of BSFL’s increasing utility for humans, their ability to harbor *S. maltophilia*, and *S. maltophilia’s* ability to cause dangerous and persistent infections, especially in those who are immune compromised creates an interesting and important field of research. Before BSFL are further explored as a waste management system, researchers should explore how to reduce the threat of *S. maltophilia* and their ability to harbor it and potentially vector it to humans.

**Bacteriology of Probiotics**

Probiotics is a term which has cemented itself within the lexicon of nutritionists and wellness-influencers. Probiotics are bacteria deemed to have helpful qualities when consumed, including but not limited to, outcompeting harmful bacteria. Probiotic bacteria have requirements which allow them modulate immunity and resist infections when consumed. In very general terms, to be considered to have probiotic potential, bacteria must be able to do the following: adhere to cells, reduce, or exclude the ability for a
pathogen to adhere to a host, persist and multiply, produce bacteriocins to impede pathogen growth, resist microbicides, be noninvasive, noncarcinogenic, nonpathogenic and overall safe to the consumer, and be able to coaggregate to form a normal, balanced flora. For many years, *Lactobacillus* species has been investigated for its probiotic potential and possibility to be used for disease treatment and prevention (Reid, 1999). Although probiotics have been extensively studied in humans, so far, probiotics within BSFL have not attracted much attention. However, the introduction of probiotic bacteria into the microbiome of BSFL could mitigate the risk of *S. maltophilia* infection and vectoring.

**Lactic Acid Producing Bacteria**

Lactic acid producing bacteria are a unique class of organisms which cannot respire to get ATP because they cannot biosynthesize cytochromes. Instead, they ferment sugars and utilize electrogenic decarboxylations and deiminations to form ATP which produces lactic acid as a byproduct. Obligate homofermentive lactic acid bacteria can produce 100% lactic acid using the Embden-Mayeroff pathway, in which a sugar is introduced and internalized by membrane transporters and then is isomerized to glucose or fructose. It should be noted that this method of metabolism is much less efficient than non-lactic acid producing pathways; however, a proton-substrate symport and lactic acid excretion can increase the energy yield for these bacteria (Pessione, 2012).

There is an incredibly wide range of lactic acid bacteria, with some of the most well-known being lactobacilli, lactococci, enterococci, and streptococci. Although similar in their fermentation abilities, they differ in morphology, pH, salt and temperature tolerance, and pathogenic potential. Because of the wide range of bacteria within this
category, it is difficult to distinguish between beneficial and virulent species of lactic acid producing bacteria. Most lactobacilli and lactococci are generally regarded as safe (Pessione, 2012).

In fact, lactic acid producing bacteria could be so helpful to humans that they have been investigated for their probiotic ability. Many types of lactic acid producing bacteria are already found on and within humans on mucous membranes, the intestines, skin, genitals, and urinary tract. Some of their beneficial impacts are a result of a symbiotic relationship between the bacteria and a human host. For example, some of these bacteria produce gamma-amino-butyrate which can relax gut smooth muscles, while others produce beta-phenylethylamine which can control satiety and mood. However, researchers have hypothesized that an increased ingestion of lactic acid producing bacteria could confer even more health benefits, ranging from immune system modulation to an increased resistance to illness (Harzallah & Belhadj, 2013).

Potential positive impacts of lactobacteria could extend far beyond what they can do in a human host. Many of them can produce anti-microbial metabolites such as bacteriocins. Bacteriocins interfere with the cell wall or membrane of target organisms which inhibit cell wall biosynthesis or cause pore formation to result in cell death. This ability to destroy potentially virulent bacteria suggests that the presence of lactic acid producing bacteria could restrict the amount of virulent bacteria which would be able to colonize within a host (O’Sullivan et al., 2002).

Although the benefits of lactic acid producing bacteria within humans is relatively studied, it suggests the question: what is known about lactic acid producing bacteria within BSFL? There is research to suggest that they may make up the majority of BSFL
microbiota (Gold et al., 2020). Lactic acid producing bacteria can produce bioactive molecules such as organic acids, hydrogen peroxide and bacteriocins which are produced when metabolizing substrate (Hadj Saadoun et al., 2020). This is particularly important because this allows BSFL to feed on a variety of different wastes and resist deadly pathogenic infections. However, as with most organisms there tends to be a limit where the antimicrobial peptides are not as efficient as they could be. However, BSFL take on much of the identity of the substrate they are fed on. Therefore, although there are data to suggest that BSFL may already contain a high amount of lactic acid producing bacteria more research is necessary to investigate the quantity of lactic acid producing bacteria within BSFL when reared on different substrates and what impacts lactic acid producing may have on BSFL and the other bacteria which may live within BSFL.
MATERIALS AND METHODS

Media Preparation

Three types of media were prepared to complete the experiment. The first was trypticase soy broth (TSB) which was prepared using 30 g of powder for 1 L of media. In a 1 L flask, ½ of the desired amount of deionized water was added, the flask was placed on a stir plate and turned on to stir while the powder was slowly added. Once the powder was in, the remaining water was added to the flask. After the powder was completely dissolved, the desired amount of broth was put into smaller flasks and covered with tin foil and autoclaved at 21°C for 15 minutes (liquid).

To prepare selective media for *S. maltophilia* (VIA), the desired amount of MSA power was prepared according to 111 g of powder for 1 L of media. In a 1 L flask, ½ of the desired amount of deionized water was added, the flask was placed on a stir plate and turned on to stir while the powder was slowly added (Kerr et al., 1996). Once the powder was in, the remaining water was added to the flask. Once the powder was completely dissolved the desired amount of broth was put into smaller flasks and covered with tin foil and autoclaved at 21°C for 15 minutes (liquid). While the media was in the autoclave, a hot water bath was turned on and once the flasks had completed autoclaving, they were placed in the water bath to cool to 50-55°C. When they had reached the desired temperature, a reconstituted and mixed solution of Vancomycin, Imipenem and Amphotericin B were added and mixed using a stir plate. The mixture was poured into labelled petri plates and allowed to solidify overnight at room temperature. The following day the plates were refrigerated.
Selective agar for lactic acid producing bacteria (MRS) was prepared using 62 g of powder for 1 L of media. In a 1 L flask ½ of the desired amount of deionized water was added, the flask was placed on a stir plate and turned on to stir while the powder was slowly added. Once the powder was in, the remaining water was added to the flask. Once the powder was completely dissolved the desired amount of broth was put into smaller flasks and covered with tin foil and autoclaved at 21°C for 15 minutes (liquid). While the mixture was autoclaving, a hot water bath was turned on and the flasks were placed in the hot water bath after autoclaving for 20-30 minutes. When the mixture was cool enough for handling it was poured into individual, labelled petri dishes, and left to solidify overnight at room temperature. The following day, the plates were refrigerated.

**Bacteria Preparation**

With a sterile loop, one separate colony of *S. maltophilia* was isolated from a quadrant streak plate of *S. maltophilia* which has been incubated for 24 hours. The loopful of culture was added into 50 mL of sterile TSB and the culture was incubated for 24 hours.

**Plate Dilution Calculation**

To discover the optimum plating dilution, these calculations were performed. Larvae were given time to feed on autoclaved potato spiked with *S. maltophilia* for 3 days. After 3 days, 5 larvae were removed and weighed to determine the amount of water required to create a 1:10 dilution, which was 10 mL. The larvae were added to 10 mL of deionized water in a test tube and crushed with a glass rod for 20 seconds. Additionally, 1 gram of potato was added to 9 mL of water and shaken for 10 minutes and vortex. The
larvae and potato were separately diluted to a gradient of dilutions ranging from $10^{-1}$ to $10^{-10}$ and plated on TSA plates as demonstrated below.

The plates were incubated at 37°C for 24 hours, after which they were removed, and the colonies were counted to determine which dilutions were most appropriate for further experimentation. It was determined that the potato was best suited to be plated to $10^{-3}$ and $10^{-4}$ dilutions, and larvae were best plated to $10^{-4}$ and $10^{-5}$ dilutions.

**Experimental Set Up**

Before experimentation began, mason jars, cheesecloth, tinfoil weigh boats, and scoopulas were autoclaved on a dry cycle to sanitize and then labeled. On day 0, 3
subsets of five larvae were removed from the set of larvae and their mass was recorded and the larvae were crushed and added to 10 mL of deionized water. The larval mixture was diluted to $10^{-4}$ and 0.001 mL were plated on MRS and incubated at 37°C for 24 hours. An additional 3 larvae were taken and added to a label microcentrifuge tube to be frozen at 0°C for future DNA extraction and PCR. The potatoes were autoclaved, once they were cooled enough to handle 165 g of potato were added to each jar and slightly mashed to expose more surface area. Then, a spectrophotometer was used to investigate the stock *Stenotrophomonas maltophilia* culture and was diluted with TSB until it had an OD of 0.3. Afterwards, 1.65 mL of bacterial culture were added to all C treatment jars, and 1.65 mL of TSB was added to all A and D treatment jars. Forty-five larvae each were added to jars A, B and C. The jars were covered with a double layer of cheese cloth and a tin foil lid with holes and placed in the chemical hood.

**Table 2.** Treatment Labels

<table>
<thead>
<tr>
<th>Treatment Label</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Potato, and larvae</td>
</tr>
<tr>
<td>B</td>
<td>Potato, TSB and larvae</td>
</tr>
<tr>
<td>C</td>
<td>Potato, <em>Stenotrophomonas maltophilia</em> and larvae</td>
</tr>
<tr>
<td>D</td>
<td>Potato and TSB</td>
</tr>
</tbody>
</table>
Figure 2. Illustration of jar treatments.

Sampling

Five larvae from treatment A, B and C were removed from each jar and added to 10 mL of water, crushed, mixed with water for 45 seconds using a glass rod, and gently vortexed. The mixture was diluted to $10^{-4}$ and 0.001 mL of the $10^{-4}$ and $10^{-5}$ dilution were plated on MRS and incubated at 37°C for 24 hours. Additionally, 3 larvae were removed from each jar from treatment A, B and C, added to a labelled microcentrifuge tube, and frozen. Then, 1 g of substrate was removed from each jar, added to 9 mL of water, and mixed for ten minutes. The mixture was diluted to the $10^{-3}$ dilution and 0.001 mL of the $10^{-3}$ and $10^{-4}$ dilution were plated on MRS and incubated for 24 hours. Additionally, 1 g
of substrate was added to a microcentrifuge tube and frozen for future applications. Sampling was performed on day 3 and 6.

Analysis

The plates were removed from the incubator and the colonies were counted; the plates were then safely disposed of in the biohazard bin. This analysis was performed on days 4 and 7.

DNA Extraction

DNA extraction was performed using the materials and protocol using the MO BIO Laboratories Inc., DNeasy PowerFood Microbial Kit (Qiagen, Germantown, MD, USA). Extraction protocols were followed directly.

Diagnostic PCR

Genomic DNA from each extraction were assessed on 0.8% agarose Tris-acetate-EDTA (TAE) gels. The gels were run at 50 V for 1.5-2 hours. Primers were selected from published literature and targeted for two areas of interest, primers to detect sequences found in lactic acid producing bacteria and primers to detect *Stenotrophomonas maltophilia*. These primers were used to probe the DNA extraction for the presence of lactic acid producing bacteria and *Stenotrophomonas maltophilia*. The specific reaction conditions required by the literature (Table) were also followed. Gels were stained prior to being imaged in 1 ng per mL solution of ethidium bromide. Primer information for diagnostic PCR.
Table 3. Primers used for PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Probing For</th>
<th>Primer Sequence (5’-&gt;3’)</th>
<th>Function</th>
<th>Amplicon Length</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>LAB</td>
<td>Lactic acid producing bacteria</td>
<td>GCTCAGGAYGAGACGCYGG</td>
<td>Forward</td>
<td>750 bp</td>
<td>(Hou et al., 2018)</td>
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<tr>
<td></td>
<td></td>
<td>CACCGCTACACATGRADTTC</td>
<td>Reverse</td>
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<td></td>
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<tr>
<td>SMAL</td>
<td><em>S. maltophilia</em></td>
<td>GGTCAGCGAATAAGCGC</td>
<td>Forward</td>
<td>531 bp</td>
<td>(Whitby et al., 2000)</td>
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<tr>
<td></td>
<td></td>
<td>GAATATTGACCTGCTTCCC</td>
<td>Reverse</td>
<td></td>
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</tbody>
</table>

Table 4. PCR conditions for each primer.

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<th>Primer Set</th>
<th>Melting Temp</th>
<th>Annealing Temp</th>
<th>Elongation Temp</th>
<th>Primer Concentration</th>
<th>Number of Cycles</th>
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</thead>
<tbody>
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<td>95°C</td>
<td>58°C</td>
<td>72°C</td>
<td>0.1 μM</td>
<td>30</td>
</tr>
<tr>
<td>SMAL</td>
<td>95°C</td>
<td>72°C</td>
<td>72°C</td>
<td>0.1 μM</td>
<td>30</td>
</tr>
</tbody>
</table>

Statistical Analysis

The normality of the plate count data was analyzed by the Wilk-Shapiro test (PROC, UNIVARIATE, SAS Institute, 2020). As the data did not follow a normal distribution, it was transformed using ranks (PROC RANK, SAS Institute, 2020). The means and standard error values which are presented in this document were calculated from untransformed data. ANOVA was used to analyze data with repeated measures (PROC MIXED, SAS Institute, 2020). Additionally, means with significant differences were separated with Tukey tests. Samples of larvae and potato were analyzed separately as to account for unbalanced design, as potato trials included 4 treatments which all included potato and larvae trials only included 3 treatments which were the only treatments containing larvae.
The main factors of this experiment were considered to be: treatment, trial and day. The treatment containing no larvae was omitted from analysis as to test for differences in bacterial counts between larvae and potato samples. Remaining data was analyzed using ANOVA and repeated measures (PROC MIXED, SAS Institute, 2020), considering the main factors of treatment, type of sample (larvae or potato), trial and day. The data was analyzed separately for each of the two dilutions and their average (Alyokhin, personal communication).
RESULTS

Potato Plating Results

The number of colonies on MRS plates, selective for lactic acid producing bacteria due to presence of sodium acetate, were counted on day 3 and 6. On day 0 an initial swab was performed and very few colonies were found, between 6 potato swab samples from both trials an average of 6.83 colonies were found on autoclaved potatoes. The number of colonies significantly increased on day 3 (Figure 3). Where there was an average colony count of $2.486 \times 10^8$ CFU per gram of substrate and the largest colony count being found in treatment D with $2.631 \times 10^8$ CFU per gram of substrate.

On day 6, there was an average colony count of $2.433 \times 10^8$ CFU. Signifying a slight decrease in the total amount of colonies between day 3 and day 6. However, this varies between the treatments.

When the $10^{-3}$ and $10^{-4}$ dilutions were averaged together, no factors became more statistically significant (P>0.1). However, statistically significant effects were found within potato substrates and their bacterial counts on MRS plates with a $10^{-3}$ dilution (df=3, 19 F= 3.22, P=0.0458). Treatment A showed smaller results than treatment B (Tukey test, t = 2.88, P=0.0438) and no differences were found among other treatments, furthermore, the interactions between these two factors were not found to be significant (df=3, 16, F= 0.82, P=0.5004). For MRS plates with a $10^{-4}$ dilution there was no difference of statistical significance found between the treatments (df=3, 19, F=0.68, P=0.5762), but day 3 data compared to day 6 data was found to be significant (df= 1, 16, F=8.56, P=0.0087). The interaction between the two factors were not found to be significant (df=3, 16 F=2.33, P=0.1126) (Alyokhin, personal communication).
Figure 3. Average total colony counts on MRS agar of potato samples. Error bars represent the standard deviation for each treatment. (A) Total colony counts for day 3 for both trials are shown and separated by treatment. The y-axis is representative of CFU per gram of substrate to the order of $10^4$. (B) Total colony counts for day 6 for both trials are shown and separated by treatment. The y-axis is representative of CFU per gram of substrate to the order of $10^4$. 
Larvae Plating Results

The number of colonies on MRS plates were counted on day 3 and 6. On day 0 larvae were crushed to investigate the initial colony count. Between 12 initial samples of randomly selected larvae from both trials an average of 60.62 colonies were found from the crushed larvae- a larger initial amount than in the potato samples. The number of colonies significantly increases on day 3 (Figure 4). Where there is an average colony count of $1.327 \times 10^8$ CFU per gram of substrate and the largest colony count being found in treatment A with $1.508 \times 10^8$ CFU per gram of substrate.

On day 6, there was an average colony count of $2.19 \times 10^8$ CFU, signifying an increase in the total amount of colonies between day 3 and day 6. However, this varies between the treatments. For treatment A, of larvae and potato there was a 52.79% increase from day 3 to day 6. For treatment B, of larvae, potato, and TSB there was a 53.44% increase from day 3 to day 6. For treatment C, of larvae, potato, and S. maltophilia there was a 97.21% increase from day 3 to day 6, signifying the largest change in a crushed larvae sample between day 3 and day 6.

For both MRS $10^{-3}$ and MRS $10^{-4}$ the different treatments did not produce statistically significant results ($df=2,14$, $F=0.88$, $P=0.4377$ for MRS $10^{-3}$ and $df=2,14$, $F=0.51$, $P=0.6111$ for $10^{-4}$), this was also true for the average of both results ($df=2,14$, $F=0.85$, $P=0.4490$). However, the difference between the two experimental days, day 3 and day 6 were found to be significant ($df=1,12$, $F=27.03$, $P=0.0002$ for MRS3; $df=1,12$, $F=38.19$, $P<0.0001$ for MRS4; and $df=1,12$, $F=49.64$, $P<0.0001$ for their average). None of the interactions were found to be statistically significant ($P>0.2$).
Potato substrate demonstrated a higher bacterial count than the crushed larvae and this indicated a statistically significant result for MRS $10^{-3}$ and for the average of the two dilutions (df=1,15, $F= 16.06$, $P=0.0011$), MRS4 (df=1,15, $F= 25.37$, $P=0.0001$ for MRS $10^{-3}$ and df=1,15, $F= 40.58$, $P<0.0001$ for the average) (Alyokhin, personal communication).

A

![Graph: Larvae Plating Data, Day 3](image)
Figure 4. Average total colony counts on MRS agar of crushed larvae samples. Error bars represent the standard deviation for each treatment. (A) Total colony counts for day 3 for both trials are shown and separated by treatment. The y-axis is representative of CFU per 3 crushed larvae to the order of $10^5$. (B) Total colony counts for day 6 for both trials are shown and separated by treatment. The y-axis is representative of CFU per 3 crushed larvae to the order of $10^5$.

Genomic DNA Extractions of Potato Sample

Gel electrophoresis of genomic DNA demonstrated an increased amount of visualized DNA between day 3 and day 6. On day 3, DNA was only visualized for lactic acid producing bacteria in treatment B, of potato, larvae and TSB for samples 1 and 2, in treatment C, of potato, larvae and *S. maltophilia* for samples 1 and 3. In treatment D, of potato and TSB for samples 1 and 3. However, after probing the day 6 samples for lactic acid producing bacteria, their DNA was found in all samples in all lanes. These data are demonstrated in Table 5 (A).
Gel electrophoresis of genomic DNA demonstrated no change in visualized DNA between day 3 and day 6 when probing for *Stenotrophomonas maltophilia* in the potato sample. There was no visualized DNA in any lanes on day 3 or on day 6 in the potato sample. This data is demonstrated in Table 5 (B).

**Table 5. (A) Lactic acid producing bacteria populations in potato sample.** Summary of genomic DNA gel electrophoresis results from the potato sample probing for lactic acid producing bacteria. (+) represents that DNA was detected in a lane for that sample, (-) represents that no DNA was detected in that lane for that sample.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Jar</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato and larvae</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
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<td></td>
<td>3</td>
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<td>+</td>
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<tr>
<td>Potato, larvae, TSB</td>
<td>1</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>2</td>
<td>+</td>
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<td>3</td>
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<td>+</td>
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<tr>
<td>Potato, larvae, <em>S. maltophilia</em></td>
<td>1</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>2</td>
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**Table 5. (B) Stenotrophomonas maltophilia populations in potato sample.** Summary of genomic DNA gel electrophoresis results from the larvae sample probing for *Stenotrophomonas maltophilia.* (+) represents that DNA was detected in a lane for that sample, (-) represents that no DNA was detected in that lane for that sample.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Jar</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
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<tbody>
<tr>
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<td>Potato, larvae, <em>S. maltophilia</em></td>
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</table>
Genomic DNA Extractions of Larvae Sample

Gel electrophoresis of genomic DNA demonstrated a decreased amount of visualized DNA between day 3 and day 6 in the larvae sample probing for lactic acid producing bacteria. On day 3, DNA was visualized for lactic acid producing bacteria in all replicates of treatment A and C and in replicate 2 and 3 of treatment B. However, after probing the day 6 samples for lactic acid producing bacteria, DNA was found in replicate 1 and 2 of treatment A, all replicates of treatment B and in no replicates of treatment C. This data is demonstrated in Table 6 (A).

Gel electrophoresis of genomic DNA demonstrated a change in visualized DNA between day 3 and day 6 when probing for *Stenotrophomonas maltophilia* in the larvae sample. On day 3, there were no bands found in any replicates in treatment A, there were bands found for replicate 1 and 2 in treatment B and bands found for replicate 1 and 2 in treatment C. On day 6 there was no DNA found for any treatment. This data is demonstrated in Table 6 (B).

**Table 6. (A) Lactic acid producing bacteria populations in larvae sample.** Summary of genomic DNA gel electrophoresis results from the larvae sample probing for lactic acid producing bacteria. (+) represents that DNA was detected in a lane for that sample, (-) represents that no DNA was detected in that lane for that sample.

<table>
<thead>
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<td><strong>Potato, larvae, TSB</strong></td>
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<td><strong>Potato, larvae, <em>S. maltophilia</em></strong></td>
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Table 6. (B) *Stenotrophomonas maltophilia* populations in larvae sample. Summary of genomic DNA gel electrophoresis results from the larvae sample probing for *Stenotrophomonas maltophilia*. (+) represents that DNA was detected in a lane for that sample, (-) represents that no DNA was detected in that lane for that sample.

<table>
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**Figure 5.** Gel electrophoresis of PCR reactions on potato samples. The (-) indicates a negative control made of nuclease free water and loading dye. Lad. Signifies the ladder which was made of NE BioLabs 1 kilobase (kb) ladder.
DISCUSSION AND FUTURE DIRECTIONS

The substrate plating data indicates how the bacteria, substrate and larvae interact and the implications of their interactions. It is interesting to note how there were lactic acid producing bacteria found on the potato even after autoclaving the potatoes. This could be indicative of several things. Firstly, this could indicate contamination from autoclaving resistant bacteria. Secondly, it could indicate another source of contamination which occurred after the potatoes were autoclaved and before they were swabbed, and that sample was plated. Although this indicates a source of error, it is also indicative of the abundance of lactic acid producing bacteria in the environment.

It is possible that those bacteria were initially present in soil and then got transferred onto the skins of potatoes. However, it is unlikely that they survived autoclaving. This indicates a source of error which should be addressed in future experimentation. Lactobacillus spp. is not found as abundantly in soil as other well documented soil bacteria, such as members of Proteobacteria and Actinobacteria phyla. However, it has still been found in soil, and in fact, has been found to survive in soil and biofertilizer used to cultivate maize, and impacted the growth of the maize (Afanador-Barajas et al., 2021; Y. Wang et al., 2020). Lactobacillus spp. has also been found to grow on the surface of potatoes skins, and even survive when put into the simulated gastric conditions and gut microbiota in an in vitro setting (Larsen et al., 2019). This has large implications for this project, as it not only supports the idea that Lactobacillus spp. can grow on the surface of potatoes, but also shows that potato fibers can protect probiotic Lactobacillus strains through the passage in the gastrointestinal tract and could selectively modulate gut bacteria populations. Although that experiment was designed to
emulate human body conditions, similar experiments could be conducted to simulate the gut conditions of BSFL to see if these outcomes would remain true within a BSFL simulated environment (Larsen et al., 2019). The baseline potato colony count data are important, as they show several implications of the relationship of lactic acid producing bacteria and potatoes, additionally, it assists in comparisons with day 3 and day 6 plating data.

No difference was found between the 4 treatments in any trial. However, there were differences of statistical significance found between day 3 and day 6, between the two trials, and between the larvae and the potato. These results counter the claim that expected to see a difference in treatments, specifically when S. maltophilia was added to treatment C. This could be attributed to several factors, firstly, the concentration of S. maltophilia may not have been high enough to produce a robust effect. This is reflected in the PCR data, as S. maltophilia DNA was not found in larvae on day 6 at all and not much was found on day 3 either (Table 6B). This circumstance could have been prevented by implementing a standard curve before the experiment to determine the threshold of detection for each primer. Stenotrophomonas maltophilia was plated on VIA agar, a selective media for S. maltophilia with several different rounds of VIA agar, however the bacteria seldom grew. This indicated that the stock of S. maltophilia may have been too low to produce the robust results which were expected. Additionally, it cannot be ignored that S. maltophilia may simply not experience the interactions with probiotic Lactobacillus spp. Bacteria which were anticipated, but further research would be required to confidently conclude this.
There were statistically significant differences between the results from day 3 and the results from day 6. This indicates there was a change between day 3 and day 6 in all treatments, days, and trials. However, these differences vary depending on the treatment and if the bacterial count increased or decreased.

For potato samples on day 6, the highest colony count was found in treatment B, with an average colony count of $2.561 \times 10^8$ CFU per gram of substrate. This represents a 5.95% increase from day 3 to day 6. Although this is not the largest increase demonstrated between day 3 and day 6, as treatment C showed a 6.38% increase between day 3 and day 6. The increase in colony count for treatment B could be attributed to a few reasons. Firstly, with the addition of trypticase soy broth, larvae can consume the broth in addition to the substrate, the addition of broth may simply provide another substrate option which larvae choose rather than the substrate without the addition of broth. Secondly, trypticase soy broth is a nutrient rich medium, which provides an aerobic environment to foster the growth of aerobic bacteria, furthermore, it is commonly used to culture bacteria (Doyle et al., 1968). Hence, the addition of trypticase soy broth could create a better living environment for the already present lactic acid producing bacteria and increase the colony count. This would be consistent with the other colony count data for day 6, as treatment A, of substrate and larvae, had a colony count of only $2.382 \times 10^8$ CFU per gram of substrate and showed a 5.5% decrease from the day 3 colony counts. This suggests that the addition of trypticase soy broth either assists in the growth of bacteria or causes the larvae to consume less lactic acid producing bacteria in favor of consuming trypticase soy broth. However, the largest change in colony count was found in treatment D, where there was a 14.09% decrease between day 3 and day 6, with the
average colony count of day 6 being $2.261 \times 10^8$ CFU per gram of substrate. Treatment D was the only treatment without the addition of larvae, although trypticase soy broth was added to investigate its impact on the bacteria, its inclusion did not allow the culture to exhibit unstoppable growth. A plausible explanation for this stark decrease in colony count could be the lactic acid producing bacteria simply following the growth curve model and exhausting their expected life span. A study of *lactobacilli* from vaginal samples found that of the *Lactobacillus* sp. sampled, all had plateaued growth by the 24 hour mark, if not before (Yoshimura et al., 2020). Although the samples used here are from potatoes and are not human samples, this research suggests that by the 6th day the population of lactic acid producing bacteria was already decreasing, as the conditions stayed the same and the population was fighting over the limited resources within the experimental vessel. Interestingly, treatment C demonstrated an increase in colony count of 6.38%, this was the largest increase seen in the substrate plating trials. This indicates that the addition of *S. maltophilia* somehow increases the amount of lactic acid producing bacteria. Perhaps, like the hypothesis regarding treatment B, the addition of *S. maltophilia* grown in trypticase soy broth offers another nutrient source for the larvae, meaning they do not only have to feed on substrate with lactic acid producing bacteria and some will inevitably feed on substrate which is spiked with *S. maltophilia*.

Further intriguing trends were found between day 3 and day 6 in the larval samples. The largest amount of lactic acid producing bacteria was, again, found in treatment A, with an average colony count of $2.304 \times 10^8$ CFU per 3 crushed larvae, which also represents a 52.79% increase in the number of colonies between day 3 and day 6. It seems that when larvae are fed on potato substrate alone, they have a higher
number of lactic acid producing bacteria. This could be attributed to the amount of *Lactobacillus* spp. which is found on potato (Larsen et al., 2019). When BSFL are left to feed on potato with no other substrate option, *Lactobacillus* spp. have been known to colonize within BSFL, so as the larvae are able to feed on the potato with *Lactobacillus* spp. They are able to continue to colonize BSFL (Klammsteiner et al., 2020). Treatment B exhibited a 53.44% increase in colony count between day 3 and day 6, a slightly larger increase than treatment A. In general, treatment A and treatment B demonstrated similar growth rates between day 3 and day 6 within the larvae. This indicates that trypticase soy broth does not significantly alter the number of lactic acid producing bacteria colonies within the larvae. However, the largest increase was demonstrated in treatment C, with a 97.21% increase between day 3 and day 6.

There were also differences of statistical significance between trial 1 and trial 2. These trials were replicates of each other with the only difference being a difference in time. However, the same materials and procedures were used, this could be indicative of a source of error, or a slight change in external conditions which allowed for more bacteria to grow under certain conditions. A first hypothesized reason for this could be the change in temperature, both trials took place during the summer of 2021 in July and August, although they took place in a temperature-controlled room, the outside temperatures could vary daily from 70° to 95° and this impacted the temperature within the building. Even the blinds to the laboratory being opened could allowed for sunlight to come in and increase the temperature of the room. The outdoor and indoor temperature was not recorded during these trials and in future experimentation should be recorded as temperature can impact the growth of bacteria.
Bacterial contamination is another source of error which could have contributed to the difference between the two trials. It is possible that bacteria remained in the pipettes which were used daily in this experiment. Additionally, although TSB was autoclaved prior to adding to jars, it is possibly that the TSB was contaminated, and this altered the bacterial content of the treatments.

Finally, there was a statistically significant difference between the larvae and potatoes, where the potato had higher bacterial counts than the crushed larvae. This could signify that larva can influence the bacterial population of the substrate and could perhaps, decrease the bacterial levels on the substrate they inhabit. *Lactobacillus* spp. has been known to exhibit probiotic qualities against other bacteria (Reid, 1999). There is limited research detailing the interactions between *Lactobacillus* spp. and *S. maltophilia*. However, the research demonstrates that *Lactobacillus acidophilus* uses bacteriocins to reduce the amount of *S. maltophilia* in an in-vitro model (Sarhan & Ibrahim, 2018). This is exciting evidence which could suggest that lactic acid producing bacteria levels are outcompeting the levels of *S. maltophilia* withing the larvae, however, more research is required in this area prior to making confident conclusions.

The PCR results also contain interesting implications. When investigating the presence of lactic acid producing bacteria within potato samples, on day 3, lactic acid producing bacteria was only found in treatment B, C and D and not in treatment A. However, by day 6 lactic acid producing bacteria was found in each replicate of each treatment (Table 5A). This suggests that there may have been lactic acid producing bacteria within all the potato samples, but the levels were not high enough to be detected in all cases. This would be consistent with the literature which has found lactic acid
producing bacteria on the surfaces of potatoes (Axel et al., 2012). Consistently, in these environments rich with nutrients from the potatoes and larvae, the lactic acid producing bacteria might have had enough nutrients to continue growth.

The PCR results investigating _S. maltophilia_ in substrate were interesting, as no levels of _S. maltophilia_ were detected on day 3 or on day 6 (Table 5B). This is particularly interesting for treatment C which included 1.65 mL of _S. maltophilia_ culture with an OD of 0.3. The lack of evidence supporting this could be attributed to several factors. Firstly, it was difficult to grow _S. maltophilia_ on VIA agar, even when following the specific guidelines from literature which had proven its effectiveness of being a selective agar for _S. maltophilia_ (Kerr et al., 1996; Pinot et al., 2011). After much wasted time, energy, patience, money, and antibiotics, it was concluded that the stock of _S. maltophilia_ used for this experiment might have been a stock of a very low concentration. This would be consistent with the PCR data which found no _S. maltophilia_, even in treatment C. Secondly, this might suggest that the primers used were inappropriate for this strain of bacteria at this concentration (Table 3). These primers have been researched and cited in recent literature, suggesting they were an appropriate choice for this experiment. However, there is relatively little research which specifically investigates _S. maltophilia_, and therefore, a low amount of experimented and published primers. Future research should investigate more primer options for detection of _S. maltophilia_. These reasons could explain the lack of DNA evidence found for _S. maltophilia_ across all samples.

In the larvae sample, DNA evidence of lactic acid producing bacteria was found in treatment A, B and C for day 3 and found in treatment A and B for day 6 (Table 6A). It
is consistent with the plating data and with current literature that lactic acid producing bacteria is found in all larvae samples from day 3 (Larsen et al., 2019). However, it is interesting that there is no evidence of lactic acid producing bacteria DNA found in treatment C on day 6. This could suggest that there are interactions occurring between \textit{S. maltophilia} and the lactic acid producing bacteria. It could suggest that \textit{S. maltophilia} implored its virulence factors to outcompete the lactic acid producing bacteria within the larvae, however, this would contradict the PCR results looking for \textit{S. maltophilia} within the larvae.

On day 6, no traces of \textit{S. maltophilia} DNA were detected in any samples, including treatment C (Table 6B). This would negate the idea that \textit{S. maltophilia} is outcompeting the lactic acid producing bacteria, and instead suggests other implications of this data. The bacteria levels of both types could be too low to detect from PCR, specifically with \textit{S. maltophilia}, as mentioned above, there is not a wide variety of published literature which explores primer options for \textit{S. maltophilia}. This could indicate that the levels of lactic acid producing bacteria and \textit{S. maltophilia} are following a growth curve and going into their death stage over time (Denet et al., 2018; \textit{Growth Curve Prediction from Optical Density Data}, n.d.). Consistently, the conditions within the jars were set, no additional nutrients were added after the jars were set up on day 0. As such, nutrients would begin to dwindle as the bacteria continues to grow and compete for these limited resources, indicating that the lower bacteria levels could be caused by bacteria exhausting their resources and dying rather than death by competition between lactic acid producing bacteria and \textit{S. maltophilia}. 

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Future investigations can be performed to expand this area of research which would use probiotic *Lactobacillus* spp. bacteria to limit the amount of *S. maltophilia* within BSFL and therefore mitigate this public health risk. Firstly, investigators should explore the microbiome of BSFL when fed on potato. Research has been performed to investigate the microbiome of BSFL when fed on grass clippings, fruits and animal feed, however, BSFL take on the microbial environment of what they are feeding on, so the investigations of BSFL feeding on different substrates are limitless (Klammsteiner et al., 2020). Potatoes are an affordable and abundant substrate source, specifically in Maine and other areas where potato agriculture is a major part of their economy. Secondly, the interactions between *S. maltophilia* and lactic acid producing bacteria should be better explored, especially on the molecular level. Research suggests that there are interactions between these two bacteria types in an in-vitro environment, and our research also suggests that there are interactions between these two bacteria (Sarhan & Ibrahim, 2018). However, no definite conclusions can be made solely with the available research, as more should be conducted. Finally, similar experiments could be completed, but they should use a higher stock concentration of *S. maltophilia*, as this would impact the results and make for stronger conclusions. These future directions could lead to a greater amount of research which could be used to mitigate this public health risk.
REFERENCES


AUTHOR’S BIOGRAPHY

Emily Marie McLaughlin was born and raised in Hudson Massachusetts, where she graduated from Hudson High School in 2017. During her undergraduate career at the University of Maine, she was a member of the Honors College and a dual degree student pursuing a B.S in Microbiology and a B.A in Spanish. Additionally, she was the two term Vice President of Academic Affairs for Delta Phi Epsilon, a fitness instructor at the Recreation Center, the secretary of Operation HEARTS and worked as an MLA and a TA for various courses. She is a member of Sigma Delta Pi, Spanish Honors Society and the recipient of the Edith McVay King scholarship, for outstanding microbiology student, and the Professor Melvin Gershman Scholarship, for an Honors College student demonstrating academic achievement through involvement in science and humanities. In her spare time, Emily enjoys music and exercise, she is also a cat mom to Theodore the cat.

Upon graduation Emily will be attending Boston University School of Public Health to obtain her Master’s in Public Health with a concentration in epidemiology and biostatistics.