Bugs, Bacteria, & Biotechnology

by

Richard Murphy

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 14, 2024

Keywords: PGPRs, *Wolbachia*, Microinjection, Bacteria, Biotechnology, Astro-Entomology

Copyright 2024 by Richard Murphy

Approved by

Dr. John F. Beckmann, Chair, Associate Department of Entomology & Plant Pathology
Dr. David Held, Department Chair Department of Entomology & Plant Pathology
Dr. Rüdiger Hauck, Associate Professor Department of Poultry Science
Kathy Lawrence, Professor Department of Entomology & Plant Pathology

Abstract

Bugs, Bacteria, and Biotechnology explores the potential of insects and microbial systems in addressing pressing challenges across agriculture, space exploration, and pest management. Chapter 1 is a literature review followed by Chapter 2 which details the screening of plant growth-promoting rhizobacteria (PGPR) strains for novel insecticidal properties. Out of 502 PGPRs strains screened, 39 demonstrated notable mortality against various pest species (7.8%), suggesting these PGPR strains, especially key species *Serratia marcescens* and *Bacillus velezensis*, hold promise as potential bioinsecticides for future sustainable pest management. Chapter 3 shifts to extraterrestrial agriculture, investigating the Black Soldier Fly (BSF) (*Hermetia illucens*) as a candidate for Martian soil composting. By utilizing a self-contained 3-D printed composter with Martian soil simulant and BSF, significant nutrient enrichment was achieved, supporting the feasibility of BSF as a soil fertility enhancer in future terraforming ventures. Chapter 4 examines the feasibility of introducing *Wolbachia* into *Alphitobious diaperinus* for population control using Incompatible Insect Technique (IIT). By optimizing microinjection conditions and methodologies, we showed successful *Wolbachia* presence in buffer, eggs, and injected larvae, supporting the idea *Wolbachia* IIT may be a viable future control option for *A. diaperinus* if a transfected population can be achieved. In Chapter 5, yeast (*Saccharomyces cerevisiae*) served as a model to study the function of *Wolbachia* effector proteins, addressing *Wolbachia*'s genetic intractability. Leveraging yeast's genetic toolkit, we explored the molecular basis of cytoplasmic incompatibility (CI) and provided insight into potential yeast expression systems. This dissertation integrates microbial and insect biology to contribute innovative biotechnological solutions with applications ranging from agriculture, pest management, and extraterrestrial environments.

Acknowledgments

I would like to thank my friends and family for their support. Thanks to Dr. Beckmann, Larry Murphy, Kim Murphy, Richard Nelson, Ruth Nelson, Hana Gleason, Ronin, Dr. Hauck, Dr. Held, Dr. Lawrence, & Dr. Wilhite.

Table of Contents

Abstract 2

Acknowledgments 2

List of Tables 5

List of Figures 6

List of Abbreviations 7

Chapter 1: Dissertation Background 8

Chapter 2: Fast Screening Libraries of Plant Growth Promoting Rhizobacteria (PGPRs) for Insecticidal Activity (Introduction [38], M&M [40], Results [48], Discussion [58]) 37

Chapter 3: Utilizing "Black Soldier Flies", *Hermetia illucens*, to Compost Martian Soil Simulant for Future Mars Terraforming Ventures (Introduction [73], M&M [76], Results [83], Discussion [87]) 73

Chapter 4: Optimizing Microinjection Technique of Wolbachia for *Alphitobius diaperinus* (Introduction [97], M&M [101], Results & Discussion [107]) .... .97

Chapter 5: (Using Baker's Yeast to Determine Functions of Novel Wolbachia (and other prokaryotic) Effectors) 127

References 20-36, 62-72, 91-96, 116-126. 147-154

List of Tables

Table 1 (Tier 1 *Drosophila melanogaster* Screening) 43

Table 1 (PGPR Significant Strains) 57

List of Figures

Figure 1 (Tier 1 *Drosophila melanogaster* Screening).. 42

Figure 2 (Tier 2 *Aphis gossypii* Screening Set Up)... 45

Figure 3 (Tier 2 *Lygus lineolaris* Screens) 46

Figure 4 (Tier 1 and Tier 2 *Drosophila melanogaster* Screening) 49

Figure 5 (Tier 2 *Spodoptera frugiperda* Screening) 53

Figure 6 (Tier 2 *Aphis gossypii* Screening Set Up)... 55

Figure 7 (Tier 2 *L. lineolaris* Screening) 56

Figure 8 (Black Soldier Fly Composter)... 81

Figure 9 (Black Soldier Fly Larvae Rocket launch G-force Test)... 84

Figure 10 (Mehlich 1 Extraction and Total Element Digestion Soil Data) 87

Figure 11 (Initial Feasibility Screening).. 109

Figure 12 (*Wolbachia* SPG Buffer Methodology Assessment)... 112

Figure 13 (Injected Beetle Egg *Wolbachia* Check) 115

Figure 14 (Schematic representation of CI operons) 131

Figure 15 (Conceptual Serial Dilution)... 133

Figure 16 (Sample yeast Plasmid) 136

Figure 17 (Sample Diagram of two Compatible Co-Expression Plasmids) 138

List of Abbreviations

PGPR	Plant Growth Promoting Rhizobacteria
SIT	Sterile Insect Technique
IIT	Incompatible Insect Technique
CI	Cytoplasmic Incompatibility
BSF	Black Soldier Flies
BUGS	Biological Unit for Gravitational Study
SEARS	South East Alabama Rocketry Society
PETG	Polyethylene Terephthalate Glycol
PLA	Polylactic Acid
ACS	Alabama Control Soil
MMS2	Mojave Mars Simulant – 2
AUSTL	Auburn University Soil Testing Laboratory

Chapter 1: Dissertation Background

Insects and bacteria are ubiquitous in nature, forming interactions that have evolved and diversified over millions of years. This dissertation explores the usage of insects and microorganisms through the utility of biotechnology, addressing diverse applications ranging from pest management, insect mediated soil reclamation and microbial techniques. Central to this work are Plant Growth Promoting Rhizobacteria (PGPRs), the insect endosymbiont *Wolbachia*, and engineered microbial systems. Each chapter explores unique approaches to leveraging biological interactions for practical and technological applications. While each chapter examines distinct aspects of microbial and insect interactions, the biotechnological potential of leveraging these systems to address ecological, agricultural, and industrial challenges unifies the purpose of the chapters. By examining these studies through the broader context of biotechnology, this dissertation bridges multiple disciplines to contribute novel innovation and discoveries to the field of biotechnological entomology.

Bacteria are one of the most primordial forms of life, having been vital in shaping ecosystems and influencing the evolution of other life forms both single and multicellular. Their immense diversity, metabolic versatility, and ability to form complex associations with plants, animals, and other microorganisms make them valuable organisms for study and technological utilization. The breadth of bacterial potential applications encompasses spaces such as agriculture, environmental management, biotechnological applications, and pest control.

They represent some of most diverse microorganisms on Earth, with species spanning vast phylogeny inhabiting virtually every habitable environment, including extreme conditions

such as hydrothermal vents, freezing temperatures, and hypersaline lakes (Lozupone and Knight, 2007). Their adaptability is due to metabolic and genetic diversity which allows bacteria to utilize a broad range of materials in biological processes. Inorganic molecules like hydrogen sulfide and ammonium, organic matter like cellulose and lignin, and even synthetic compounds in pesticides can be broken down and utilized by certain bacterial species (Singh et al., 1999; Chandra et al., 2007; Falkowski et al., 2008; Xu et al., 2018). The diverse metabolic capabilities of bacteria allow them to drive key ecological processes, such as nutrient cycling, nitrogen fixation, and organic matter decomposition. Nitrogen-fixing bacteria like *Azospirillum* for example, can convert unusable atmospheric nitrogen into ammonia, a form of nitrogen that plants can utilize for growth, due to nitrogen fixing structural genes (nif) (Steenhoudt and Vanderleyden, 2000).

Bacterial environmental success in reproduction and niche acquisition can be heavily attributed to innate biological mechanisms such as horizontal gene transfer. Horizontal gene transfer is the exchange of genetic materials between bacteria, which once exchanged can be passed on to descendants. This contributes to bacterial adaptability, allowing for rapid acquisition of new phenotypes, such as antibiotic resistance, salinity tolerance, or the ability to degrade specific compounds (Soucy et al., 2015). This genetic plasticity has significant implications for biotechnological innovation, allowing for the harnessing of bacteria and their traits for applications in agriculture, waste management, and environmental remediation, insect population control, and much more.

Bacterial interactions with plants are vital to plant success. Some bacteria are able to produce metabolites which have the ability synthesis of phytohormones, aid in nitrogen fixation

and phosphate solubilization, bolster plant defenses for the inhibition of pathogens, and induce systemic resistance in plants (Kloepper et al., 1980; Kaymak, 2011; Saharan and Nehra, 2011; Bhattacharyya and Jha, 2012; Ahemad and Kibret, 2014).

Plant growth-promoting rhizobacteria (PGPR) are bacteria which reside in and/or colonize the rhizosphere of plants and aid in plant growth through a variety of direct and indirect mechanisms (Kloepper et al., 1980; Lugtenberg and Kamilova, 2009; Ahemad and Kibret, 2014). Dr. Joseph Kleopper, Auburn University, discovered, classified, and popularized them in 1980 with his work relating *Pseudomonas fluorescens-putida* (Kloepper et al., 1980). PGPRs encompass a large array of beneficial rhizobacteria. They are comprised of a wide phylogeny of bacteria species such as, *Pseudomonas, Bacillus, Burkholderia, Azospirillum, Arthrobacter, Azotobacter, Streptomyces, Klebsiella, Enterobacter, Alcaligenes,* and *Agrobacterium,*(Kloepper et al., 1989; Joseph et al., 2012; Okon et al., 2015). These bacterial clades have shown unique interactions when exposed to plants, roots, or the rhizosphere. *Pseudomonas* for example produce hydrolytic enzymes, and phytohormones, can aid in heavy metal uptake, and can serve as a biofilm providing antibiotic resistance (Meliani, 2016; Panpatte et al., 2016). *Burkholderia* isolates have been shown to synthesize auxin, a key phytohormone involved in regulation of plant shoot and root growth. Additionally, these PGPR isolates can exhibit chitinase activity, which enable the degradation of chitin from arthropod exoskeletons contributing to enhanced nutrient availability (Shaharoona et al., 2007; Aroumougame et al., 2020). *Bacillus*, abundant in crop soil, can aid crop health by boosting host plant defenses through suppressing pathogenic organisms (Kumar et al., 2011; Sivasakthi et al., 2014). *Bacillus* also assists in phosphate solubilization in plant roots which improves host plant nutrient recruitment (Kashyap et al., 2019).

PGPRs are currently being explored for a wide array of applications, including their use in agriculture, as bioinoculants in bioformulations, in nanotechnology, as fertilizers, and in nutrient management systems (El Zemrany et al., 2007; Gholami et al., 2009; Kaymak, 2011; Mahmood et al., 2016; Verma et al., 2019). A technological usage example of PGPRs include inoculation of seeds which have been shown to enhance root growth and development in crops such as maize, increases crop yield in barley and wheat, and can provide protection for germinated plants from toxic and low quality soils (El Zemrany et al., 2007; Baris et al., 2014; Grobelak et al., 2015). Their additional usage in bioformulations include the promotion of plant growth in crops such as chili, tomatoes, and cauliflower (Latha et al., 2009; Kalita et al., 2015). PGPRs are also effective in providing protection against diseases like blister blight in tea plants, and in managing fruit rot (Bharathi et al., 2004; Saravanakumar et al., 2007). Their versatility extends to numerous other agricultural applications, underscoring their broad utility in sustainable crop production (Arora et al., 2011; Kumari et al., 2019; Adoko et al., 2022). The potential of PGPRs as insecticidal agents remains largely underexplored. To date, few PGPR-based insecticides have been commercially developed, of which *Bacillus thuringensis* (*Bt*) is primarily incorporated. *Bt* is known to produce Cry proteins that are toxic to a wide range of insect larvae, providing a natural insecticidal mode of action (Schnepf et al., 1998). Advancements in genetic engineering have further enhanced the efficacy of *Bt*, enabling their use in agriculture allowing for crop resistance to insect pests (Shelton et al., 2002). Alternative studies have documented reductions in insect herbivory, production of chitinase activity, and the generation of plant defense-enhancing metabolites (Coy et al., 2019; Mathur et al., 2019; Regaiolo et al., 2020). These studies, however, have not specifically fed PGPRs to insects to evaluate insecticidal effects.

The insecticidal potential of PGPRs may be of great value as synthetic insecticides, while commonplace in society, do not come without drawbacks. Synthetic insecticides are chemical formulations designed to control or kill insect pest populations. These compounds are used extensively worldwide in agricultural, rural, and urban areas to mitigate pest infestations and improve conditions. While they have been instrumental in controlling vector-borne diseases (van den Berg et al., 2012), reducing agricultural crop losses (Metcalf, 1980), and managing pest populations (Oberemok et al., 2015), their widespread and indiscriminate use does not come without environmental and health concerns (Ansari et al., 2014).

Synthetic insecticide and pesticide effectiveness revolve around their ability to exhibit high lethality to target pests while sparing non-target organisms. However, in reality, achieving this is challenging as off-target effects frequently occur (Bird et al., 1996; Jergentz et al., 2005; Munjanja et al., 2020). Off target effects of insecticides generally refer to non-target organisms coming in contact or being afflicted, run-off of insecticide into rivers or non-applied lands, environmental contamination, and downstream human health effects. The extent of non-targeted organisms afflicted by pesticides can range from natural plant microorganisms, insects, birds, and larger marine mammals such as river dolphins (Kannan et al., 1997; Aktar et al., 2009). These unintended off target consequences can harm natural ecosystems and affect biodiversity.

In addition to non-target effects, insecticide resistance in insect species is of great concern (Hemingway et al., 2002; Ffrench-Constant, 2013). Insecticide resistance occurs when individuals of a population evolve selective mechanisms which reduce the effectiveness of the synthetic compound. Over time this positive selection pressure reduces the total effectiveness of the synthetic compound against that pest population. Resistance has become pervasive enough

for the inception of the Insecticide Resistance Action Committee (IRAC). IRAC operates with a focus on providing up to date insecticide modes of actions information, with an additional goal of managing insecticide resistance through long term management and awareness strategies (Sparks and Nauen, 2015).

Chapter 2 focuses on exploring the insecticidal potential of PGPR strains from a library collection against insect pests. The Auburn University PGPR library contains glycerol stock isolates (stored in -80°C) of 8 collections of PGPR strains totaling over 6,800 individual strain samples. The physical sample collection is also accompanied by a digital collection which includes strain sample number, strain genus and species identification (when applicable and what percentage match), GPS coordinates where it was found, plant or crop it was collected near, as well as any plant colony interactions recorded or assays involving a particular strain noted. This collection has been accrued for over 40.

While chapter 2 focuses on PGPRs derived from terrestrial soil environments in relation to insect pests, Chapter 3 pivots into the use of black soldier flies (BSF), *Hermetia illucens*, for Martian soil reclamation through biotechnological novel approaches.

Humans have gazed with wonder towards the stars for thousands of years. As technology advances, so does our curiosity and ability to achieve the previously unimaginable. The great space race of the 1960s led to the first humans stepping foot on the moon on July 20th, 1969. The enduring curiosity of civilization in relation to the cosmos has led to many continued advancements in astronomical technology and discoveries. The continued success and desire to explore has led pundits to setting their sights on the future colonization of Mars. The prospect of human colonization of Mars currently presents significant challenges. One such challenge is the

establishment of self-sufficient food production and soil fertility systems for long-term missions. Agriculture on Earth benefits from arable soils enriched through biological processes, while extraterrestrial regolith such as Martian soil lacks the organic matter, water, and conditions essential for optimal plant growth (Wamelink et al., 2014). Efforts to tackle these challenges must focus on resource-efficient soil enhancement strategies to support potential Martian agriculture for colonists (Silverstone et al., 2003, 2005).

Martian regolith is primarily characterized by its red coloration, a lack of organic content, low nitrogen levels, iron oxides and the presence of toxic perchlorates (Bell III et al., 2000; Mancinelli and Banin, 2003; Carrier and Kounaves, 2015; Stern et al., 2015). Much of what has been learned about the soil composition of Mars has come from the 1979 and 1997 spacecrafts Viking 1 and Pathfinder which took and analyzed soil samples (Bell III et al., 2000; Larsen et al., 2000). Although soil-free agricultural technologies such as hydroponics and aeroponics can bypass soil constraints, low gravity constraints, particular inputs of water, energy, and nutrients resources scarce on Mars challenge these agriculture technologies (Moffatt et al., 2019; Khatri et al., 2024). The success of initial agriculture and crop cultivation on Mars depends upon developing systems that minimize mass and resource demands while enabling in-situ nutrient cycling.

One potential solution for addressing the challenges of soil fertility for Martian agriculture may be the usage of biological processes to enrich the top-soil regolith with essential nutrients and organic material. Among potential strategies, composting may serve as an innovative and resource-efficient technological solution. Organic amendment, or the introduction of organic materials to soil, can increase the conditions of the soil whether chemically or

physically while also providing potential energy for microorganisms (Larney and Angers, 2012). Composting at its core is the biological process of degrading organic matter. Composted organic material incorporated into soil can substantially improve plant growth as well as provide other benefits (Epstein, 2019).

Using insects to compost organic materials offers a sustainable and alternative option to enhance soil quality by recycling nutrients and reducing organic waste. Insects like black soldier fly (BSF), *Hermetia illucens*, larvae are particularly effective in bioconversion processes due to their ability to break down a wide range of organic substrates, including waste into biomass without environmental pollution (Aziz *et al.*, 2022). During digestion, insects transform organic waste through the digestive tract with the assistance of anaerobic gut microorganisms into nutrient-rich frass, which can be used as soil amendment (Klammsteiner et al., 2020). Deposition of chitin into soil can also be beneficial as chitin degrades into ammonia which as stated prior provides usable nitrogen for plants (Sharp, 2013; Klammsteiner et al., 2020). Martian soil is low in nitrogen, the addition of chitin as a biproduct of chitinolytic gut bacteria or molted exuvia may be beneficial for soil reclamation efforts.

BSF are holometabolic, true fly, insects. Their lifecycle is categorized by the life stages of egg, larvae, pupa, and adult. Following hatching, there are 5 larval instars followed by a pupal stage which emerges into the winged adult (Terrell and Ingwell, 2022). The life cycle of a BSF rages around 38-45 days depending on conditions of growth (Cannella et al., 2016; Terrell and Ingwell, 2022). BSF have been touted with promise in the agricultural communities due to their composting efficiency, high protein yield, animal feed viability, potential in space missions, and much more (Barragan-Fonseca et al., 2017; Siddiqui et al., 2022; Guglielmi et al., 2024; Romano

et al., 2024). BSF larvae have high protein composition, with dry matter contents estimated from 40% up to 63% (Wang and Shelomi, 2017; Guglielmi et al., 2024). BSF larvae also yields up to ~38% fat while being able to accumulate numerous micronutrients such as manganese and calcium (Wang and Shelomi, 2017). Relating to space missions, protein, fats, and micronutrients are vital for astronauts due to reductions in body mass experienced by both short and long term missions. BSF may be a potentially "renewable" nutrient source for space missions due to their nutritional yields and ability to compost manure.

The utilization of BSF for space related missions is still underexplored. The ability to compost organic materials, complete a lifecycle in under 50 days, and yield high protein in conjunction with other vital nutrients such as fats and calcium, may provide untold benefits for future space related missions. Questions about the feasibility and potential of BSF still remain, such as the effects of microgravity on growth, environmental and special constraints, radiation viability, transport logistics, and long-term sustainability. The potential of BSF to compost organic material for the purpose of soil amendment will be explored and examined in chapter 3.

Chapters 5 and 6 explore the bacteria, *Wolbachia*, through unique interactions with insects, potential for incompatible insect technique, and gene effector techniques.

Wolbachia are obligate intercellular gram-negative alpha-protobacterium infecting up to 75% of all insect species (Jeyaprakash and Hoy, 2000). They are recognized for their ability to manipulate host reproduction to enhance its transmission through host populations gene drive. The ability to manipulate host reproduction of insects makes their research valuable for biotechnological and pest control practices. Unfortunately, these bacteria are currently unculturable and have not been directly manipulated before. Regarding reproductive

manipulation, *Wolbachia* can directly affect the success or failure of insect offspring through the mechanism known as Cytoplasmic Incompatibility (CI). CI occurs when a *Wolbachia* infected male mates with a female either uninfected or of another strain resulting in failure for the embryo to develop resulting in sterility (Laven, 1967a, 1967b; Bordenstein et al., 2001; Shropshire et al., 2020). The mechanism of CI has allowed for the technological development of Sterile Insect technique (SIT) and Incompatible Insect technique (IIT) which have been used to control wild pest insect population numbers (Zhang, Lees, et al., 2015; Zhang, Zheng, et al., 2015; Nikolouli et al., 2018). SIT and IIT are pest management methods which involve the release of sterile male individuals into a wild population for mating, resulting in invalid offspring.

Hermann Muller first discovered radiation treated males caused mutation, and high doses could lead to partial sterility among *Drosophila* (Muller, 1927). This work was later expounded upon by Knipling who suggested the release of irradiated male insects into wild populations as a means to reduce population numbers through sterility (Knipling, 1955, 1959). Since then SIT applications has been employed globally to combat numerous pest species (Dyck et al., 2021). SIT is often associated with irradiated males for sterility, while IIT associates pest population reduction specifically through reproductive incompatibility which is the case involving *Wolbachia*. The genetic mechanism underlying *Wolbachia's* ability to cause CI are termed CI factors (*cifs*). The premise revolves around a toxin-antidote system by which an infected male has a toxin *cif* which will cause sterility if mated with an uninfected female, but if mated with an infected female will lead to rescue and embryo viability due to infected females containing the antidote *cif* (Beckmann et al., 2017, 2019). These mechanisms are discussed in detail in Chapter 5's review on *Wolbachia* effectors. *Wolbachia* has been successfully exploited in pest management applications involving IIT for numerous species including *Culex*

pipiens/quinquefasciatus/aegypti/albopictus (mosquitoes), *Cadra cautella* (cherry fruit flies), and *Rhagoletis cerasi* (almond moth) (Zabalou et al., 2004; Atyame et al., 2015). The success of *Wolbachia* based IIT has driven efforts to explore its potential application to a wider array of pest species for future pest population control.

One potential utilization of *Wolbachia* for IIT may be in controlling poultry house litter beetles, *Alphitobious diaperinus*. Known as the lesser mealworm or litter beetle, *A. diaperinus*, is an economically significant pest in poultry production systems. They have up to 11 instars and reach adulthood in generally 40 to 100 days, while females have the ability to lay up to 2,000 eggs (Dunford and Kaufman, 2006). They are able, under favorable conditions, such as those present in poultry houses to reach adulthood in as little as ~30 days, depending on the key factors like temperature and humidity which can effect survivorship and development (Zafeiriadis et al., 2023; Rueda and Axtell, 1996). They are ubiquitous pests in poultry houses, residing everywhere from feed, manure, to even inside the structural components of poultry houses. Additionally, adult females have been known to lay their eggs everywhere from manure, cracks and crevices, to even poultry litter itself, all while larvae consume organic matter, poultry feed, and deposited waste (Salin et al., 2000; Retamales et al., 2011). The structural damage caused by *A. diaperinus* larvae cannot be understated as larvae are known to burrow into the insulation and wooden structures of poultry houses, (Vaughan et al., 1984; Geden and Axtell, 1987). Insulation damage has been associated with increased energy costs up to 67% more than uninfected poultry houses, this can be attributed to the reduction in thermal efficiency resulting in the use of more energy (Geden and Axtell, 1987; Axtell and Arends, 1990). The pest species, *A. diaperinus*, have spread globally from their sub/tropic origins (Geden and Hogsette, 1994; Mozaffar *et al.*, 2004) making their control and management a major concern.

A. diaperinus infestations are critical in poultry farms due to their ability to thrive in warm, humid environments which are rich in organic material such as poultry litter. Beetle infestations can reduce feed efficiency in poultry and if consumed can cause poultry to have nutritional imbalances, leading to reduced growth (Despins et al., 1994; Despins and Axtell, 1995). They pose additional risks to poultry operations as they can act as a disease vector for harmful pathogens (Despins et al., 1994; Dinev, 2013). The beetles' affinity to serve as reservoirs for pathogens cannot be understated. Bacteria such as *E.coli, Salmonella* spp., and *Campylobacter jejuni*, have been shown to be not only carried but transmitted by *A. diaperinus* (de las Casas et al., 1968; McAllister et al., 1994; Skov et al., 2004; Strother et al., 2005; Smith et al., 2022). *A. diaperinus* can also carry harmful *Eimeria* spp., and spread infectious bursal disease (IBD) both of which pose serious health risks to poultry (De Las Casas et al., 1976; Reyna et al., 1983; McAllister, 1993; Goodwin and Waltman, 1996; Gussem, 2007). Transmission of pathogen to poultry can occur through ingestion of the beetle by poultry (Despins and Axtell, 1995; Beckmann et al., 2021). This has been demonstrated before as *Salmonella* was detectable from a cloacal swab from a newborn chicken after a 24hr period from the consummation of one infected larvae (McAllister et al., 1994).

Effective management strategies for *A. diaperinus* are critical when addressing the significant economic and health challenges they pose. Chemical insecticides may not be ideal control measures as concerns for downstream human health effects may arise given the nature of the relationship between poultry and humans. Additionally due to prolonged insecticide usage of insecticides utilizing the same mode of action, insecticide resistance has occurred in some populations (Sammarco et al., 2023). Alternatively, sanitation measures such waste removal and frequent cleanings of poultry houses must occur as infrequent sanitation can result in the

development large populations of beetles (Vaughan, 1982). Entomopathogenic measures such as fungi and nematodes, show management promise in trials, however, viability concerns remain (Santoro et al., 2008; Alves et al., 2012; Del Valle et al., 2016). Due to the nature of the pest beetles being confined to poultry houses, which are tied to human health, strategic and intentional management methods must be employed. *Wolbachia* based IIT may yield a unique solution to management of *A. diaperinus* in poultry houses as no chemical insecticides are used and successful IIT can result in population reduction in poultry house beetles. Chapter 4 evaluates *Wolbachia* in relation to *A. diaperinus* as well as initial work in establishing a laboratory colony of *Wolbachia* infected *A. diaperinus*.

This review highlighted some of the dynamic nature of bacteria, insects, and their immense potential in biotechnological applications, laying the foundation for the subsequent chapters of this dissertation. By exploring diverse topics such as the insecticidal potential of Plant Growth Promoting Rhizobacteria (PGPRs), the use of black soldier flies (BSF) for Martian soil reclamation, and *Wolbachia* based IIT pest control strategies, this work encompasses entomological, agricultural, and technological disciplines. Each chapter underscores the transformative potential of entomological biotechnology.

References:

Adoko, M.Y., Noumavo, A.D.P., Agbodjato, N.A., Amogou, O., Salami, H.A., Aguégué, R.M., et al. (2022) Effect of the application or coating of PGPR-based biostimulant on the growth, yield and nutritional status of maize in Benin. *Front Plant Sci* **13**: 1064710.

Ahemad, M. and Kibret, M. (2014) Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *J King Saud Univ - Sci* **26**: 1–20.

Aktar, W., Sengupta, D., and Chowdhury, A. (2009) Impact of pesticides use in agriculture: their benefits and hazards. *Interdiscip Toxicol* **2**: 1–12.

Alves, V.S., Neves, P.M.D.O., Alves, L.F., Moino Jr, A., And Holz, N. (2012) Entomopathogenic nematodes (*Rhabditida*: *Heterorhabditidae* and *Steinernematidae*) screening for lesser mealworm *Alphitobius diaperinus* (*Coleoptera*: *Tenebrionidae*) control. *Rev Colomb Entomol* **38**: 76–80.

Ansari, M.S., Moraiet, M.A., and Ahmad, S. (2014) Insecticides: Impact on the Environment and Human Health. In *Environmental Deterioration and Human Health: Natural and anthropogenic determinants*. Malik, A., Grohmann, E., and Akhtar, R. (eds). Dordrecht: Springer Netherlands, pp. 99–123.

Arora, N.K., Khare, E., and Maheshwari, D.K. (2011) Plant Growth Promoting Rhizobacteria: Constraints in Bioformulation, Commercialization, and Future Strategies. In *Plant Growth and Health Promoting Bacteria*. Maheshwari, D.K. (ed). Berlin, Heidelberg: Springer, pp. 97–116.

Aroumougame, S., Geetha, T., and Thangaraju, M. (2020) Exploitation of PGPR endophytic *Burkholderia* isolates to enhance organic agriculture. *Am J Biosci* **8**:3, 57-64.

Atyame, C.M., Cattel, J., Lebon, C., Flores, O., Dehecq, J.-S., Weill, M., et al. (2015) *Wolbachia*-based population control strategy targeting *Culex quinquefasciatus* mosquitoes proves efficient under semi-field conditions. *PLOS ONE* **10**: e0119288.

Axtell, R.C. and Arends, J.J. (1990) Ecology and management of arthropod pests of poultry. *Annu Rev Entomol*. **35**:1, 101-126

Aziz, H.A., Lee, W.S., Hasan, H.A., Hassan, H.M., Wang, L.K., Wang, M.-H.S., and Hung, Y.-T. (2022) Composting by Black Soldier Fly. In *Solid Waste Engineering and Management: Volume 3*. Wang, L.K., Wang, M.-H.S., and Hung, Y.-T. (eds). Cham: Springer International Publishing, pp. 299–373.

Baris, O., Sahin, F., Turan, M., Orhan, F., and Gulluce, M. (2014) Use of Plant-Growth-Promoting Rhizobacteria (PGPR) seed inoculation as alternative fertilizer inputs in wheat and barley production. *Commun Soil Sci Plant Anal* **45**: 2457–2467.

Barragan-Fonseca, K. b., Dicke, M., and van Loon, J. j. a. (2017) Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – a review. *J Insects Food Feed* **3**: 105–120.

Beckmann, J.F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Merçot, H., et al. (2019) The toxin–antidote model of cytoplasmic incompatibility: genetics and evolutionary implications. *Trends Genet* **35**: 175–185.

Beckmann, J.F., Dormitorio, T., Oladipupo, S.O., Bethonico Terra, M.T., Lawrence, K., Macklin, K.S., and Hauck, R. (2021) *Heterakis gallinarum* and *Histomonas meleagridis* DNA persists in chicken houses years after depopulation. *Vet Parasitol* **298**: 109536.

Beckmann, J.F., Ronau, J.A., and Hochstrasser, M. (2017) A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nat Microbiol* **2**: 1–7.

Bell III, J.F., McSween Jr., H.Y., Crisp, J.A., Morris, R.V., Murchie, S.L., Bridges, N.T., et al. (2000) Mineralogic and compositional properties of Martian soil and dust: Results from Mars Pathfinder. *J Geophys Res Planets* **105**: 1721–1755.

van den Berg, H., Zaim, M., Yadav, R.S., Soares, A., Ameneshewa, B., Mnzava, A., et al. (2012) Global trends in the use of insecticides to control vector-borne diseases. *Environ Health Perspect* **120**: 577–582.

Bharathi, R., Vivekananthan, R., Harish, S., Ramanathan, A., and Samiyappan, R. (2004) Rhizobacteria-based bio-formulations for the management of fruit rot infection in chillies. *Crop Prot* **23**: 835–843.

Bhattacharyya, P.N. and Jha, D.K. (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J Microbiol Biotechnol* **28**: 1327–1350.

Bird, S.L., Esterly, D.M., and Perry, S.G. (1996) Off-target deposition of pesticides from agricultural aerial spray applications. *J Environ Qual* **25**: 1095–1104.

Bordenstein, S.R., O'Hara, F.P., and Werren, J.H. (2001) *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in Nasonia. *Nature* **409**: 707–710.

Cannella, L., Nassef, A.R.A., Bailey, D., and Ferrarezi, R.S. (2016) Alternative sources of food for aquaponics in the US Virgin Islands: a case study with black soldier flies. *Agric Exp Stn* **34**: 36–39.

Carrier, B.L. and Kounaves, S.P. (2015) The origins of perchlorate in the Martian soil. *Geophys Res Lett* **42**: 3739–3745.

de las Casas, E., Pomeroy, B.S., and Harein, P.K. (1968) Infection and quantitative recovery of *Salmonella typhimurium* and *Escherichia coli* from within the lesser mealworm, *Alphitobius diaperinus* (Panzer).1,2. *Poult Sci* **47**: 1871–1875.

Chandra, R., Raj, A., Purohit, H.J., and Kapley, A. (2007) Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. *Chemosphere* **67**: 839–846.

Coy, R.M., Held, D.W., and Kloepper, J.W. (2019) Rhizobacterial treatments of tall fescue and bermudagrass increases tolerance to damage from white grubs. *Pest Manag Sci* **75**: 3210–3217.

De Las Casas, E., Harein, P.K., Deshmukh, D.R., and Pomeroy, B.S. (1976) Relationship between the lesser mealworm, fowl pox, and newcastle disease virus in poultry12. *J Econ Entomol* **69**: 775–779.

Del Valle, E.E., Frizzo, L.S., Malmierca, M., Zbrun, M.V., Lax, P., and Doucet, M.E. (2016) Biological control of *Alphitobius diaperinus* with *Steinernema rarum* CUL and *Heterorhabditis bacteriophora* SMC and feasibility of application in rice hull. *J Pest Sci* **89**: 161–170.

Despins, J.L. and Axtell, R.C. (1995) Feeding behavior and growth of broiler chicks fed larvae of the darkling beetle, *Alphitobius diaperinus*. *Poult Sci* **74**: 331–336.

Despins, J.L., Axtell, R.C., Rives, D.V., Guy, J.S., and Ficken, M.D. (1994) Transmission of Enteric Pathogens of Turkeys by Darkling Beetle Larva (*Alphitobius diaperinus*). *J Appl Poult Res* **3**: 61–65.

Dinev, I. (2013) The darkling beetle (*Alphitobius diaperinus*)—A health hazard for broiler chicken production. *Trakia J Sci* **1**: 1–4.

Dunford, J.C. and Kaufman, P.E. (2006) Lesser mealworm, litter beetle, *Alphitobius diaperinus* (Panzer)(Insecta: *Coleoptera*: *Tenebrionidae*): EENY-367/IN662, rev. 6/2006. *EDIS* **2006**:.

Dyck, V., Hendrichs, J., and Robinson, A. (2021) *Sterile Insect Technique Principles and Practice in Area-Wide Integrated Pest Management.* (p.1216)

El Zemrany, H., Czarnes, S., Hallett, P.D., Alamercery, S., Bally, R., and Jocteur Monrozier, L. (2007) Early changes in root characteristics of maize (*Zea mays*) following seed inoculation with the PGPR Azospirillum lipoferum CRT1. *Plant Soil* **291**: 109–118.

Epstein, E. (2019) The Science of Composting, Boca Raton: CRC Press.

Falkowski, P.G., Fenchel, T., and Delong, E.F. (2008) The microbial engines that drive earth's biogeochemical cycles. *Science* **320**: 1034–1039.

Ffrench-Constant, R.H. (2013) The molecular genetics of insecticide resistance. *Genetics* **194**: 807–815.

Geden, C.J. and Axtell, R.C. (1987) Factors affecting climbing and tunneling behavior of the lesser mealworm (*Coleoptera*: *Tenebrionidae*). *J Econ Entomol* **80**: 1197–1204.

Geden, C.J. and Hogsette, J. (1994) Research and Extension Needs for Integrated Pest Management for Arthropods of Veterinary Importance. *Publ USDA-ARS UNL Fac 1039*.

Gholami, A., Shahsavani, S., and Nezarat, S. (2009) The effect of Plant Growth Promoting Rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *International Journal of Agricultural and Biosystems Engineering*, **3**:1, 9-14.

Goodwin, M.A. and Waltman, W.D. (1996) Transmission of Eimeria, Viruses, and Bacteria to Chicks: Darkling Beetles (Alphitobius diaperinus) as Vectors of Pathogens. *J Appl Poult Res* **5**: 51–55.

Grobelak, A., Napora, A., and Kacprzak, M. (2015) Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. *Ecol Eng* **84**: 22–28.

Guglielmi, L., Cesare, A.S., Siviero, A., Castagneri, D., Palumbieri, A., Hedin, L., et al. (2024) Comparative analysis of systems for sustainable food production during long-term missions. *IAC-24 A* **5**: 4.

Gussem, M.D. (2007) Coccidiosis in poultry: review on diagnosis, control, prevention and interaction with overall gut health. *Proc 16th Eur Symp Poult Nutr* 253–261.

Hemingway, J., Field, L., and Vontas, J. (2002) An overview of insecticide resistance. *Science* **298**: 96–97.

Jergentz, S., Mugni, H., Bonetto, C., and Schulz, R. (2005) Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. *Chemosphere* **61**: 817–826.

Jeyaprakash, A. and Hoy, M.A. (2000) Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* **9**: 393–405.

Joseph, B., Patra, R.R., and Lawrence, R. (2012) Characterization of plant growth promoting rhizobacteria associated with chickpea (Cicer arietinum L.) *International Journal of Plant Production*, **1**:2, 141-152.

Kalita, M., Bharadwaz, M., Dey, T., Gogoi, K., Dowarah, P., Unni, B.G., et al. (2015) Developing novel bacterial based bioformulation having PGPR properties for enhanced production of agricultural crops. *Indian J Exp Biol*. 0975-1009. p. 56-60.

Kannan, K., Senthilkumar, K., and Sinha, R.K. (1997) Sources and accumulation of butyltin compounds in Ganges River Dolphin. *Appl Organomet Chem* **11**: 223–230.

Kashyap, B.K., Solanki, M.K., Pandey, A.K., Prabha, S., Kumar, P., and Kumari, B. (2019) Bacillus as Plant Growth Promoting Rhizobacteria (PGPR): A Promising Green Agriculture Technology. In *Plant Health Under Biotic Stress: Volume 2: Microbial Interactions*. Ansari, R.A. and Mahmood, I. (eds). Singapore: Springer, pp. 219–236.

Kaymak, H.C. (2011) Potential of PGPR in Agricultural Innovations. In *Plant Growth and Health Promoting Bacteria*. Maheshwari, D.K. (ed). Berlin, Heidelberg: Springer, pp. 45–79.

Khatri, L., Kunwar, A., and Bist, D.R. (2024) Hydroponics: Advantages And Challenges In Soilless Farming. *Big Data Agric.(BDA)*, **6**: 81-88.

Klammsteiner, T., Turan, V., Fernández-Delgado Juárez, M., Oberegger, S., and Insam, H. (2020) Suitability of black soldier fly frass as soil amendment and implication for organic waste hygienization. *Agronomy* **10**: 1578.

Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* **286**: 885–886.

Kloepper, J.W., Lifshitz, R., and Zablotowicz, R.M. (1989) Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnol* **7**: 39–44.

Knipling, E.F. (1955) Possibilities of insect control or eradication through the use of sexually sterile males1. *J Econ Entomol* **48**: 459–462.

Knipling, E.F. (1959) Sterile-male method of population control. *Science* **130**: 902–904.

Kumar, A., Prakash, A., and Johri, B.N. (2011) Bacillus as PGPR in Crop Ecosystem. In *Bacteria in Agrobiology: Crop Ecosystems*. Maheshwari, D.K. (ed). Berlin, Heidelberg: Springer, pp. 37–59.

Kumari, B., Mallick, M.A., Solanki, M.K., Solanki, A.C., Hora, A., and Guo, W. (2019) Plant Growth Promoting Rhizobacteria (PGPR): Modern Prospects for Sustainable Agriculture. In *Plant Health Under Biotic Stress: Volume 2: Microbial Interactions*. Ansari, R.A. and Mahmood, I. (eds). Singapore: Springer, pp. 109–127.

Larney, F.J. and Angers, D.A. (2012) The role of organic amendments in soil reclamation: A review. *Can J Soil Sci* **92**: 19–38.

Larsen, K.W., Arvidson, R.E., Jolliff, B.L., and Clark, B.C. (2000) Correspondence and least squares analyses of soil and rock compositions for the Viking Lander 1 and Pathfinder landing sites. *J Geophys Res Planets* **105**: 29207–29221.

Latha, P., Anand, T., Ragupathi, N., Prakasam, V., and Samiyappan, R. (2009) Antimicrobial activity of plant extracts and induction of systemic resistance in tomato plants by mixtures of PGPR strains and Zimmu leaf extract against *Alternaria solani*. *Biol Control* **50**: 85–93.

Laven, H. (1967a) A possible model for speciation by cytoplasmic isolation in the Culex pipiens complex. *Bull World Health Organ* **37**: 263.

Laven, H. (1967b) Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility. *Nature* **216**: 383–384.

Lozupone, C.A. and Knight, R. (2007) Global patterns in bacterial diversity. *Proc Natl Acad Sci* **104**: 11436–11440.

Lugtenberg, B. and Kamilova, F. (2009) Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* **63**: 541–556.

Mahmood, A., Turgay, O.C., Farooq, M., and Hayat, R. (2016) Seed biopriming with plant growth promoting rhizobacteria: a review. *FEMS Microbiol Ecol* **92**: fiw112.

Mancinelli, R.L. and Banin, A. (2003) Where is the nitrogen on Mars? *Int J Astrobiol* **2**: 217–225.

Mathur, A., Koul, A., and Hattewar, J. (2019) Plant Growth-Promoting Rhizobacteria (PGPRs): Significant Revolutionary Tools for Achieving Long-Term Sustainability and Combating the Biotic Stress Caused by the Attack of Pathogens Affecting Crops in Agriculture. In *Plant Growth Promoting Rhizobacteria for Sustainable Stress Management : Volume 2: Rhizobacteria in Biotic Stress Management*. Sayyed, R.Z. (ed). Singapore: Springer, pp. 379–388.

McAllister, J.C. (1993) Determination of the potential of lesser mealworms, *Alphitobius diaperinus (Coleoptera: Tenebrionidae),* to transmit poultry pathogens. University of Arkansas.

McAllister, J.C., Steelman, C.D., and Skeeles, J.K. (1994) Reservoir competence of the lesser Mealworm (*Coleoptera*: *Tenebrionidae*) for *Salmonella typhimurium* (*Eubacteriales*: *Enterobacteriaceae*). *J Med Entomol* **31**: 369–372.

Meliani, A. (2016) Biofilm-mediated heavy metals bioremediation in PGPR *Pseudomonas. J Bioremediation Biodegrad* **3**: 370.

Metcalf, R.L. (1980) Changing Role of Insecticides in Crop Protection. *Annu Rev Entomol* **25**: 219–256.

Moffatt, S.A., Morrow, R.C., and Wetzel, J.P. (2019) Astro GardenTM Aeroponic Plant Growth System Design Evolution. *49th Int Conf Environ Syst*. ICES-2019-195

Mozaffar, H., Khan, A., and Hosen, M. (2004) Growth and development of the lesser mealworm, *Alphitobius diaperinus* (Panzer) (*Coleoptera*: *Tenebrionidae*) on cereal flours. *Pak J Biol Sci* 7(9), 1505-1508.

Muller, H.J. (1927) Artificial transmutation of the gene. *Science* **66**: 84–87.

Munjanja, B.K., Naudé, Y., and Forbes, P.B.C. (2020) A review of sampling approaches to off-target pesticide deposition. *Trends Environ Anal Chem* **25**: e00075.

Nikolouli, K., Colinet, H., Renault, D., Enriquez, T., Mouton, L., Gibert, P., et al. (2018) Sterile insect technique and Wolbachia symbiosis as potential tools for the control of the invasive species Drosophila suzukii. *J Pest Sci* **91**: 489–503.

Oberemok, V.V., Laikova, K.V., Gninenko, Y.I., Zaitsev, A.S., Nyadar, P.M., and Adeyemi, T.A. (2015) A short history of insecticides. *J Plant Prot Res*: 55(3).

Okon, Y., Labandera-Gonzales, C., Lage, M., and Lage, P. (2015) Agronomic applications of *Azospirillum* and other PGPR. *Biological nitrogen fixation*, 925-936.

Panpatte, D.G., Jhala, Y.K., Shelat, H.N., and Vyas, R.V. (2016) Pseudomonas fluorescens: A Promising Biocontrol Agent and PGPR for Sustainable Agriculture. In *Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 1: Research Perspectives*. Singh, D.P., Singh, H.B., and Prabha, R. (eds). New Delhi: Springer India, pp. 257–270.

Regaiolo, A., Dominelli, N., Andresen, K., and Heermann, R. (2020) The biocontrol agent and insect pathogen *Photorhabdus luminescens* interacts with plant roots. *Appl Environ Microbiol* **86**: e00891-20.

Retamales, J., Vivallo, F., and Robeson, J. (2011) Insects associated with chicken manure in a breeder poultry farm of Central Chile. *Arch Med Vet* **43**: 79–83.

Reyna, P.S., McDougald, L.R., and Mathis, G.F. (1983) Survival of *Coccidia* in poultry litter and reservoirs of infection. *Avian Dis* **27**: 464–473.

Romano, D., Di Giovanni, A., and Stefanini, C. (2024) Assessing black soldier fly pupation and survival in lunar regolith simulant: Implications for sustainable controlled habitats on the Moon. *Acta Astronaut* **223**: 505–511.

Rueda, L. M. and Axtell, R.C. (1996) Temperature-dependent development and survival of the lesser mealworm, *Alphitobius diaperinus*. *Med Vet Entomol* **10**: 80–86.

Saharan, B.S. and Nehra, V. (2011) Plant growth promoting rhizobacteria: a critical review. *Life Sci Med Res* **21**: 30.

Salin, C., Delettre, Y.R., Cannavacciuolo, M., and Vernon, P. (2000) Spatial distribution of *Alphitobius diaperinus* (Panzer) (*Coleoptera*: *Tenebrionidae*) in the soil of a poultry house along a breeding cycle. *Eur J Soil Biol* **36**: 107–115.

Sammarco, B.C., Hinkle, N.C., and Crossley, M.S. (2023) Biology and management of lesser mealworm *Alphitobius diaperinus* (*Coleoptera*: *Tenebrionidae*) in broiler houses. *J Integr Pest Manag* **14**: 2.

Santoro, P.H., Neves, P.M.O.J., Alexandre, T.M., Sartori, D., Alves, L.F.A., and Fungaro, M.H.P. (2008) Selection of *Beauveria bassiana* isolates to control *Alphitobius diaperinus*. *J Invertebr Pathol* **97**: 83–90.

Saravanakumar, D., Vijayakumar, C., Kumar, N., and Samiyappan, R. (2007) PGPR-induced defense responses in the tea plant against blister blight disease. *Crop Prot* **26**: 556–565.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., et al. (1998) Bacillus thuringiensis and Its Pesticidal Crystal Proteins. *Microbiology and molecular biology reviews*, **62**:3, 775-806.

Shaharoona, B., Jamro, G.M., Zahir, Z.A., Arshad, M., and Memon, K.S. (2007) Effectiveness of various Pseudomonas spp. and Burkholderia caryophylli containing ACC-Deaminase for improving growth and yield of wheat (*Triticum aestivum* I.). *J Microbiol Biotechnol* **17**: 1300.

Sharp, R.G. (2013) A review of the applications of chitin and its derivatives in agriculture to modify plant-microbial interactions and improve crop yields. *Agronomy* **3**: 757–793.

Shelton, A.M., Zhao, J.-Z., and Roush, R.T. (2002) Economic, ecological, food safety, and social consequences of the deployment of *Bt* transgenic plants. *Annu Rev Entomol* **47**: 845–881.

Shropshire, J.D., Leigh, B., and Bordenstein, S.R. (2020) Symbiont-mediated cytoplasmic incompatibility: What have we learned in 50 years? *eLife* **9**: e61989.

Siddiqui, S.A., Ristow, B., Rahayu, T., Putra, N.S., Widya Yuwono, N., Nisa', K., et al. (2022) Black soldier fly larvae (BSFL) and their affinity for organic waste processing. *Waste Manag* **140**: 1–13.

Silverstone, S., Nelson, M., Alling, A., and Allen, J. (2003) Development and research program for a soil-based bioregenerative agriculture system to feed a four person crew at a Mars base. *Adv Space Res* **31**: 69–75.

Silverstone, S., Nelson, M., Alling, A., and Allen, J.P. (2005) Soil and crop management experiments in the Laboratory Biosphere: An analogue system for the Mars on Earth® facility. *Adv Space Res* **35**: 1544–1551.

Singh, B.K., Kuhad, R.C., Singh, A., Lal, R., and Tripathi, K.K. (1999) Biochemical and Molecular Basis of Pesticide Degradation by Microorganisms. *Crit Rev Biotechnol* **19**: 197–225.

Sivasakthi, S., Usharani, G., and Saranraj, Pjaj. (2014) Biocontrol potentiality of plant growth promoting bacteria (PGPR)-Pseudomonas fluorescens and Bacillus subtilis: A review. *Afr J Agric Res* **9**: 1265–1277.

Skov, M.N., Spencer, A.G., Hald, B., Petersen, L., Nauerby, B., Carstensen, B., and Madsen, M. (2004) The role of litter beetles as potential reservoir for *Salmonella enterica* and thermophilic *Campylobacter spp.* between broiler flocks. *Avian Dis* **48**: 9–18.

Smith, R., Hauck, R., Macklin, K., Price, S., Dormitorio, T., and Wang, C. (2022) A review of the lesser mealworm beetle (*Alphitobius diaperinus*) as a reservoir for poultry bacterial pathogens and antimicrobial resistance. *Worlds Poult Sci J* **78**: 197–214.

Soucy, S.M., Huang, J., and Gogarten, J.P. (2015) Horizontal gene transfer: building the web of life. *Nat Rev Genet* **16**: 472–482.

Sparks, T.C. and Nauen, R. (2015) IRAC: Mode of action classification and insecticide resistance management. *Pestic Biochem Physiol* **121**: 122–128.

Steenhoudt, O. and Vanderleyden, J. (2000) Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol Rev* **24**: 487–506.

Stern, J.C., Sutter, B., Freissinet, C., Navarro-González, R., McKay, C.P., Archer, P.D., et al. (2015) Evidence for indigenous nitrogen in sedimentary and aeolian deposits from the Curiosity rover investigations at Gale crater, Mars. *Proc Natl Acad Sci* **112**: 4245–4250.

Strother, K.O., Steelman, C.D., and Gbur, E.E. (2005) Reservoir competence of lesser mealworm (*Coleoptera*: *Tenebrionidae*) for *Campylobacter jejuni* (*Campylobacterales*: *Campylobacteraceae*). *J Med Entomol* **42**: 42–47.

Terrell, C., & Ingwell, L. (2022). Black Soldier Fly Composting Guide. *Purdue University, Extension Entomology*. https://extension.entm.purdue.edu/publications/E-276/E-276.html

Vaughan, J. A. (1982). *Biology and control of the lesser mealworm: Alphitobius diaperinus, a structural pest in poultry houses* (Doctoral dissertation, Virginia Polytechnic Institute and State University).

Vaughan, J.A., Turner, E.C., and Ruszler, P.L. (1984) Infestation and damage of poultry house insulation by the lesser mealworm, *Alphitobius diaperinus* (Panzer)1,2. *Poult Sci* **63**: 1094–1100.

Verma, M., Mishra, J., and Arora, N.K. (2019) Plant Growth-Promoting Rhizobacteria: Diversity and Applications. In *Environmental Biotechnology: For Sustainable Future*. Sobti, R.C., Arora, N.K., and Kothari, R. (eds). Singapore: Springer, pp. 129–173.

Wamelink, G.W.W., Frissel, J.Y., Krijnen, W.H.J., Verwoert, M.R., and Goedhart, P.W. (2014) Can plants grow on mars and the moon: a growth experiment on mars and moon soil simulants. *PLoS ONE* **9**: e103138.

Wang, Y.-S. and Shelomi, M. (2017) Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. *Foods* **6**: 91.

Xu, R., Zhang, K., Liu, P., Han, H., Zhao, S., Kakade, A., et al. (2018) Lignin depolymerization and utilization by bacteria. *Bioresour Technol* **269**: 557–566.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C., and Bourtzis, K. (2004) Wolbachia-induced cytoplasmic incompatibility as a means for insect pest population control. *Proc Natl Acad Sci* **101**: 15042–15045.

Zafeiriadis, S., Baliota, G.V., and Athanassiou, C.G. (2023) The effect of temperature and moisture content on population growth of *Alphitobius diaperinus* (Panzer) (*Coleoptera*: *Tenebrionidae*). *Agronomy* **13**: 2535.

Zhang, D., Lees, R.S., Xi, Z., Gilles, J.R.L., and Bourtzis, K. (2015) Combining the sterile insect technique with *Wolbachia*-based approaches: II- a safer approach to *Aedes albopictus* population suppression programs, designed to minimize the consequences of inadvertent female release. *PLOS ONE* **10**: e0135194.

Zhang, D., Zheng, X., Xi, Z., Bourtzis, K., and Gilles, J.R.L. (2015) Combining the Sterile insect technique with the incompatible insect technique: I-Impact of *Wolbachia* infection on the fitness of triple- and double-infected strains of *Aedes albopictus*. *PLOS ONE* **10**: e0121126.

Chapter 2:

Fast Screening Libraries of Plant Growth Promoting Rhizobacteria (PGPRs) for Insecticidal Activity

---

Abstract

Plant growth-promoting rhizobacteria (PGPR) are bacteria which colonize the rhizosphere. PGPRs are currently being investigated for a variety of applications involving their usage in agriculture, bioinoculants, bioformulations, nanotechnology, fertilizers, and nutrient management systems. Over the years, many PGPR libraries have been assembled. Our study aimed to discover insecticidal bacteria derived from PGPR libraries. We screened more than 500 PGPRs for a killing phenotype against *Drosophila melanogaster* larvae. Strains that killed *D. melanogaster* larva were funneled into an experimental pipeline including multiple tiered bioassays against multiple insect pests including *Drosophila suzukii*, *Spodoptera frugiperda*, *Aphis gossypii,* and *Lygus lineolaris*. We screened 502 endophytic PGPR strains and identified 39 strains with statistically significant mortality. Of those, three strains were lethal to four pest species and seven were lethal to three insect species, primarily consisting of *Serratia marcescens* and *Bacillus velezensis*. Our fast screening methodologies were successful to rapidly screen bacterium for insecticidal activity to identify key strains for further testing with harder to rear pest insects.

Introduction

Plant growth-promoting rhizobacteria (PGPR) colonize roots and the rhizosphere (Kloepper et al. 1980, Ahemad 2014, Lugtenberg 2009). The rhizosphere refers to the area of physical, biological and/or chemical reach of root development (Pinton et al. 2007). PGPRs were termed by Joseph Kloepper who initially discovered *Pseudomonas* strains that protected potatoes from plant pathogens with siderophores (Kloepper et al 1980). PGPRs can produce chemical secretions that promote growth of plants and roots (Kloepper 1980, Kloepper 1989, Ahemed 2014, Kaymak 2011). PGPR metabolites can aid in plant acquisition of key abiotic nutrients such as phosphorus, vital minerals, sulfur, and nitrogen which enhances plant metabolic growth and processes (Ahemed 2014). PGPRs also produce plant hormones and can exclude plant pathogens from the rhizosphere (Saharan 2011).

PGPRs generally come from the following bacterial genera, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Azospirillum*, *Arthrobacter*, *Azotobacter*, *Streptomyces*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, and *Agrobacterium* (Kloepper 1989, Joseph 2012, Okon 2015). These key species have unique properties when exposed to plants, their roots, or the rhizosphere around them. *Pseudomonas* strains for example have been shown to aid in heavy metal uptake while acting as a biofilm with antibiotic resistance (Meliani 2016) and produce hydrolytic enzymes and phytohormones (Panpatte 2016). *Bacillus* has been shown to be abundant in crop soil, aid in crop health by bolstering host plant defenses, while also suppressing pathogenic pests and organisms (Kumar1970, Sivasakthi 2014). *Bacillus* is also involved in phosphate solubilization in plant roots which improves host plant nutrient recruitment (Kashyap 2019). *Burkholderia* isolates have been shown to produce auxin (a key plant hormone for growth and elongation of roots) and chitinases (encoding the ability to degrade insect chitin); in addition, they colonize roots and solubilize

phosphorus (Shaharoona 2007, Aroumougame 2020). Another study, Zehnder et al. 1997, demonstrated PGPR-induced resistance against spotted cucumber beetle, *Diabrotica undecimpunctata howardi* (Barber), and the striped cucumber beetle, *Acalymma vittatum* (Fabricius). In this case, the PGPR was deemed more effective than insecticides for controlling cucumber beetles and associated cucurbit wilt disease on cucumber (Zehnder et al. 1997).

In relation to PGPR insecticides, there are currently no commercial PGPR insecticides excluding *Bacillus thuringiensis*. No studies have directly checked PGPR active insecticidal potential through direct feeding. Although reports of reductions in insect herbivory, chitinase potential, and plant defense bolstering metabolites are published (Coy et al 2019, Mathur 2019, Regaiolo 2020). Specifically, Coy et al. 2019 did assess mortality of white grubs who fed on PGPR treated Bermuda grass, no significant change in grub mortality or morbidity was recorded.

Our research aimed to develop a rapid screening protocol for insecticidal potential of PGPR strains. We screened strains from a PGPR library at Auburn University collected by Dr. Kloepper and his team. The PGPR library contains over ~6,800 PGPR strains collected from all over the United States and other regions. The library contains over 40 years of collected identified bacteria strains, location data, as well as plant/soil annotations. We tested 502 endophyte PGPRs against *Drosophila melanogaster* then selected key crop pests such as *Drosophila suzukii* (spotted wing *Drosophila*) [Matsumura], *Spodoptera frugiperda* (fall army worm (FAW)) [Smith], *Aphis gossypii* (cotton aphids) [Glover], and *Lygus lineolaris* (tarnished plant bug) [Palisot de Beauvois] for additional testing against insecticidal PGPRs. We specifically screened endophytes for this study due to their attribute of residing within certain plants, which in natural application theory would allow them to be up taken through consumption by insect herbivores. We created a screening

pipeline which first tested the insecticidal potential of PGPRs against *Drosophilia melanogaster*, a model insect organism, for survivorship after exposure and contact with the PGPR. If the mortality threshold was reached by a PGPR strain, it was retested with higher (*n*) to rule out false positives. Strains which showed initial mortality were also subjected to further testing utilizing the insect crop pests above. After experiments, the killing potential of all valuable bacteria were tabulated. Notably, some strains killed pests from diverse insect orders.

Materials and methods

PGPR Strain Acquisition

PGPR strains are maintained as frozen isolates at -80ºC in Auburn University's PGPR Library. The library contains glycerol stocks of 8 collections of PGPR strains totaling over 6,800 individual samples. The JM endophyte collection was procured from this library and used in this study. Collections are annotated and contain detailed data on location and notable traits. To initiate studies, PGPR strains were streaked out on Luria broth (LB) (1L: yeast extract 5g, tryptone 10g, NaCl 5g, dH_2O to 1 L, autoclave: Tomko) plates and clones were picked to inoculate LB liquid cultures.

Fly food and Fly Rearing, D. melanogaster & D. suzukii

Glucose Fly Food, Archon Scientific, (Durham, NC) was used to rear flies in the lab in 10 mL food tubes. Both *D. melanogaster* and *D. suzukii* are reared at ~23°C. The *D. melanogaster* strain is *white* Canton-S. Fly tubes are passed biweekly and maintained. *D. suzukii* flies are maintained under similar conditions with a folded paper to assist pupation.

Drosophila melanogaster Screening (Tier 1)

D. melanogaster Tier 1 (T1) screening is a bioassay measuring if a strain is a killing phenotype of fly larvae. PGPR culture strains were obtained from strain library noted above. Bacterial strains were picked and grown in liquid tryptic soy broth (TSB) media for 72 hrs at 37°C to promote growth and allow for any secondary metabolite production. To collect fly eggs, apple juice media is used (1 Liter: 100% apple juice 405mL, Dextrose 52.2g, Sucrose 26.06g, Agar 19.8g, 1.25M NaOH 24mL, dH2O 484mL). Once mixed, apple juice media is autoclaved then poured into inverted 2 oz (59.15 mL) souffle cups. Yeast paste, Fleischmann's ActiveDry Yeast, is added to the plate to sexually invigorate the adult fruit flies resulting in higher egg fecundity. Fruit flies (50-100) are transferred to apple juice plates. After 24 hrs, fruit flies are anesthetized with CO_2 and the apple juice plate lid swapped to initiate a fresh egg clutch. Saturated 72-hr TSB bacterial cultures are pipetted (50 µl) onto triplicate fresh apple juice plate lids and spread via sterile glass rod. Then ten *D. melanogaster* eggs (24 hrs after the plate swap) are placed onto the freshly inoculated apple juice lid by paintbrush to initiate the experiment which lasts for 96 hrs (four days) (**Fig. 1**). After four days, living larvae are counted under microscope. Internal controls were included in every trial. The negative control groups are plates where eggs are not exposed to bacteria and plates exposed to non-entomopathogenic *Top10F' E.coli*. Experiments where the internal control's survivorship was less than 7.6 were triaged to enforce screen quality. Strains which yielded a replicate mean survival of less than 6 larvae were moved into the next tiers of screening. For simple experimental timeline, see ***Table 1.*** To eliminate false positives, a secondary confirmation screen, *D. melanogaster* Tier 2 (T2) screening, was performed which followed the same procedure and increased the biological replicates by 10.

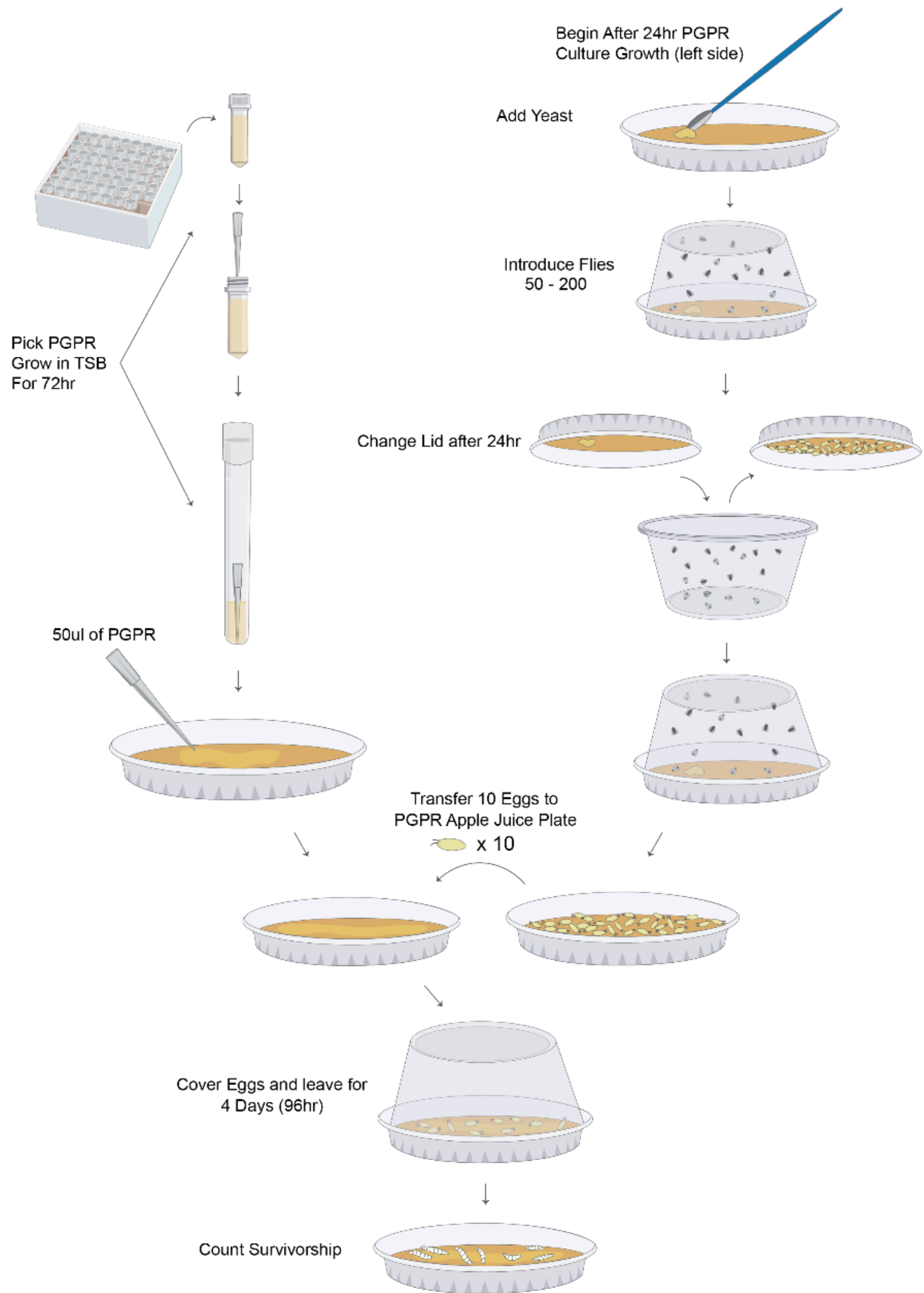

Begin After 24hr PGPR
Culture Growth (left side)
Add Yeast
Introduce Flies
50 - 200
Pick PGPR
Grow in TSB
For 72hr
Change Lid after 24hr
50ul of PGPR
Transfer 10 Eggs to
PGPR Apple Juice Plate
x 10
Cover Eggs and leave for
4 Days (96hr)
Count Survivorship

Fig 1. Tier 1 *Drosophila melanogaster* Screening. Visual representations of T1 screening process for *D. melanogaster*. The experiment involves a seven day window. Bacterial cultures are inoculated from frozen glycerol stocks. Bacteria are grown for 72 hours and spread on apple juice plates. Ten fruit fly eggs are transplanted to the plate. Plates are incubated for four days at 23°C. After the experiment, survivors are counted.

Table 1. Tier 1 *Drosophila melanogaster* Screening Timeline. Displays T1 procedure throughout 7-day preparation and experimentation cycle.

Tier 1 *Drosophila melanogaster* Screening Timeline

Procedure	Day (*t*)	Time
Culture PGPR in Liquid TSB from glycerol stock	1	0 hr Day 0
Set Flies on apple juice plate lid with yeast	2	24 hr Day 1
Change apple juice plate lid	3	48 hr Day 2
Apply 50 µl PGPR culture to new apple juice lid and transfer 10 fly eggs per strain per plate (3 reps per strain)	4	72 hr Day 3
After 4 day (96 hr) exposure period count survivorship on PGPR egg plate	8	168 hr Day 7

Drosophila Suzukii (Tier 3) Pest Screening

Tier 3 (T3) pest screening was initiated for strains that killed more than four *D. melanogaster* larvae in the T2 experiments. This screen utilized the same procedure with the exception of a modified egg transfer protocol. *D. suzukii* femles inserted eggs in the agar as opposed to on top

the agar. As a result, screening is more intensive as each individual egg must be gently extracted out of the apple juice agar media with either a firm paintbrush or small scoop prior to being transferred to the strain inoculated apple juice plate.

Spodoptera frugiperda (FAW)Tier 2 *Screens*

Strains that killed more than four larvae in the *D. melanogaster* T1 screen were subsequently tested against FAW. First and 3rd instar FAW were obtained from Benzon Research (Carlisle, PA). Starter cultures were inoculated as described above (*D. melanogaster* Screening (Tier 1)). Experiments were conducted in souffle cups (as above). Cups were filled halfway with artificial diet purchased from Southland Products Inc, (Lake Village, AK). PGPR solutions are prepared by inoculating a culture of TSB using a glycerol stock of the desired strain and incubating the culture for 72 hrs at 37°C as described in (*Drosophila melanogaster* Screening (Tier 1)). A souffle cup is filled halfway (volume) with the artificial diet and inoculated with 250 µl of desired PGPR strain. After inoculation, ten 1st or 3rd instar FAW were placed inside the cup and covered with perforated lid for air. Rearing trays were then placed in a growth chamber at 27°C with a 14-hr photoperiod. The experiment involved 3 inoculated plates per strain tested with 10 individuals per plate. Plates were checked after a 5-day period after which survivorship was recorded.

Aphis gossypii Tier 2 Screens

Aphids (*Aphis gossypii*) were originally collected in Tallassee, AL in 2019. For the duration of these experiments, aphid colonies were maintained in a plant growth chamber. The chamber is set to a consistent temperature range of 25-27°C with approximately 65% humidity, under a light-dark cycle of 16:8 hours. Cotton seedlings are nurtured to emergence, which typically takes about one week. Subsequently, 1-2 wingless adult aphids are transferred onto individual true leaves of

these seedlings using a fine artist's paintbrush. The colonies are enclosed in mesh tents and are systematically replaced after about three weeks or upon substantial production of alate aphids.

Bacteria strains were grown as above in (*D. melanogaster* Screening (Tier 1)). For screening, E.coli strain TOP10F' serves as a non-lethal negative control. Survival quality threshold set at 80%. Screening involves the careful uprooting of entire cotton plants to access the aphids. The plants are then segmented to facilitate aphid collection. Using a fine artist's paintbrush, wingless adults are transferred into pre-labeled 1.5ml Eppendorf tubes, which are initially sealed with lids and subsequently replaced with a tightly stretched 2.54 x 2.54 cm square of parafilm (**Fig. 2**). Each tube receives 10 µl of either the test solution or control on top of the parafilm, which is then covered with another layer of parafilm to secure the solution. The aphids are incubated for 48 hours at room temperature (20-22°C) under the standard 16/8 light-dark cycle. The outcome is determined by examining each aphid under a microscope to note survival, recording the results as binary data.

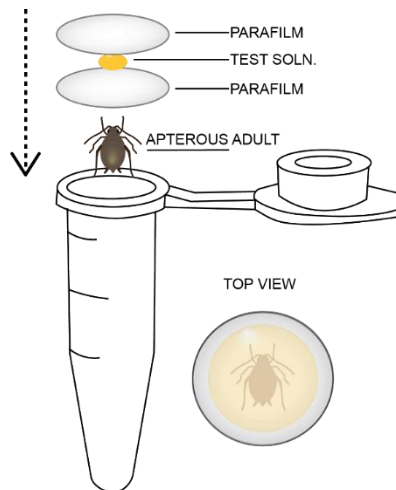
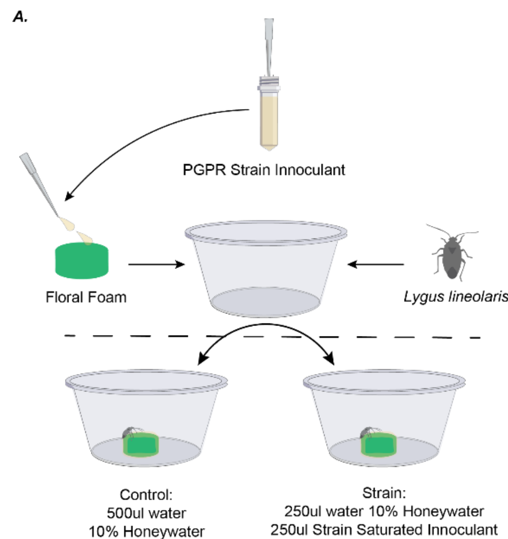


Fig 2. Tier 2 *Aphis gossypii* Screens set up. Wingless adult aphids are transferred into a 1.5 mL Eppendorf tubes, which are initially sealed with lids and subsequently replaced with a tightly stretched 2.54 cm x 2.54 cm square of parafilm.

Lygus lineolaris (*L. lineolaris*) *Tier 2* Screens

L. lineolaris were temporarily available for screening against T2 strains. Plant bugs were collected from *Ergeron annuus* (daisy fleabane) at field sites around Auburn University and transported to the lab and tested following PGPR 72 hr growth (*D. melanogaster* Screening (Tier 1)). *L lineolaris* were screened in souffle cups (as above) with cylindrical 1 x 1cm cutouts of floral foam (OASIS Maxlife) with either control or PGPR inoculant. Floral foam acts to absorb the liquid nutrient for insect stylet piercing and feeding. One tarnished plant bug was used per container with a minimum of 10 replicates (one individual per container) per PGPR strain. Plates were counted for survival after 7 days. Any plates with fungal growth were discarded. Control floral foam cutouts received 500 µl of 10% honey water solution which was directly applied and absorbed by the floral foam. Experimental replicates received 250 µl of 10% honey water and 250 µl of 72 hr grown T2 PGPR strain (**Fig. 3**).

Fig 3. Tier 2 *Lygus lineolaris* Screens. Individual Lygus plant bug is added to a souffle cup with



a 1 x 1 cm floral foam cutout inoculated with either 500 µl 10% honey water (negative control) or 250 µl of 10% honey water and 250 µl bacterial culture in TSB.

Key Strain Sequencing and Identification

The JM library contains strains identified via the following 16s rDNA Primers, 8F -5'- AGA GTT TGA TCC TGG CTC AG - 3', 907R - 5'- CCG TCA ATT CCT TTG AGT TT -3' (Only needed when samples are sequenced), and 1492R - 5'- ACG GCT ACC TTG TTA CGA CTT -3'. For confirmation and future studies, we also followed the PGPR screening results with full genome sequencing of key JM strains which were grown and sent to SEQCENTER (Microbial Genome Sequencing Center, LLC, Pittsburgh, PA). Samples were DNA extracted and sequenced via 650 mb Illumina Sequencing. Once the fastq sequences arrived back, sequence alignment and genome construction were conducted on bv-brc.org following BVBRC (Bacterial and Viral Bioinformatics Resource Center) genome assembly service protocol and service. Following genome assembly, NCBI BLAST was used to perform alignment similarity queries for strain identification. Up to 6 genome sections per strain were queried in NCBI BLAST for strain identification with high identity species names recorded. Some of the key strains also underwent quality assessments of raw reads, both pre- and post-trimming/filtering (by BBduk) was performed by fastqc. De-novo assembly was completed using SPAdes in metagenomic mode. The assembly's quality was scrutinized using BUSCO, followed by the extraction of 5S, 16S, and 23S rRNA sequences with BarrNAP. These sequences were then compared against the RefSeq RNA database using BLAST, with ANI calculations performed to confirm the species identification further. The results were further scrutinized by KRAKEN2, which taxonomically identifies each read. Finally, BV-BRC's similar genome tool was used to further confirm the BLAST, ANI, and KRAKEN results.

Statistics

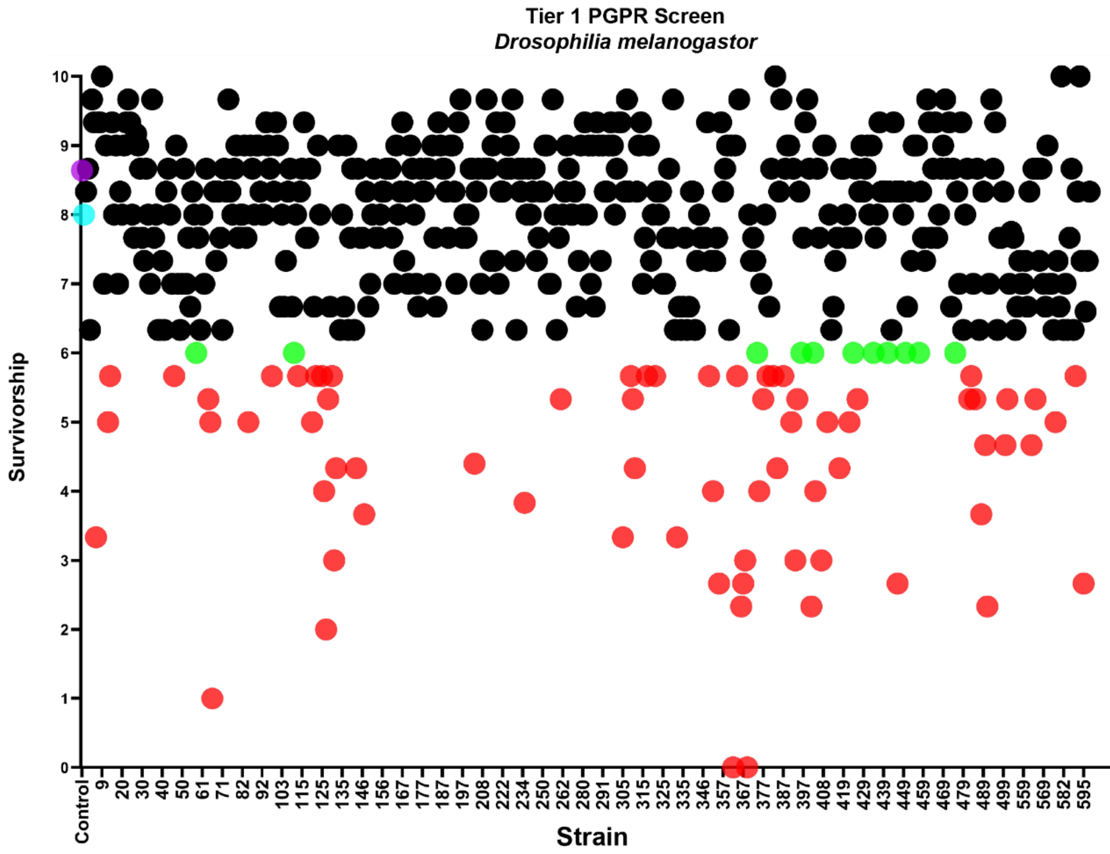
D. melanogaster T1 screening, *D. suzukii,* and FAW screening were analyzed via ordinary Dunnett's multiple comparison AVOVA test due to smaller replicate sizes and wider variance. *D.*

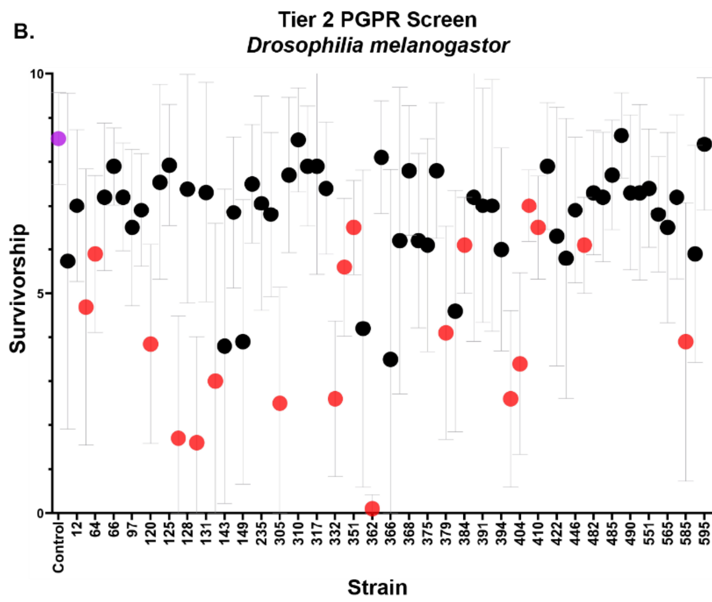
melanogaster T2 screening was analyzed via Dunnett's T3 multiple comparisons ANOVA test due to larger replicate sizes supporting the test's reliability. Lygus and aphids were analyzed via Fishers Exact test due to the binary nature of the individual replicates and binary outcome. Statistics were analyzed using GraphPad Prism 9.3.1.

Results

Tier 1 Drosophila melanogaster Screens

In the initial *D. melanogaster* screen 502 endophytic PGPR from the JM collection were screened via this methodology (**Fig. 4a.**). The mean survivorship for negative control plates lacking bacteria (empty) was 86% while the other negative control with non-pathogenic *Top10F'* bacteria was 80%. These values provide a base comparison for which to determine insecticidal activity of strains. 26 out of 502 PGPR strains were statistically significant when compared against the empty negative control. Thus ~5% of all JM strains tested showed significant insecticidal activity. As a result, the threshold for continued screening of any strain was greater than 40% mortality. Of note, strain 362 (*Serratia marcescens*) and 369 (*Rhizobium wenxiniae*) yielded 100% mortality across 3 initial replicates. Seventy strains met the threshold for continued T2 screening (**Fig. 4b).**


A.
Tier 1 PGPR Screen
Drosophilia melanogastor
Survivorship
0
1
2
3
4
5
6
7
8
9
10
Control
9
20
30
40
50
61
71
82
92
103
115
125
135
146
156
167
177
187
197
208
222
234
250
262
280
291
305
315
325
335
346
357
367
377
387
397
408
419
429
439
449
459
469
479
489
499
559
569
582
595
Strain


B.
Tier 2 PGPR Screen
Drosophilia melanogastor
Survivorship
0
5
10
Control
12
64
66
97
120
125
128
131
143
149
235
305
310
317
332
351
362
366
368
375
379
384
391
394
404
410
422
446
482
485
490
551
565
585
595
Strain

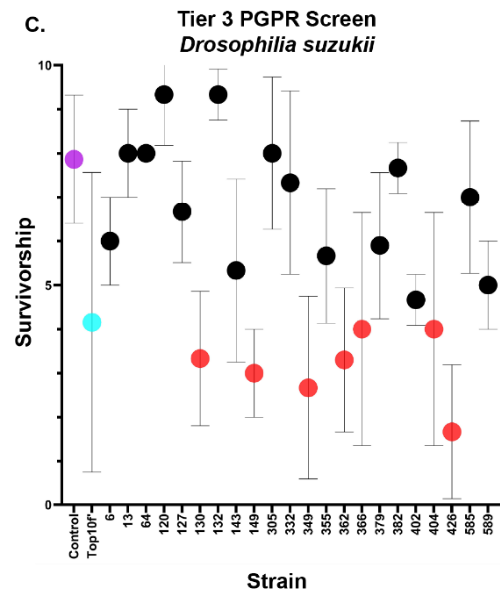

C.
Tier 3 PGPR Screen
Drosophilia suzukii
Survivorship
0
5
10
Control
Top10f
6
13
64
120
127
130
132
143
149
305
332
349
355
362
366
379
382
402
404
426
585
589
Strain

Fig 4. Tier 1 and Tier 2 *Drosophila melanogaster* Screening followed by Tier 2 *Drosophila suzukii* screening. **a.** Screen of 502 endophytic PGPR strains from the JM collection. Dots represent mean survivorship of 3 replicates. Data points are as follows: negative control lacking bacteria (purple dot), negative control with non-pathogenic *Top10F' E. coli* (blue dot), strains at the threshold with mean survivorship values equal to 6 (green dots), strains meeting the threshold, with mean survivorship values less than 6 (70 red dots). **b.** Seventy JM strains showing insecticidal phenotypes were re-screened with a higher (*n*) of 10 replicates per strain in Tier 2 screening (red dots show significance). Of the 70, 19 strains replicated insecticidal phenotypes consistently with statistical significance when compared against the empty negative control. **c.** Seven final strains from Tier 2 were screened against *D. suzukii* and showed significant insecticidal killing (red dots show significance).

Tier 2 Drosophila melanogaster Screens

One flaw of screening methodology is false positives. To rule out false positives we re-screened strains with higher replicates in a T2 screen. Replicates for 70 strains were increased to 10. T2 screening revealed 19 strains were significant when compared to the negative empty control. Fourteen strains had greater than 40% mortality. Of note, JM362 had 99% mortality. Eight strains showed significant insecticidal activity in both T1 and T2. Eleven strains were not significant in T1 but became significant with higher replicates (*n*) in T2; which suggests that utilizing arduous statistics with low (*n*), as a threshold, can result in loss of valuable strains early in a screen. Overall, in *D. melanogaster*, T1 had a predictive true discovery rate of insecticidal strains of 1.6% of samples processed, a ~69% false positive rate (meaning a hit in T1 but invalidated in T2), and a 25% false negative rate (meaning a miss in T1, but confirmation in T2). In toto – combining T1

with T2 reveals a true discovery rate of insecticidal strains at 3.8%. This justifies the multiple tiered screening methodology.

Tier 2 Drosophila suzukii Screens

We subjected positive T2 strains to testing against an agricultural pest, *D. suzukii*. Our hypothesis was that strains with significant mortality against *D. melanogaster* would show significant mortality against *D. suzukii*. Of the 22 strains tested, 7 were significant against *D. suzukii* (red dots in **Fig. 4c**). These results show that libraries of bacteria can be screened for insecticidal activity against a fast model (like *D. melanogaster*), prior to the actual pest screening. This validates the rapid high throughput methodology.

Tier 2 Aphis gossypii, Spodoptera frugiperda, & Lygus lineolaris Screens

Following successful congeneric screening against a fast model and a pest, our hypothesis was these strains might also show inter-order insecticidal activity against other pests. Thus, we subjected the 70 T2 bacteria to continued testing against *A. gossypii*, FAW, and *L. lineolaris*. Of the T2 strains tested against *A. gossypii*, 13 were statistically significant killers (**Fig. 6**). Of the PGPR strains tested against *L. lineolaris*, all 13 had significant mortality when compared to the empty control, with 8 showing 100% mortality (**Fig. 7**). Of the 70 T2 strains tested against *S. frugiperda*, 13 killed significantly more after 5 days compared to the control (**Fig. 5b**). In *S. frugiperda* screens, control values are low as a result of cannibalism (Chapman 1999 Sokame 2023, Mbuji 2022). Despite this, 13 strains showed significant mortality in the 3rd instar screening. One reason for significance among 3rd instar and none in 1st instar screening could be strain concentration consumption variance given the differences in head and body size between 1st and 3rd instars (Odeyemi 2021). For pests other than *Drosophila*, the screening identified 29 out of 70,

~41%, of T2 strains as causing significant mortality against at least one agricultural pest. Three strains had insecticidal activity against 4 pests, 7 had activity against 3 pests, and 5 had activity against 2 pests (**Table 2**). This data shows that our screening methodology uncovers important inter-order insecticidal PGPRs as shown in **Table 2**.

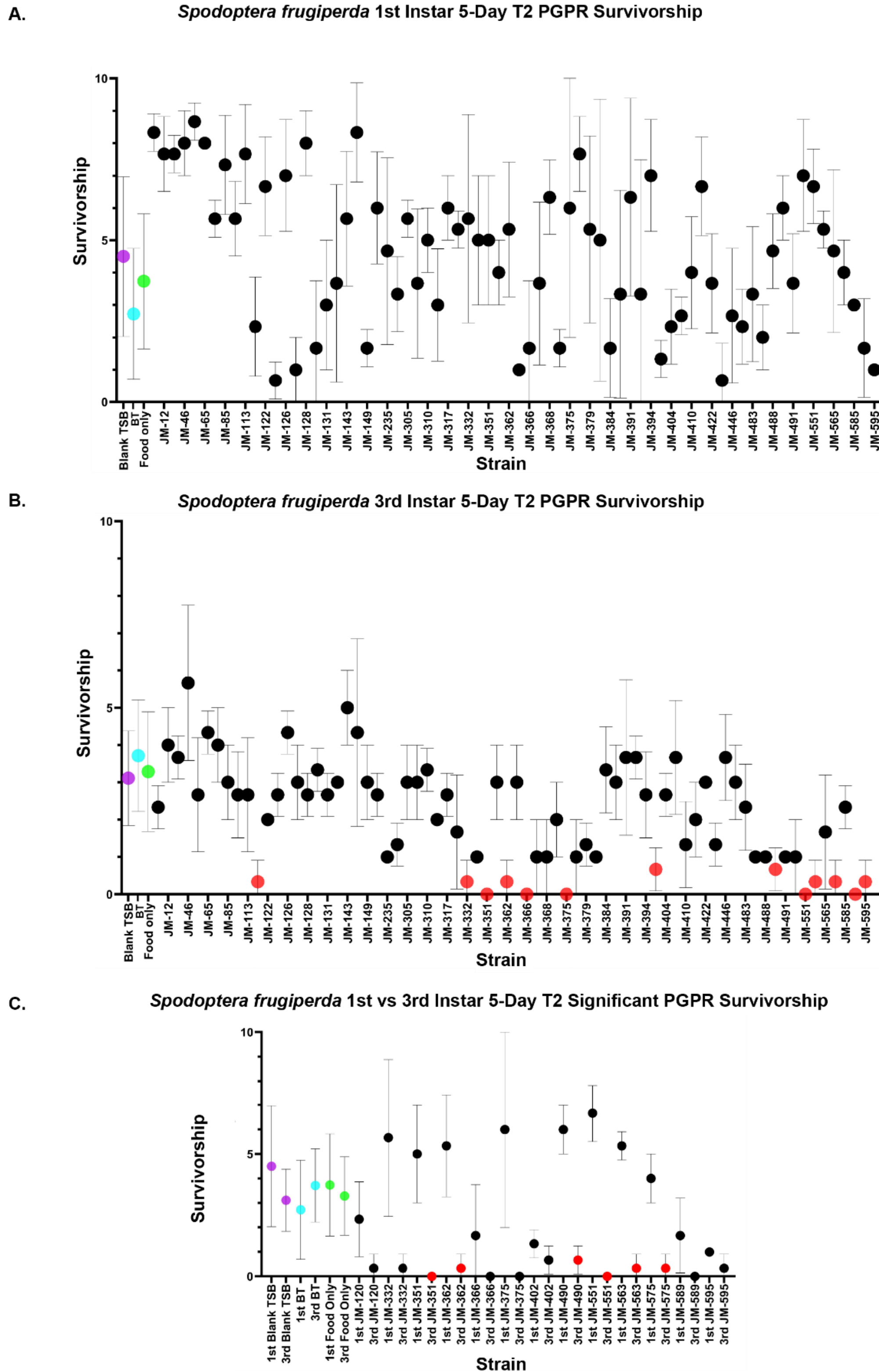


Figure 5. Tier 2 *Spodoptera frugiperda* Screening **a.** T2 PGPR strains screened against *Spodoptera frugiperda* 1st instar 5-day survivorship. (Purple dot) = Negative control Blank TSB, (Blue dot) =

Positive control *Bacillus thuringiensis*, (Green dot) = Negative control Food only, significant hits in red. **b.** T2 PGPR strains screened against *Spodoptera frugiperda* 3rd instar 5-day survivorship. 13 T2 strains showed significant mortality compared to the control due to cannibalism. **c.** Comparison results for 1st and 3rd instar larvae.

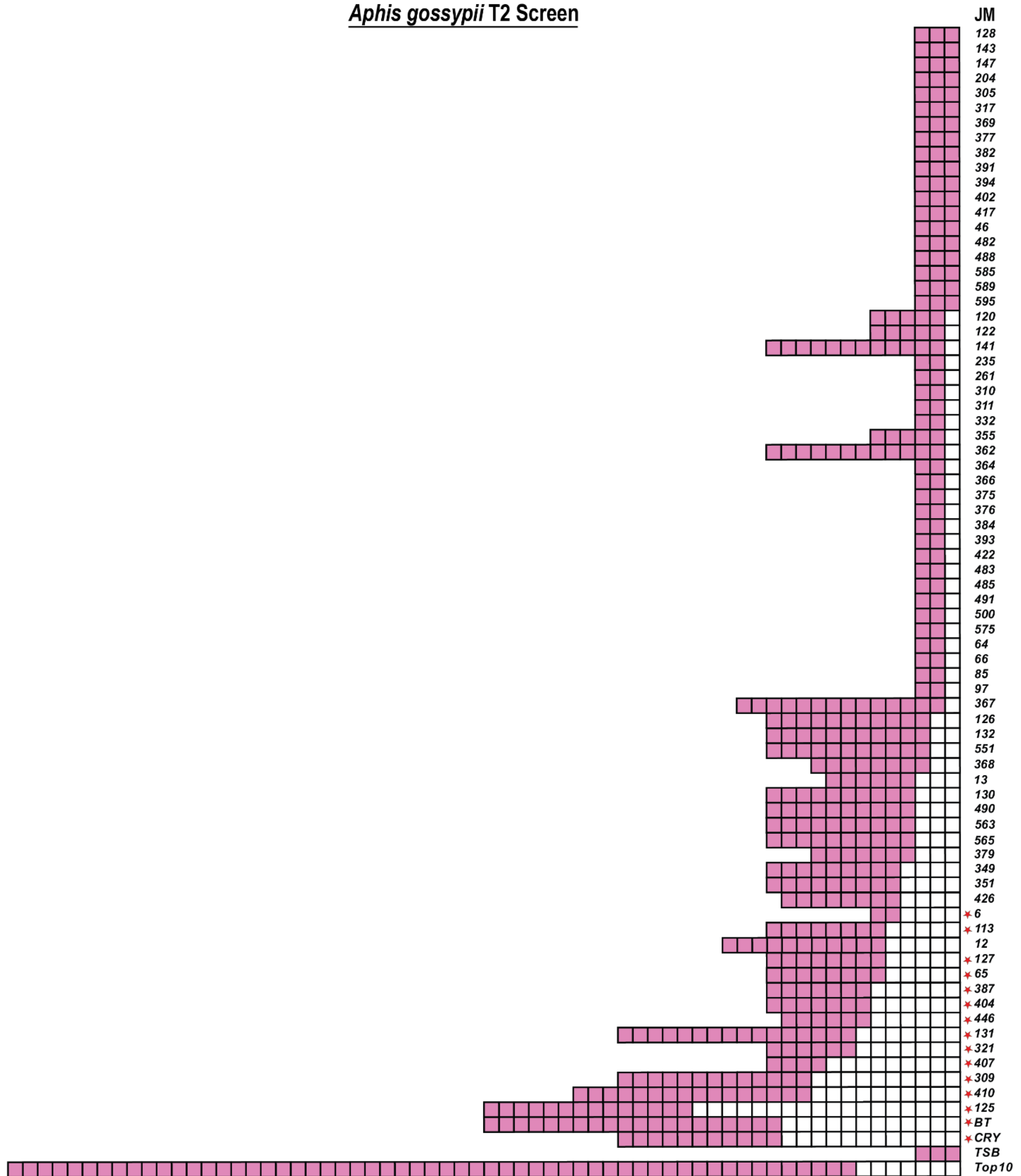


Fig. 6. Tier 2 *Aphis gossypii* Screening. The 69 PGPR JM strains screened against Aphis gossipii. Top10f', TSB, BT, and CRY controls are shown as well. 13 of the 73 PGPR strains yield significance. Each box signifies 1 Aphid. A white box indicated a dead aphid while a pink box

depicts a live aphid post screening. The red star symbol next to the strain number indicated statistical significance from the Top10f' control.

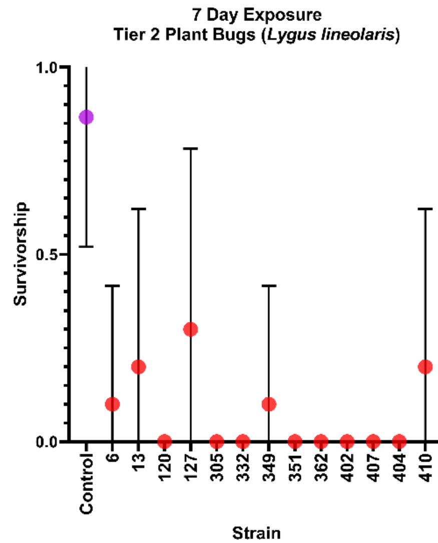


Figure 7. Tier 2 *L. lineolaris* Screening. *L. lineolaris* were field collected, limiting the number of tests. **a.** A subset of 13 killing specimens from Tier 2 *D. melanogaster* screens were chosen for screening against *L. lineolaris.* All strains tested resulted in significant mortality compared to the honey water control (red dots) and 8 strains had 100% mortality within the 7 day experimental timeline.

Table 2. PGPR Significant Strains **a.** Displays all strains of significance as well as their mortality across specified pest species (*D. mel, D. suzukii, Aphis gossipii, Lygus lineolaris*) including species ID. Light red highlight indicated the highest mortality strain for the given insect pest. **b.** Displays strains which possessed significant insect killing across 4 pest species tested. **c.** Displays strains which possessed significant insect killing across 3 pest species tested. **d.** Displays strains which possessed significant insect killing across 2 pest species tested.

A.

Total Species Killing PGPR Mortality %						
Strain #	***D. mel***	***D. Suzukii***	***A. gossipii***	***L. lineolaris***	***S. frugiperda***	***Identification***
JM 6			67	90		*Microbacterium hydrothermale*
JM 13	53			80		*Rhizobium wenxiniae*
JM 46	39					*Ralstonia pickettii*
JM 64	41					*Paenibacillus hispanicus*
JM 65			38			*Bacillus amyloliquefaciens*
JM 113			38			*Microbacterium arborescens*
JM 120	61			100	97	*Bacillus velezensis, Bacillus sp.*
JM 125			56			*Bacillus velezensis*
JM 127	83		38	70		*Staphylococcus epidermidis, Bacillus velezensis*
JM 130	84	66				*Bacillus cereus, Bacillus thuringiensis, Bacillus proteolyticus*
JM 131			30			*Rhizobium nepotum*
JM 132	70					*Bacillus velezensis, Rhizobium pusense*
JM 143						*Burkholderia cenocepacia, Bacillus velezensis*
JM 149		70				*Bacillus velezensi, Burkholderia sp.*
JM 305	75			100		*Bacillus velezensis, Staphylococcus sp.*
JM 309			43			*Pseudomonas baetica*
JM 321			54			*Serratia marcescens sakuensis*
JM 332	74			100	97	*Serratia marcescens, Bacillus velezensis, Burkholderia sp., Bacillus sp.*
JM 349	44	73		90		*Serratia marcescens sakuensis*
JM 351	35			100	100	*Serratia marcescens sakuensis*
JM 362	99	67		100	97	*Serratia marcescens*
JM 366		60			100	*Bosea robiniae*
JM 375					100	*Bacillus safensis*
JM 379	59					*Bacillus velezensis*
JM 384	39					*Leclercia adecarboxylata*
JM 387			46			*Kosakonia pseudosacchari*
JM 402	74	53		100	93	*Enterobacter ludwigii, Bacillus velezensis, Serratia marcescens, Staphylococcus sp*
JM 404	66	60	46	100		*Bacillus velezensis*
JM 407	30		69	100		*Proteus morganii*
JM 410	35		38	80		*Bacillus velezensis*
JM 426		83				*Serratia marcescens sakuensis*
JM 446			50			*Enterobacter asburiae*
JM 490					93	*Pantoea vagans*
JM 551					100	*Rhizobium freirei/multihospitium*
JM 563					97	*Tardiphaga robiniae*
JM 575					97	*Rhizobium freirei/multihospitium*
JM 585	61					*Staphylococcus epidermidis*
JM 589					100	*Bosea thiooxidans*
JM 595					97	*Enterobacter asburiae*

B.

Quadruple Species Killing PGPR Mortality %						
Strain #	***D. mel***	***D. Suzukii***	***A. gossipii***	***L. lineolaris***	***S. frugiperda***	***Identification***
JM 362	99	67		100	96.7	*Serratia marcescens*
JM 402	74	53		100	93.3	*Enterobacter ludwigii, Bacillus velezensis, Serratia marcescens, Staphylococcus sp.*
JM 404	66	60	46	100		*Bacillus velezensis*

C.

Tripple Species Killing PGPR Mortality %						
Strain #	***D. mel***	***D. Suzukii***	***A. gossipii***	***L. lineolaris***	***S. frugiperda***	***Identification***
JM 120	61			100	96.7	*Bacillus velezensis, Bacillus sp.*
JM 127	83		38	70		*Staphylococcus epidermidis, Bacillus velezensis*
JM 332	74			100	96.7	*Serratia marcescens, Bacillus velezensis, Burkholderia sp., Bacillus sp.*
JM 349	44	73		90		*Serratia marcescens sakuensis*
JM 351	35			100	100	*Serratia marcescens sakuensis*
JM 407	30		69	100		*Proteus morganii*
JM 410	35		36	80		*Bacillus velezensis*

D.

Double Species Killing PGPR Mortality %						
Strain #	***D. mel***	***D. Suzukii***	***A. gossipii***	***L. lineolaris***	***S. frugiperda***	***Identification***
JM 6			66	90		*Microbacterium hydrothermale*
JM 13	53			80		*Rhizobium wenxiniae*
JM 130	84	66				*Bacillus cereus, Bacillus thuringiensis, Bacillus proteolyticus*
JM 305	75			100		*Bacillus velezensis & Staphylococcus sp*
JM 366		60			100	*Bosea robiniae*

Identification of Key PGPR Insecticidal Strains

Following the PGPR screening results (**Table 2**), DNA was extracted from key valuable insecticidal PGPR strains and sequenced via 650mb Illumina Sequencing at SeqCenter Microbial Genome Sequencing Center, LLC. Genomes were constructed and aligned (see Materials and Methods). For other less valuable strains, we relied on prior research identifications annotated in the Auburn PGPR Library; these data derived from 16s rDNA PCR amplicon sequencing. From these data, bacterial genus and species identifications were assigned (**Table 2**, last column). Of the identified strains, the quadruple and triple PGPR killers are primarily constituted of *Serratia marcescens* and *Bacillus velezensis* with instances of *Staphylococcus epidermidis*, *Enterobacter ludwigii*, and *Proteus morganii*.

Discussion

Uncovering Inter-Order Insecticidal PGPR Strains Using Rapid D. melanogaster Screening.

The most prolific and ubiquitous PGPR is *Bacillus thuringiensis (Bt)*, which has well described insecticidal properties and has become useful and important in biotechnology (Sansinenea 2012, Prieto-Samsonov et. al 1997, Jouzani 2017). Our goal was to discover if there were other PGPR strains with insecticidal phenotypes, like *Bt*, which existed in The Auburn University PGPR Library. The search for novel bioinsecticides provides alternatives to synthetic insecticides. Our study focused on exploiting an endophytic PGPR library for this purpose. We chose an endophyte library because these bacteria reside within plant tissue and might be consumed by pest insect herbivores. This provides a natural pathway for production and delivery of insecticidal metabolites

should an endophytic PGPR strain be identified. Our study also aimed to develop a rapid screening methodology. Our screening methodology fits within a 7-day time window and is capable of simultaneous processing of sets of ~50 bacterial strains. Our methodology can be extended to other insects, in addition to *Drosophila*. The limiting factor on screen speed is usually the insect rearing. To validate the methodology herein, we screened 502 endophytic strains. This is a small starter portion of the total library. The screening methodology is an unbiased approach, which complements more targeted PGPR studies of the past (Kaymak 2011, Tariq 2014, Zhang 2002). Our T1 screening utilized the *D. melanogaster* model insect because it is cheap, easy to rear, short generation time, with ease of handling (Hales 2015, Huang 2023).

Of the 502 endophytic bacteria screened, 70 strains showed greater than 40% mortality against *D. melanogaster*. The 70 T1 strains were re-tested with higher (*n*) of 10 replicates with 10 individuals per plate. In final, T2 *D. melanogaster* screening yielded a ~4% confirmation rate of insecticidal activity. T2 selections became the primary strains tested against intra and inter-order pest species. Having tiered screening systems, such as ours, has advantages and disadvantages. Screening *D. mel* first provided an initial "weed out" for insecticidal strains of interest. Disadvantages to this methodology include false-negatives and false positives. For example, a strain that kills Lepidopterans may not be discovered in a T1 Dipteran screen. While these outcomes are possible, the speed benefits of the T1 *D. melanogaster* screening methodologies outweigh the cons. About half of the successful strains showed general insecticidal activity against multiple insect orders, evidencing against the above criticism. We have identified insecticidal strains against: *D. melanogaster*, *D. suzukii*, *S. frugiperda*, *A. gossypii,* and *L. lineolaris*. The ability to take a massive library of bacteria and rapidly assess potential insecticidal activity in a fast manner. We screened 500 by hand with limited personnel but note that the methodology could

be upscaled to screen thousands and tens of thousands of microbes. Importantly, our methods are easily adaptable to other insect pests and might also be applied to diverse microbial libraries (not just PGPRs).

Key Strains Reproduce Prior Annotations and Validate the Screening Methodology

Serratia marcescens and *Bacillus velezensis* were lethal to multiple species, identified as either quadruple or triple species strain killers. In addition, we detected traces of *Staphylococcus epidermidis*, *Enterobacter ludwigii*, and *Proteus morganii*. *Serratia marcescens* (*S. marcescens*) is a rod-shaped Gram-negative bacterium belonging to the family *Enterobacteriaceae*. It is commonly found in the environment, including water, soil, and plants, and is often considered as an opportunistic pathogen (Grimont 2006, Hejazi & Falkiner 1997). Validating our findings, *S. marcescens* has been described in the 1950s as being pathogenic to grasshoppers (Stevenson 1954, Stevenson 1959a/b, Bucher 1959, Zelazny 1997). *S. marcescens* caused the infection of a disease outbreak in a colony of desert locus with internal tissues below the abdominal integument becoming discolored, viscous, and soft (Stevenson 1954, Stevenson 1959a). Mortality caused by *S. marcescens* was seen rapidly within 24 hours with a majority of mortality occurring 2 weeks after locus consumption. Later Bucher found *S. marcescens* could cause infection upon high concentration consumption (Bucher 1959). Bucher detailed *S. marcescens* as being capable of spreading through the hemocoel of insects creating a systemic infection leading to death (Bucher 1960). *S. marcescens* is also heralded for its ability as an endophytic PGPR able to produce siderophores, chitolytic enzymes, antibiotic and antimicrobial substances, as well as produce systemic resistance in plants (McInroy & Kloepper 1994, Ordentlich 1988, Kalbe 1996, Khan 1977). As a note, strain diversity in bacteria can be quite high, meaning that a strain of S. marcescens from Alabama can encode many genetic differences when compared to another strain

from Colorado. While our findings reproduce prior annotations, they still represent unique strain collections.

Bacillus velezensis (*B. velenzensis*) was the other prominent PGPR killer amongst the quadruple and triple species killing strains. Unlike *S. marcescens, B. velezensis*, is a gram positive, endospore forming PGPR. *B. velezensis* is known to promote plant growth, produce biosynthesis promoting metabolites, as well as aiding in systemic plant resistance (Rabbee 2019, Ye 2018, Meng 2016). *B. velezensis* has been shown to reduce the density of *Meloidogyne incognita* (Kofoid & White, 1919), a plant-parasitic nematode, while also enhancing the growth of cotton (Xiang et. al 2017). In addition to root inoculation, seedlings can be inoculated for beneficial growth effects and show the same effect as nitrogen fertilizers as seen with strawberries. *B. velezensis* inoculated roots have increased plant defenses against Brown Planthopper, *Nilaparvata lugens* [Stål] (Harun-Or-Rashid 2018). Similarly to *S. marcescens, B. velezensis*, has also been known to produce hydrolytic chitinases and proteases which have shown effectiveness against subterranean termites, *Reticulitermes speratus kyushuensis* [Morimoto] as well as a dipteran pest, *Dasineura jujubifolia* [Jiao and Bu] (Moon 2023, Choi et al. 2023, Ajuna 2023). Future work might include continued exploration of endophytic or non-endophytic bacterial libraries, introduction and expansion of other novel pest species, continued strain efficacy tests, field tests for viability within a natural or agricultural setting, or studies exploring the causative gene/s creating insecticidal activity. Genetic comparisons against similar species, lacking insecticidal phenotypes will be key to identify the molecular mechanisms.

In conclusion, the rapid screening methodology of 502 endophytic PGPR strains from Auburn University's library unveiled the significant insecticidal potential of 39 strains. Recall that ~4% of strains were active against *D. melanogaster*, however the total number of active strains against all

the five species tested was actually double this number at ~8%. Thus 8% of bacteria tested had at least some insecticidal value. The screening methodology's efficiency in identifying potent strains is evident. Some of these strains will likely be useful in field applications, but this will need to be tested. Ongoing investigations could yield environmentally friendly pest management solutions rooted in naturally occurring soil bacteria. Finally, the endophytes derived from this library were all cultivated from the same Cotton and Corn field plot in Alabama, evidencing the utility of local bacterial collections. In total, 39 strains of bacteria demonstrated insecticidal activity against one or more insect pest species. As society progresses in a more environmentally conscious direction, we are constantly searching for alternatives to synthetic insecticides. It's possible that local soil bacteria under our feet can provide these solutions.

References

Adoko MY, Noumavo AD, Agbodjato NA, Amogou O, Salami HA, Aguégué RM, Adjovi Ahoyo N, Adjanohoun A, Baba-Moussa L (2022) Effect of the application or coating of PGPR-based biostimulant on the growth, yield and nutritional status of maize in Benin. *Front Plant Sci 13*, 1064710.

Ahemad M, Kibret M (2014) Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *J King Saud Univ* - Sci **26**:1–20

Ajuna HB, Lim HI, Moon JH, Won SJ, Choub V, Choi SI, Ahn YS (2023) The prospect of hydrolytic enzymes from *Bacillus* species in the biological control of pests and diseases in forest and fruit tree production. *Int J Mol Sci* **24**:16889

AR N, Joseph B, Jose A, Krishnankutty R (2020) Nanotechnological advances with PGPR applications. *Sustainable Agriculture Reviews 41: Nanotechnology for Plant Growth and Development*, 163-180.

Arora N, Khare E, Maheshwari D (1970) Plant growth promoting rhizobacteria: constraints in bioformulation, commercialization, and future strategies. *Plant growth and health promoting bacteria*, 97-116.

Aroumougame S, Geetha TM, Thangaraju M (2020) Exploitation of PGPR endophytic *Burkholderia* isolates to enhance organic agriculture. *Am J BioSci* **8**:57–64

Bariş Ö, Sahin F, Turan M, Orhan F, Gulluce (2014) Use of plant-growth-promoting rhizobacteria (PGPR) seed inoculation as alternative fertilizer inputs in wheat and barley production. *Commun Soil Sci Plant Anal* **45**:2457–2467

Bharathi R, Vivekananthan R, Harish S, Ramanathan A, Samiyappan R (2004) Rhizobacteria based bio-formulations for the management of fruit rot infection in chillies. *Crop Prot* **23**:835–843

Bucher GE (1959) Bacterium of grasshoppers of western Canada: III. Frequency of occurrence, pathogenicity. *J Insect Pathol* **1**:391–405

Bucher GE (1960) Potential bacterial pathogens of insects and their characteristics. *J Insect Pathol* **2**:172–195

Chapman JW, Williams T, Escribano A, Caballero P, Cave RD, Goulson D (1999) Fitness consequences of cannibalism in the fall armyworm, *Spodoptera frugiperda*. *Behav Ecol* **10**:298–303

Chebotar VK, Chizhevskaya EP, Vorobyov NI, Bobkova VV, Pomyaksheva LV, Khomyakov YV, Konovalov SN (2022) The quality and productivity of strawberry (*Fragaria*× *ananassa* Duch.) improved by the inoculation of PGPR *Bacillus velezensis* BS89 in field experiments. *Agronomy* **12**:2600

Choi SI, Ajuna HB, Won SJ, Choub V, Kim CW, Moon JH, Ahn YS (2023) The insecticidal potential of *Bacillus velezensis* CE 100 against *Dasineura jujubifolia* Jiao & Bu (*Diptera*: *Cecidomyiidae*) larvae infestation and its role in the enhancement of yield and fruit quality of jujube (*Zizyphus jujuba* Miller var. inermis Rehder). *Crop Prot* **163**:106098

Coy RM, Held DW, Kloepper JW (2019) Rhizobacterial treatments of tall fescue and bermudagrass increases tolerance to damage from white grubs. *Pest Manag Sci* **75**:3210–3217

El Zemrany H, Czarnes S, Hallett PD, Alamercery S, Bally R, Jocteur Monrozier L (2007) Early changes in root characteristics of maize (*Zea mays*) following seed inoculation with the PGPR *Azospirillum lipoferum* CRT1. *Plant Soil* ***291***:109–118

Gholami A, Shahsavani S, Nezarat S (2009) The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *International Journal of Agricultural and Biosystems Engineering,* **3**:1, 9-14.

Grimont F, Grimont PA (2006) The genus *Serratia*. In The Prokaryotes 219–244. *Springer*

Grobelak A, Napora A, Kacprzak M (2015) Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. *Ecol Eng* **84**:22–28

Hales KG, Korey CA, Larracuente AM, Roberts DM (2015) Genetics on the fly: a primer on the *Drosophila* model system. *Genetics* **201**:815–842

Harun-Or-Rashid M, Kim HJ, Yeom SI, Yu HA, Manir MM, Moon SS, Chung YR (2018) *Bacillus velezensis* YC7010 enhances plant defenses against brown planthopper through transcriptomic and metabolic changes in rice. *Front Plant Sci* ***9***:1904

Hejazi A, Falkiner FR (1997) *Serratia marcescens*. *J Med Microbiol* **46**:903–912

Huang J, Lee Y (2023) The power of *Drosophila* genetics in studying insect toxicology and chemical ecology. *Crop Health* **1**:12

Joseph B, Ranjan Patra R, Lawrence R (2012) Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *Int J Plant Prod* **1**:141–152

Jouzani GS, Valijanian E, Sharafi R (2017) *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. *Appl Microbiol Biotechnol* **101**:2691–2711

Kalbe C, Marten P, Berg G (1996) Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. *Microbiol Res* **151**:433–439

Kalita M, Bharadwaz M, Dey T, Gogoi K, Dowarah P, Unni BG, Ozah D, Saikia I (2015) Developing novel bacterial based bioformulation having PGPR properties for enhanced production of agricultural crops. *Indian J Exp Biol* **53**:56–60

Kashyap B, Joseph B, Jose A, Krishnankutty R (2019) *Bacillus* as plant growth promoting rhizobacteria (PGPR): a promising green agriculture technology. *Plant health under biotic stress: volume 2: microbial interactions*, 219-236.

Kaymak H (2011) Potential of PGPR in agricultural innovations. *Plant growth and health promoting bacteria*, 45-79.

Khan AR, Park GS, Asaf S, Hong SJ, Jung BK, Shin JH (2017) Complete genome analysis of *Serratia marcescens* RSC-14: a plant growth-promoting bacterium that alleviates cadmium stress in host plants. *PloS one*, *12*(2), e0171534.

Kloepper JW, Leong J, Teintze M, Schroth MN (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* **286**:885–886

Kloepper JW, Lifshitz R, Zablotowicz RM (1989) Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnol* **7**:39–44

Kumar A, Prakash A, Johri B (1970) *Bacillus* as PGPR in crop ecosystem. In *Bacteria in Agrobiology: Crop Ecosystems* 37–59. doi:10.1007/978-3-642-18357-7_2

Kumari B, Mallick MA, Solanki MK, Solanki AC, Hora A, Guo W. (2019) Plant growth promoting rhizobacteria (PGPR): modern prospects for sustainable agriculture. *Plant Health Under Biotic Stress: Volume 2: Microbial Interactions*, 109-127.

Latha P, Theerthagiri A, Ragupathi N, Prakasam V, Ramasamy S (2009) Antimicrobial activity of plant extracts and induction of systemic resistance in tomato plants by mixture of PGPR strains and Zimmu leaf extract against *Alternaria solani*. *Biol Control* **50**:85–93

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* **63**:541–556

Mahmood A, Turgay OC, Farooq M, Hayat R (2016) Seed biopriming with plant growth promoting rhizobacteria: a review. FEMS *Microbiol Ecol* 92.8, fiw112

Mathur A, Koul A, Hattewar J (2019) Plant growth-promoting rhizobacteria (PGPRs): significant revolutionary tools for achieving long-term sustainability and combating the biotic stress caused by the attack of pathogens affecting crops in agriculture. *Plant Growth Promoting Rhizobacteria for Sustainable Stress Management: Volume 2: Rhizobacteria in Biotic Stress Management*, 379-388.

Mbuji AL, Gong X, Li F, Yang L, Shi X, Peng Z, Wu S (2022) A study on the cannibalism behaviour and predation of the larvae of fall armyworm (*Spodoptera frugiperda*). *Entomol Res* **52**:251–258

McInroy JA, Kloepper JW (1994) Studies on indigenous endophytic bacteria of sweet corn and cotton. *Mol Ecol Rhizosphere Microorg: Biotechnol Release GMOs* 19–28

Meliani A, Bensoltane A (2016) Biofilm-mediated heavy metals bioremediation in PGPR *Pseudomonas*. *J Bioremediat Biodegrad* **7**:370

Meng Q, Jiang H, Hao JJ (2016) Effects of *Bacillus velezensis* strain BAC03 in promoting plant growth. *Biol Control* **98**:18–26

Moon JH, Ajuna HB, Won SJ, Choub V, Choi SI, Yun JY, Ahn YS (2023) Entomopathogenic potential of *Bacillus velezensis* CE 100 for the biological control of termite damage in wooden architectural buildings of Korean cultural heritage. *Int J Mol Sci* **24**:8189

Odeyemi OO, Alamu OT, Yekinni GA, Oloyede-Kamiyo QO (2021) Development and determination of larval stages of fall armyworm on some staple crops. *Afr Crop Sci J* **29**:445–457

Okon Y, Labandera-Gonzales C, Lage M, Lage P (2015) Agronomic applications of *Azospirillum* and other PGPR. *Biological nitrogen fixation* 925–936. doi:10.1002/9781119053095.ch90

Ordentlich A, Elad Y, Chet I (1988) The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* **78**:84–87

Panpatte D, Jhala Y, Shelat H, Vyas RV (2016) *Pseudomonas fluorescens*: a promising biocontrol agent and PGPR for sustainable agriculture. *Microbial inoculants in sustainable agricultural productivity: Vol. 1: Research perspectives*, 257-270.

Pinton, R, Varanini, Z, Nannipieri, P (2007). *The Rhizosphere: biochemistry and organic substances at the soil-plant interface*. CRC Press.

Prieto-Samsonov DL, Vazquez-Padron RI, Ayra-Pardo C, Gonzalez-Cabrera J, De la Riva GA (1997) *Bacillus thuringiensis*: from biodiversity to biotechnology. *J Ind Microbiol Biotechnol* **19**:202–219

Rabbee MF, Ali MS, Choi J, Hwang BS, Jeong SC, Baek KH (2019) *Bacillus velezensis*: a valuable member of bioactive molecules within plant microbiomes. *Molecules* **24**:1046

Regaiolo A, Dominelli N, Andresen K, Heermann R (2020) The biocontrol agent and insect pathogen *Photorhabdus luminescens* interacts with plant roots. *Applied and Environmental Microbiology*, *86*(17), e00891-20.

Saharan B, Nehra V (2011) Plant growth promoting rhizobacteria: a critical review. *Life Sci Med Res* **21**:1–30

Sansinenea E (2012) *Bacillus thuringiensis* biotechnology. Netherlands: *Springer*

Saravanakumar D, Vijayakumar C, Kumar N, Samiyappan R (2007) PGPR-induced defense responses in the tea plant against blister blight disease. *Crop Prot* **26**:556–565

Shaharoona B, Jamro G, Zahir Z, Arshad M, Memon K (2007) Effectiveness of various *Pseudomonas* spp. and *Burkholderia caryophylli* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.). *J Microbiol Biotechnol* **17**:1300–1307

Sivasakthi S, Usharani G, Saranraj P (2014) Biocontrol potentiality of plant growth promoting bacteria (PGPR) - *Pseudomonas fluorescens* and *Bacillus subtilis*: A review. Air *J Agric Res* **9**:1265–1277

Sokame BM, Musyoka B, Mohammed SA, Tamiru A, Bruce A, Anderson P, Calatayud PA (2023) Cannibalism and intraguild predation involved in the intra- and inter-specific interactions of the invasive fall armyworm, *Spodoptera frugiperda*, and lepidopteran maize stemborers. *J Pest Sci* **96**:1455–1464

Stevenson JP (1954) An epizootic among laboratory stocks of the desert locust, *Schistocerca gregaria* Forsk. *Nature* **174**:222

Stevenson JP (1959a) An infection of the desert locust, *Schistocerca gregaria* Forskal, with a nonchromogenic strain of *Serratia marcescens* Bizio. *J Insect Pathol* **1**:129–141

Stevenson JP (1959b) Epizootiology of a disease of the desert locust, *Schistocerca gregaria* (Forskal), caused by nonchromogenic strains of *Serratia marcescens* Bizio. *J Insect Pathol* **1**:232–241

Tariq M, Ali Q, Khan A, Khan GA, Rashid B, Rahi MS, Husnain T (2014) Yield potential study of *Capsicum annuum* L. under the application of PGPR. *Adv Life Sci* **1**:202–207

Verma M, Mishra J, Arora N (2019) Plant growth-promoting rhizobacteria: diversity and applications. *Environmental biotechnology: for sustainable future*, 129-173.

Xiang N, Lawrence KS, Kloepper JW, Donald PA, McInroy JA, Lawrence GW (2017) Biological control of *Meloidogyne incognita* by spore-forming plant growth-promoting rhizobacteria on cotton. *Plant Dis* **101**:774–784

Ye M, Tang X, Yang R, Zhang H, Li F, Tao F, Wang Z (2018) Characteristics and application of a novel species of *Bacillus*: *Bacillus velezensis*. *ACS Chem Biol* **13**:500–505

Zelazny B, Goettel MS, Keller B (1997) The potential of bacteria for the microbial control of grasshoppers and locusts. Mem Entomol Soc Can 129:S171:147–156. doi:10.4039/entm129171147-1

Zhang S, Reddy MS, Kloepper JW (2002) Development of assays for assessing induced systemic resistance by plant growth-promoting rhizobacteria against blue mold of tobacco. *Biol Control* **23**:79–86

Chapter 3:

Transforming Martian Regolith with Black Soldier Flies, *Hermetia illucens*

Abstract

The sustainability of long-term Martian colonization hinges on the ability to produce food from the soil of the planet. Current space missions rely on pre-packaged food, a model unsuitable for extended missions to Mars due to mass, supply, and logistical constraints. This study explores the feasibility of Black Soldier Flies, *Hermetia illucens* (*Diptera*: *Stratiomyidae*):as a soil fertility enhancer via composting organic matter. We designed a 3D printed, compact BSF composter to process Martian soil simulant into arable soil. Additionally, we subjected BSF larvae to G-force testing and demonstrated high survivability under rocket launch conditions and centrifugal forces of up to 40 G's. Composting experiments involved introducing BSF to Martian soil simulant and an organic food source (mung beans) over a one-month composting duration. Soil analyses showed significant nutrient increases, notably in the plant-availability of potassium (+1179%), magnesium (+67%), phosphorus (+803%), and manganese (+7847%), enhancing the soil's suitability for crop cultivation. These findings underscore the resilience and potential of BSF for extraterrestrial agriculture. This study lays the groundwork for BSF-based composting systems as a sustainable solution for soil fertility management and nutritional supplementation on Mars, fostering future research on astro-entomology and in-situ resource utilization.

Introduction

Yuri Alekseyevich Gagarin was the first human to venture into Space, and on his mission, he brought pureed beef and liver paste (Uri, 2020). He demonstrated eating and swallowing is possible in zero-g environments, despite the food coming from an aluminium tube

(Uri, 2020). Subsequently, the 2-week-long Gemini 7 mission required more care to be taken with regard to dietary and caloric requirements, while also remaining in an extremely tight mass envelope (Reid et al., 1968; Johnston et al., 1975). This envelope only allowed 0.77kg of food per astronaut per day (Johnston et al., 1975). Because of this the food taken ended up consisting almost exclusively of dehydrated food that most closely resembled military survival rations. This was acceptable for the Gemini missions, as their primary purpose was to ensure man could survive for an extended duration in a space environment. This envelope; however, was determined to be inadequate for the Apollo missions because it was too small to maintain astronaut weight and there were undesirable physiological and psychological responses to consuming dehydrated food for two weeks straight (Johnston et al., 1975). Though the Apollo program introduced significant improvements to the astronaut's culinary experience, including the introduction of cheese, sausage, and scrambled eggs, the majority of astronauts did not consume sufficient nutrients (Johnston et al., 1975). Astronauts aboard the ISS today have much-improved meals, even having specialty food items such as sushi, burgers, turkey, and pizza (Jiang et al., 2020; Uri, 2020). Despite this, fresh fruits and vegetables are always in demand as they have limited shelf lives. The ISS is restocked multiple times a year, which allows for the large and varied dietary menu that astronauts enjoy (Smith et al., 2005). The ISS can be restocked so often because of its proximity to Earth and the relative ease and low cost of resupply missions. This will not be the case with future Lunar and Martian colonization efforts (Linck et al., 2019).

The large distances and high cost of Lunar and Martian missions mean resupplies must be kept to a minimum. This is coupled with the need to reduce mass wherever possible due to the larger Delta-V requirements for these missions (Linck et al., 2019). Extraterrestrial colonization

efforts must be as self-sufficient as possible to reduce the need for constant resupplies, and any supplies taken must be lightweight to fit into strict mass envelopes (Douglas et al., 2020). This means the pre-packaged sustenance model used for all previous missions is no longer acceptable in the new age of space exploration. The ISS food envelope is too large, and the Gemini envelope is both physically and psychologically unsustainable (Oluwafemi et al., 2018). For future colonization efforts to be a success, astronauts must be capable of growing and producing their own food on whatever celestial body they aim to inhabit (Perchonok et al., 2012; Oluwafemi et al., 2018; Douglas et al., 2021).

The need for agriculture in space has been recognized by NASA, and they have begun the VEGGIE food production system to experiment with growing vegetables aboard the ISS. The project has successfully demonstrated that leafy greens, such as kale and mustard, can be grown and consumed aboard the ISS (Bunchek et al., 2021). While these experiments pave the way for growing crops beyond Earth, they alone are insufficient to begin sustainable agriculture on foreign bodies. The VEGGIE program grows its crops in soil taken from Earth and uses artificial fertilizers to feed the produce (Bunchek et al., 2021). The use of artificial fertilizers itself is not a bad thing, but if used alone they represent an increase in mass that needs to be transported for the colonization effort. Additionally, it currently takes an average of roughly 5 Hectare, or 50,000m^2 of arable land to feed 100 men (Connor and Mínguez, 2012). It is unreasonable to expect to take this amount of soil to the Lunar Surface, let alone Mars. This means that astronauts will need a way to convert Lunar and Martian regolith into farmable soil similar to Earth. We believe the fertilization and the soil conversion problems may be alleviated with the usage of Black Soldier Flies, *Hermetia illucens* [Linnaeus].

Black Soldier Flies (BSF) are acclaimed for their ability to compost organic matter

(Kumar et al., 2018; Liu et al., 2019; da Silva and Hesselberg, 2020; Klammsteiner et al., 2020; Anyega et al., 2021). Byproduct of BSF compost can be used as an organic fertilizer that reduces or eliminates the need for chemical fertilizers. Composting also significantly reduces the health concerns associated with direct application of manure or biological waste (Liu et al., 2019). We believe that a lightweight, transportable BSF soil composting system, could be taken to space or celestial bodies to assist with the fertilization of arable soil and convert extraterrestrial regolith into farmland bit by bit. Additionally, regarding astronaut nutrition, BSF can be consumed for additional protein and basic nutrients, especially considering their larvae can yield above 40% protein and ~30% fat (Wang and Shelomi, 2017; Bessa et al., 2020). Black Solider Flies have amino acid profiles comparable with common meats such as chicken and could be used as a more feasible alternative to bringing livestock to the Martian surface (Bessa et al., 2020). Our goal was to compost Martian soil simulant through the usage of 3-D printed BSF composters to increase the nutrient contents of the simulant. This initial system aims to add to the sustainability outlook of future colonization efforts directed towards Mars.

Methods & Materials

Experimentation with BSF comprised primarily two arms of research: the BSF's endurance under high-G stress and BSF ability to effectively compost extraterrestrial regolith within a one-month duration. The high-G testing consisted of several different experiments using centrifuges to induce equivalent conditions to different launches as well as a real-flight Tripoli Level 1 rocket test.

Black Soldier Fly Colony

BSF colonies were purchased from a commercial insectary (Symtom Inc.) G-force experiments utilized 5th instar and prepupal BSF measuring ~10 mm in size. BSF were purchased

and allowed to grow naturally in initial container until approximate 5th instar utilization. Composting experiments utilized 1st/2nd instar larvae ~5 mm in size. Small larvae ~5 mm in size were selected upon arrival for utilization. Composters were introduced to 300 larvae each with 100 g of beansprouts (LEASA) initially and 100 g more bean sprouts on the beginning of the 2nd week of the experiment.

G-Force Centrifuge Assessment & Tripoli Level 1 Rocket Test

The high-G testing consisted of several different experiments using a centrifuge to induce extraneous G conditions. A Heraeus Multifuge X1R (ThermoFisher Scientific) centrifuge was used for BSF head-up and head-down g-force tests. Centrifuge G-force testing involved placing individual BSF 5th instar larvae ~10mm and pre-pupa BSF ~15mL in 1.5 mL conical tubes and spun at either 10, 25, or 40 G for a 5-minute spin duration followed by a 10-minute survivability check (mortality). Ten individuals per g-force with three replicates were assessed.

The linear G-force assessment utilized a Tripoli Level 1 rocket test. Specifications for the rocket include an AeroTech H283 ammonium perchlorate-based rocket motor with a burn time of 0.7 seconds. The motor produces a peak thrust of 325 N, an average thrust of 283 N, and a total impulse of 201 N*s. The airframe tubes of the rocket were constructed from 0.889 mm-thick cardboard tubing, the fins were manufactured using 3.127-mm-thick plywood, and the nosecone was 3D printed using PETG filament. The nosecone was printed with a thickness of 6.35 mm, 100% infill in the shoulder and tip, 25% infill in the rest of the nosecone, and 3 outer walls. The launch was conducted at the South East Alabama Rocketry Society (SEARS) launch site in Samson, Alabama. The parachute used had a diameter of 0.762 m and was constructed from ripstop nylon.

Contained within the payload section, eight biological replicates for head-down and nine

biological replicates for head-up were used each oriented in their own individual holding 1.5 mL angled tubes. Additionally, three replicates of five larvae were combined in 2 mL tubes. The BSF tubes were secured in a specially 3d-printed and designed payload module called the Biological Unit for Gravitational Study (BUGS). It consists of four sections, can hold twenty-four individual 2 mL centrifuge tubes, as well as the Altus Metrum - EasyMini computer. The four sections of BUGS are the Battery Container, the Sample and Electronics Enclosure, the Upper Sample Enclosure, and the Sample Enclosure Cap, and each was 3D printed using PLA filament. The battery container housed the 9V battery used to power the EasyMini, and the Sample and Electronics Enclosure contained 12, 2 mL tube slots and the EasyMini computer. Above that, the Upper Sample Enclosure housed an additional 12, 2 mL tube slots, and the Sample Enclosure Cap retained these tubes. This system was secured using a ¼”-20 steel threaded rod through the center of each of the four sections. For the test flight, BUGS was placed within the payload tube of the Tripoli Level 1 rocket **Fig 2A**. The Altus Metrum EasyMini computer took real flight data during the launch through landing. Due to size limitations of the level 1 rocket and payload size constraints, eight biological replicates for head-down and nine biological replicates for head-up were used each oriented in their own individual holding 1.5 mL angled tubes.

Composter CAD Design and Function

The BSF composter consists of several compartment drawers contained by a main chassis **Fig 1**. Access from one compartment to another is restricted by several slides that can be inserted or removed when it is desirable for access to be denied or granted. The BSF larvae begin in the compost tray, which contains soil and beansprouts to sustain the BSF through their larval stage **Fig 1A**. The rear of the compost tray contains a ramp which the larva will climb up and out of the compost tray once they have reached older instar stages and the slots are pulled open **Fig 1E**.

The tray also contains a removable bottom, which can be pulled out to drop the contents of the composter tray into the tray below it for extra storage **Fig 1C**.

Once the larvae have developed to an appropriate stage (prepupa), they will move up the ramp at the rear of the compost tray and into one of two slot channels. Access to these channels is restricted by slides that may be inserted or removed if desired to limit the larvae from progressing until they have sufficiently developed. The larger drop section leads directly to the larval collection tray **Fig 1D**, which contains no soil or nutrients and from which the larvae cannot escape, to be held until they are collected for harvest or set for re-use with new soil. The smaller slot leads to the 'recycling track' in which the larvae will remain in the composter and develop into adults as the angular, funnel shaped bottom is designed to make escape difficult. Following pupation, the winged adults can fly upward into the top breeding chamber where they may reproduce allowing for continued colony propagation within the composter. Once an adult has reached the adult breeding chamber, the design allows mating and the laying of eggs in one of the egg laying cartridges fitted inside the breeding compartment. These cartridges contain many small holes designed for the flies to deposit eggs into and may be removed for ease of cleaning or to be replaced with a cartridge of a different design if desired. The bottom of the breeding compartment is perforated with a number of holes ~2 mm for a new larva to fall through but small enough to restrict an adult from fall through. The design is such that new larvae may exit the breeding compartment and fall though into the composting compartment, where they may begin the cycle again. Access to the composting compartment through these holes is controlled by a slide contained by the chassis underneath the breeding compartment. The breeding compartment is covered by another slide, which may be made out of a clear material in order to provide a view into the compartment for inspection and also allow light to enter the

breeding chamber. This aids in manual visualization of adults within the top bug breeder compartment.

Fused Deposition Modeling 3-D printing (FDM) was chosen as the method of manufacture for the composters. This provided a low cost, ease of manufacturing, rapid prototyping and iteration, and allowed the creation of complex internal geometries not possible with other manufacturing methods. The multiple internal channels and passageways in the main chassis of the composter would have been unreasonably difficult to machine in a single frame using traditional subtractive manufacturing. The large chambers on the front top and bottom rear edges of the otherwise cubic chassis are so that it may be printed at a 45° angle. This minimized the amount of bridging and support material that needed to be generated by the printer, reducing production time and improving build quality.

Polyethylene terephthalate glycol (PETG) filament was chosen as the material for the composter for its low cost, wide availability, ease of printing and resistance to degradation. Compared to other common 3-D printing filaments, namely polylactic acid (PLA), PETG exhibits much greater resistance to degradation from both moisture and UV light. This was critical as the composter will contain moist soil and bio-matter, and it is desirable for it to be able to be operated in direct sunlight. In addition, PETG is more heat resistant, and would be less

likely to deform if exposed to high temperatures and sunlight for extended periods of time.

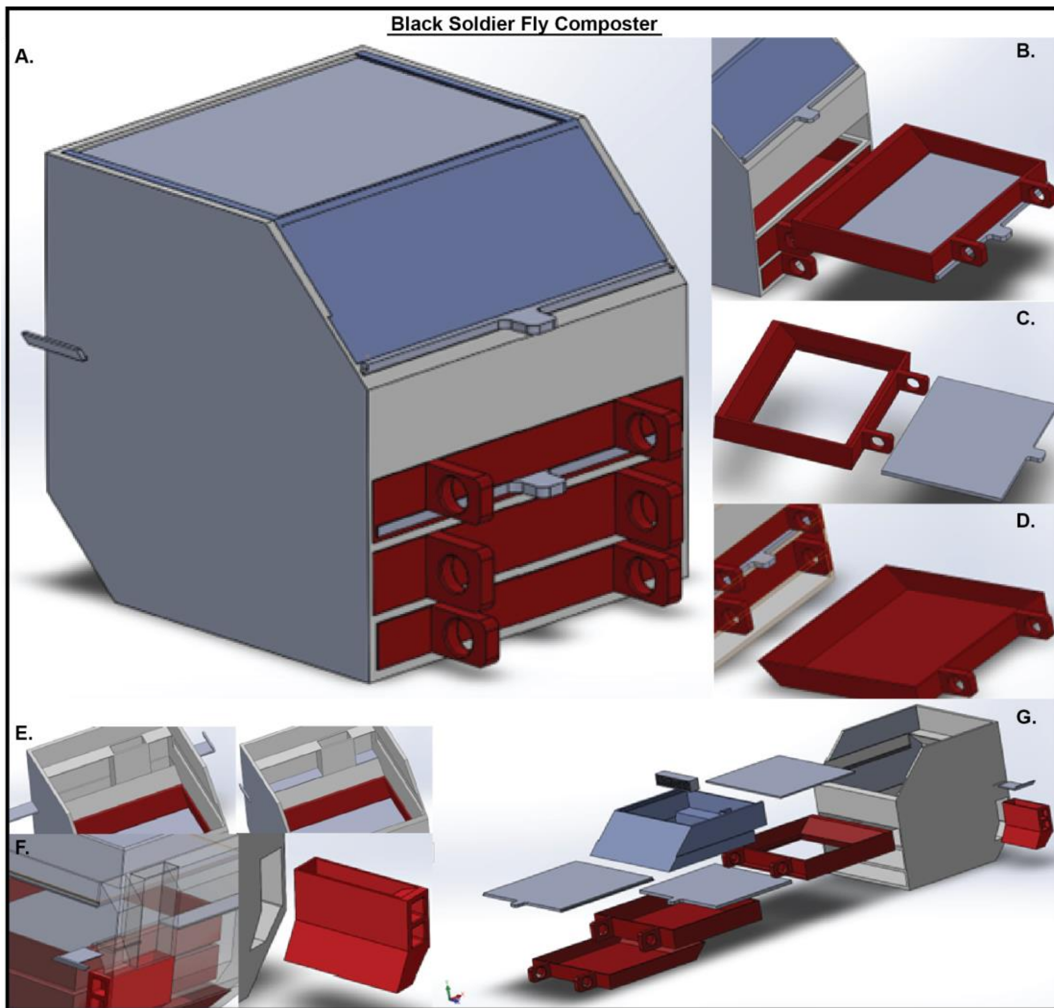


Fig 1. Black Soldier Fly Composter. This figure depicts the SolidWorks design for the BSF composter. **A.** Enclosed total cube-sat composter. **B.** Primary soil draw for soil, BSF larvae, and food source (bean sprouts). **C.** Removable slot to allow soil to drop down to drawer 2 for soil storage. **D.** This is the bottom pupa collection drawer where larvae fall to pupate following slot opening. **E.** Inside view of composter where two closable slots lead to either bottom pupa collection draw (left), or recycling track. **F.** Visual of recycling track internally and removed. This compartment traps larvae with inverted funnel design preventing escape until pupation where adult flies can fly up to top section. **G.** Visualization of opened composter with top of composter (breeding chamber) visible. The breeding chamber is the angular top compartment

which houses adult flies for breeding and egg deposition into rectangular egg deposition cartridges. The top of the breeding chamber is outfitted with clear PETG lid for visualization of adult flies. Twelve identical composters were produced with these specifications for the composting experiments.

Composting & Soil Data Acquisition

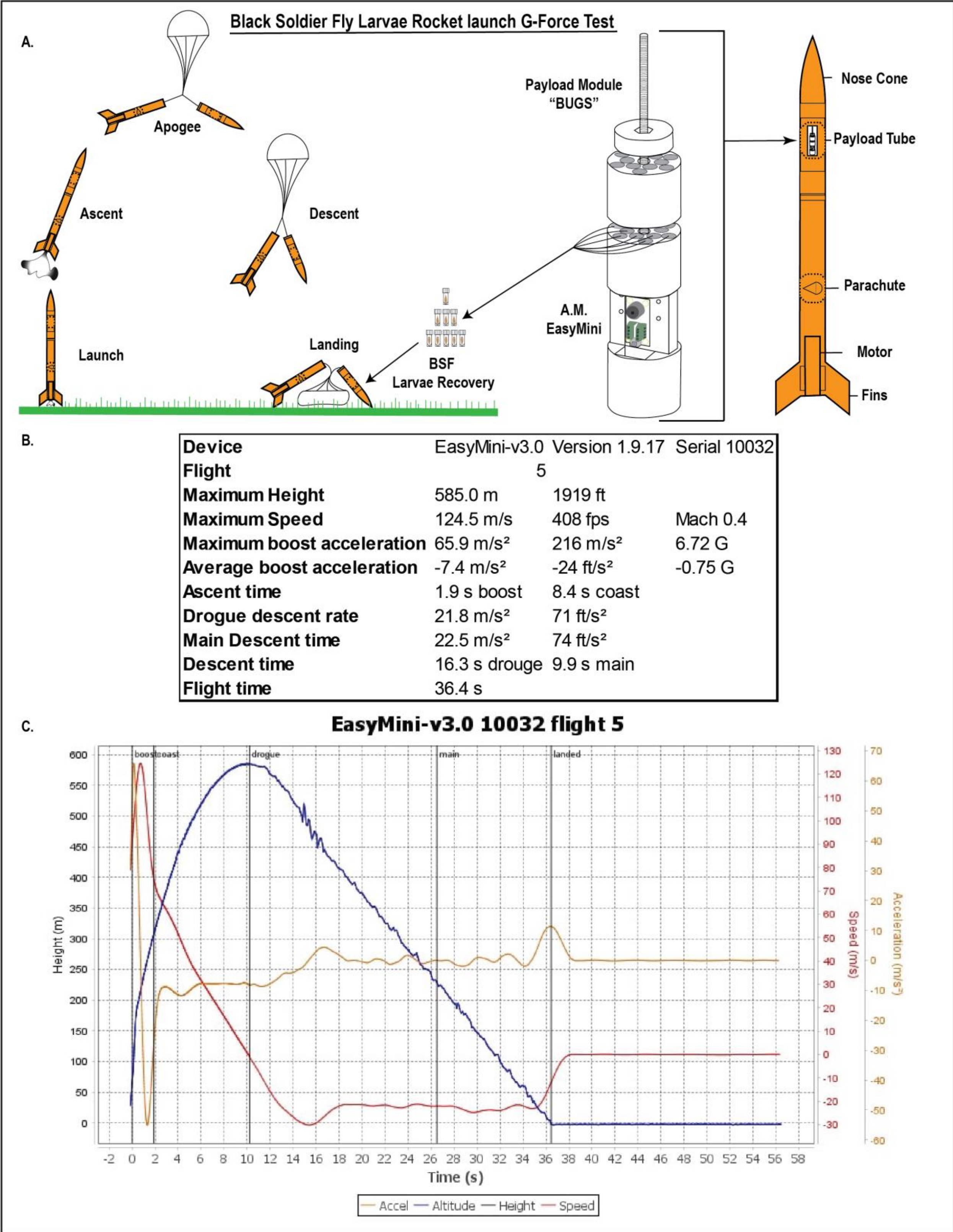
Composting experiments consisted of 12 total BSF composters. Each was introduced to 300 larvae each with 100 g of beansprouts (LEASA) initially and 100 g more bean sprouts on the beginning of the 2nd week of the experiment. Six composters utilized Alabama Control Soil (ACS) and six utilized MMS-2 purchased from The Martian Garden (Austin, Texas). ACS was collected from (32.59410°N, 85.48267°W) (Marvyn/Kinston soil) then baked for 24 hr at (204°C) for sterilization and total drying (Fisher IsoTemp Oven 100 Series Model 126G). ACS was then sieved finely and stored in a sealed tub until use. Fifty g of each soil type was given to Auburn University Soil Testing Laboratory (AUSTL) for Mehlich 1 and Total Element Extraction. To each composter was introduced to 150 g of soil type, 100 g of crushed bean sprouts (LEASA), 300 1st/2nd instar larvae ~5mm in length, and 50 mL of DIW were initially introduced. Each composter was misted once weekly during the duration of the experiment with 3 mL of water. Composters were held at (28°C) and (70%) for 4 weeks in laboratory rearing room. On day 14, 100 more grams of bean sprouts were added to each composter for BSF consumption. At the start of the 3rd week of composting both slits **Fig 1C** were opened to allow BSF self-sorting. Upon one-month composter completion, soil was removed from the composters and weighed. Larvae were allowed to remain in the composter with no additional food or water until total death. Carcasses were then counted and assessed. 50 g of soil from each composter was sent to AUSTL for Mehlich 1 and Total Element Extraction. Following soil

extraction one-way t-test analysis were performed comparing initial soil reading values to the six replicate soil values per soil type.

Results

BSF G-Force Testing

G-force testing BSF is crucial to preliminary feasibility of composting on Mars, as BSF must first be able to survive the G-force of a rocket. Initial G-force testing of 5th instar BSF resulted in both head-up and head-down surviving at 100% after a 5-minute centrifuge time at 10, 25, and 40. Only 2 pre-pupa individuals out of the total 90 pre-pupa tested did not respond following testing. Following the centrifugal g-force assessment the linear vertical g-force test was conducted **Fig 2** A. The rocket launch lasted 36.4 seconds from launch to landing and resulted in a maximum height of 585.0 meters (1919 feet), a maximum speed of 124.5 m/s^2 (408 fps), and a peak g-force of 6.74 G (**Fig 2 B**, **Fig 2 C**). Upon descent it was noted our parachute become tangled leading to a faster descent than anticipated, with the final 10 seconds of decent resulting in ~225 m loss in altitude. As a result, the nose cone penetrated the soil ~7 cm into the sod upon landing. Upon BSF recovery, all tubes and larvae were recovered. One 2 mL containing 5 larvae had a cracked lid from the impact resulting in a singular larva to be ejected through the payload tube and into the nose cone. That larva and all other larvae were successfully recovered and were alive. All larvae survived initially and were still alive one week post launch. Both centrifugal 10-40 G and linear vertical 6.74 maximum G testing demonstrated total survivorship of all BSF larvae following g-force exposure.



Device	EasyMini-v3.0	Version 1.9.17	Serial 10032
Flight	5		
Maximum Height	585.0 m	1919 ft	
Maximum Speed	124.5 m/s	408 fps	Mach 0.4
Maximum boost acceleration	65.9 m/s²	216 m/s²	6.72 G
Average boost acceleration	-7.4 m/s²	-24 ft/s²	-0.75 G
Ascent time	1.9 s boost	8.4 s coast	
Drogue descent rate	21.8 m/s²	71 ft/s²	
Main Descent time	22.5 m/s²	74 ft/s²	
Descent time	16.3 s drouge	9.9 s main	
Flight time	36.4 s		

Fig 2. Black Soldier Fly Larvae Rocket launch G-force Test. **A.** Visual representation of the

rocket launch containing the custom 3d-printed BSF Payload Module which housed our experimental BSF larvae for testing within the payload tube. **B.** EasyMini-v3.0 real time date collected during the rocket launch through landing. **C.** EasyMini-v3.0 visualized flight data rocket launch.

Composting Soil Analysis

Two soil analysis were conducted, Mehlich 1 extraction and Total Element Digestion. Mehlich 1 extraction is useful for assessing how nutrients can be up taken by a plant/crop, while Total Element Digestion assesses the total composition of elements within the soil. When comparing the Mehlich 1 for Alabama Control Soil (ACS) to the 6 ACS composted replicates, the one-month BSF composted ACS revealed statistically significant differences in Calcium, Potassium, Phosphorus, Aluminium, Boron, Magnesium, Manganese, Sodium, Zinc, Nitrogen, pH, and organic matter **Fig 3A**. Only Copper and Iron from the composted ACS did not statistically differ. Calcium, Phosphorus, Aluminium, Boron, Iron, Manganese, and Zinc went down, while Potassium, Magnesium, Copper, Sodium, Nitrogen, pH, and organic matter went up. When comparing the Mehlich 1 for MMS-2 (MMS) to the 6 MMS composted replicates, one-month composted MMS revealed statistical significance regarding Calcium, Potassium, Phosphorus, Aluminium, Boron, Magnesium, Manganese, Copper, Iron, Zinc, Nitrogen, pH, and organic matter, with the only non-significant element change being Sodium. MMS composting resulted in an increase Mehlich 1 change in Potassium, Magnesium, Phosphorus, Copper, Manganese, Nitrogen, and organic matter **Fig 3A**. Mehlich 1 differences revealed MMS showing an increase of phosphorus and Manganese compared to MMS control, while ACS composting resulted in the opposite with Phosphorus and Manganese decreases compared to ACS control. The primary takeaway from MMS Mehlich 1 testing is the change in Potassium by 1179%,

Phosphorus by 803%, Magnesium by 67%, and Manganese by 7847%.

Regarding the Total Element Digest, both ACS and MMS saw statistical significance in post composting soil composition compared to their pre-composting soil composition in Calcium, Potassium, Magnesium, Phosphorus, Aluminium, Arsenic, Boron, Barium, Cadmium, Chromium, Iron, Manganese, Molybdenum, Sodium, Nickel, Lead, and Zinc. The only non-statistical Total Element Digest change for both was in the element Copper **Fig 3B**. Both ACS and MMS post composting shared increases in Potassium, Phosphorus, Arsenic, Boron, Cadmium, Copper, Iron, Molybdenum, and Nickel. Both ACS and MMS shared decreases in Calcium, Magnesium, Barium, and Manganese. MMS post composting saw increases in Aluminium, Chromium, Lead, and Zinc, while ACS post composting saw a decrease. Only Sodium increased in ACS post composting compared to MMS decrease post composting. Total Element Digestion of MMS2 post composting revealed a 132% increase in Potassium, 82% increase in Magnesium, and 26% in Zinc. Both soil data types reveal successful one-month composting as soil compositional change occurred statistically throughout both soil composting types.

Additionally, upon soil recovery, the mean weight for ACS was 157.8g and 158g for MMS2. Adding a total of 8g more soil mass than the starting 150g of each soil type. Following the experimentation period and allowance for total death, a mean of 261.17 larvae from ACS and 267 larvae from MMS2 were recovered. Surprisingly despite the removal of food and soil after the 4 weeks, 40 adult carcasses from ACS and 7 from MMS2 composters were later recovered. This is of note as it typically takes a black soldier fly ~45 days to reach adulthood. Larvae only fed for 4 weeks with some being able to reach adulthood demonstrates the resilience of BSF and warrant for future longer-term composting testing to assess BSF maturation with a continuous

food source.

Mehlich 1 & Total Element Digenstion
Post-Composting Data

A. Post-Composting Mehlich 1 Soil Data

Mehlich Soil Change Comparisons

	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	%	pH	%
Sample ID	**Calcium**	**Potassium**	**Magnesium**	**Phosphorus**	**Aluminum**	**Boron**	**Copper**	**Iron**	**Manganese**	**Sodium**	**Zinc**	**Nitrogen**	**pH**	**Organic Matter**
Alabama Control Soil (ACS)	1054	64	171	76	371	0.8	0.5	227	86	59	15	0.203	5.3	2.8
ACS (1-6) Rep Average	711.50	741.83	209.00	50.25	234.83	0.63	0.58	167.67	67.92	98.35	12.95	0.27	6.73	4.00
ACS Average Change	-342.50	677.83	38.00	-25.75	-136.17	-0.17	0.08	-59.33	-18.08	39.35	-2.05	0.07	1.43	1.20
ACS Percent Change	-32%	1059%	22%	-34%	-37%	-21%	17%	-26%	-21%	67%	-14%	32%	27%	43%
Alabama Composting Change	-	+	+	-	-	-	+	-	-	+	-	+	+	+
Sample ID	**Calcium**	**Potassium**	**Magnesium**	**Phosphorus**	**Aluminum**	**Boron**	**Copper**	**Iron**	**Manganese**	**Sodium**	**Zinc**	**Nitrogen**	**pH**	**Organic Matter**
MMS-2 Control	5010	37	2014	4.2	3.2	15	0.2	3.8	0.5	397	1.9	Below Det	9.9	1.1
MMS2 (1-6) Rep Average	4330.50	695.33	3357.67	37.92	1.28	7.08	0.28	1.73	39.73	364.83	0.77	0.11	8.40	2.57
MMS2 Average Change	-679.50	658.33	1343.67	33.72	-1.92	-7.92	0.08	-2.07	39.23	-32.17	-1.13	0.11	-1.50	1.47
MMS2 Percent Change	-14%	1779%	67%	803%	-60%	-53%	42%	-54%	7847%	-8%	-60%	Large	-15%	133%
MMS2 Composting Change	-	+	+	+	-	-	+	-	+	-	-	+	-	+

B. Post-Composting Total Element Digestion Soil Data

Total Element Digestion Soil Change Comparison

	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
Sample ID	**Calcium**	**Potassium**	**Magnesium**	**Phosphorus**	**Aluminum**	**Arsenic**	**Boron**	**Barium**	**Cadmium**	**Chromium**	**Copper**	**Iron**	**Manganese**	**Molybdenum**	**Sodium**	**Nickel**	**Lead**	**Zinc**
Alabama Control (ACS)	1319	229	466	335	7854	<0.1	7	260	<0.1	15	11	19356	135	<0.1	27	<0.1	30	45
ACS (1-6) Rep Average	775.50	978.50	279.17	640.33	7004.17	3.93	10.15	27.57	0.37	14.12	11.28	21025.50	95.50	0.83	33.35	1.45	20.18	39.37
ACS Average Change	-543.50	749.50	-186.83	305.33	-849.83	3.93	3.15	-232.43	0.37	-0.88	0.28	1669.50	-39.50	0.83	6.35	1.45	-9.82	-5.63
ACS Percent Change	-41%	327%	-40%	91%	-11%	Large	45%	-89%	Large	-6%	3%	9%	-29%	Large	24%	Large	-33%	-13%
Alabama Composting Change	-	+	-	+	-	+	+	-	+	-	+	+	-	+	+	+	-	-
Sample ID	**Calcium**	**Potassium**	**Magnesium**	**Phosphorus**	**Aluminum**	**Arsenic**	**Boron**	**Barium**	**Cadmium**	**Chromium**	**Copper**	**Iron**	**Manganese**	**Molybdenum**	**Sodium**	**Nickel**	**Lead**	**Zinc**
MMS-2 Control	28798	876	22521	559	6670	<0.1	42	122	<0.1	14	17	31369	613	<0.1	1211	40	<0.1	34
MMS2 (1-6) Rep Average	25897.83	2028.67	19108.67	1015.67	7640.83	9.92	51.93	75.53	0.40	15.98	17.07	36180.00	585.83	0.44	867.72	40.53	0.98	42.72
MMS2 Average Change	-2900.17	1152.67	-3412.33	456.67	970.83	9.92	9.93	-46.47	0.40	1.98	0.07	4811.00	-27.17	0.44	-343.28	0.53	0.98	8.72
MMS2 Percent Change	-10%	132%	-15%	82%	15%	Large	24%	-38%	Large	14%	0%	15%	-4%	Large	-28%	1%	Large	26%
MMS2 Composting Change	-	+	-	+	+	+	+	-	+	+	+	+	-	+	-	+	+	+

Fig 3. Mehlich 1 Extraction and Total Element Digestion Soil Data. **A.** Mehlich 1 soil data of Alabama Control Soil (ACS) and MMS2 (MMS) pre and post composting. **B.** Total Element Digest soil data of Alabama Control Soil (ACS) and MMS2 (MMS) pre and post composting.

Discussion

Humanity's innate desire to explore the cosmos is a quest comprised of incremental small steps which add up to major accomplishments. Before humans can become a multi-planet species and colonize Mars, we must be able to solve the steps of logistics and practicality regarding terraforming and human survival on the red planet. Our goal revolved around the potential of improving soil fertility of Martian regolith, through the usage of Martian soil simulant, with the assistance black soldier flies (BSF) composting. Prior to BSF soil composting, we evaluated BSF

under high G-forces to assess initial transport feasibility regarding rocket G-force exposure, which would be experienced during an actual launch. We conducted high G-force centrifugal testing as well as a linear G-force actual rocket launch using a Tripoli Level 1 rocket with success. Both centrifugal and linear G-force assessments yielded ~100% survivorship. We purposefully tested up to 40 G which is beyond what would be experienced by any assent or re-entry to indicate that they would be viable in any reasonable scenario. Most currently used human-rated governmental and commercial launch vehicles only experience 2-3Gs of sustained acceleration and 5Gs of maximum acceleration during ascent (Wilkins and Sowers, 2010; Smith, 2018; Wagner et al., 2021). Some launch vehicles, such as Starship, which is developed for both human and cargo missions, can experience max acceleration above 5Gs, but even this is well within what we have demonstrated BSF can handle (SpaceX, 2020). Additionally, it is also possible to cryofreeze BSF, which may be pertinent to long term transit (Giliad et al. 2023). This may be important when considering the potential regarding travel time to Mars as frozen embryos could be sent.

The composter design was successful in such a way in that BSF were able to compost the Martian soil simulant during the one-month duration in a contained all-inclusive unit. The composters require minimal training to operate and little to no oversight when being used outside of misting. This minimizes the potential for user error, allowing consistent results from even untrained individuals. The innovation regarding the design and total self-containment of the composting unit can save astronauts time which can then be devoted to other experiments. The composters were also designed to fit into a standard cube sat format. This means they can be efficiently packed and stacked with both each other and any other item that fits these standard dimensions. This will allow for easy transport of the boxes from Earth to whatever celestial body

they are being employed on. Additionally, the boxes are easily scalable. If a larger BSF colony and more composted soil is desired, larger boxes can be assembled by scaling the dimensions of the design. This will allow the composters to continue to fit the needs of a growing extraterrestrial colony.

Regarding the composting itself, we were successfully able to compost organic material on Martian soil simulant to discern statistically significant differences of one-month composted soil in 17 key soil nutrients. Of note regarding the plant available nutrients, provided by Mehlich 1 testing, Potassium, Magnesium, Phosphorus, and Manganese showed the greatest percent change in availability post composting. Total Element Digestion revealed the highest MMS2 post composting increases in soil concentrations of Potassium, Phosphorus, and Zinc. Potassium is a vital mineral for plants assisting in cytosol in plant cells, osmotic potential relating to turgor pressure, K+ channels, enzyme activation, phloem transport of sucrose, and much more (White and Karley, 2010; Marschner, 2011; Prajapati, 2012). Magnesium also plays a vital role in plants regarding photosynthesis, phosphorylation, enzymes, growth and cellular signalling and functions (Verbruggen and Hermans, 2013; Kleczkowski and Igamberdiev, 2021; Cakmak and Yazeie, 2010). Deficiencies of magnesium in plants have been shown to be of concern (Guo et al., 2016). Phosphorus is another essential soil mineral vital to root development (Shen et al., 2011; Lambers, 2022). The ability of a plant or crop to acquire phosphorus is vital to that plant's establishment within the soil and growth. Phosphorus is also scarce as it is often lacking macronutrient relating plant development (Schachtman et al., 1998). Mehlich 1 testing revealed a mean increase in plant availability of phosphorus by 803% after only one-month of composting. Another macronutrient of note is Manganese. Initially Mehlich 1, MMS2 soil was low in Manganese at 0.5ppm. Following composting, availability went up a staggering mean of 7,847%

to 39.73ppm. Manganese is a vital to plant kinetics, photosynthesis, hydrolyzation, as well as oxygen utilization within cells (Burnell, 1988; Schmidt and Husted, 2019). The increase of Zinc in the MMS2 soil post composting is also beneficial. Zinc is important in plants for its role regarding shoot growth, root growth, and primarily enzymatic functioning (Lindsay, 1972; Brown et al., 1993; Broadley et al., 2007). The Zinc concentration in the MMS2 composted soil experienced a 26% increase.

Following the successful composting of Martian soil simulant (MMS2), further work could investigate improving composted soil and plant growth assays. One way to improve the soil amendment properties of composted soil and potential crop growth metrics, may include the addition of Plant Growth Promoting Rhizobacteria (PGPR). Utilizing PGPRs for composted regolith inoculation, or seed inoculation may provide continued crop benefits, especially due to their beneficial secondary metabolites and stress-mitigating capabilities which can provide a promising strategy for enhancing plant resilience and growth in such challenging substrates (Nelson, 2004; Nadeem et al., 2014; Prasad et al., 2015; Khatoon et al., 2020). Future testing could explore inoculation of seeds with a PGPR consortium comprised of *Bacillus megaterium, Bacillus velenzensis, Variovorax paradoxus,* and *Enterobacter asburiae*. This bacterial mixture for example may provide a capacity to improve root architecture, plant growth, ion homeostasis, cytokinin signalling, antioxidant levels, and other stress response mechanisms (Mahdi et al., 2020; Toukabri et al., 2021; Chen et al., 2022; Lee et al., 2024). These traits may enhance plant viability in the composted Martian soil simulant, promoting adaptation to otherwise adverse soil conditions. These tests could involve comparing plant growth, nutrient content, and stress responses in PGPR-treated composted Martian soil versus non-treated controls. Key metrics to assess could include root and shoot biomass, nutrient uptake efficiency, and indicators of stress

tolerance such as peroxidase and catalase activity. By systematically evaluating these parameters, the efficacy of PGPR consortia as an amendment in Martian soil, may increase the viability of in-situ agricultural methods for future Martian terraforming ventures.

The goal of this study was to compost Martian soil simulant for a one-month duration with BSF. In doing so we assessed the high G-force exposure of BSF while also designing an all-inclusive, compact, confined, and easy to use composter. Our findings reasonably demonstrate BSF should be able to withstand any G-forces experienced by a typical government or commercial rocket. We also showed BSF have the ability to compost Martian soil simulant in a one-month duration and significantly alter the mineral content of the soil. Both astro-entomological assessments suggest further testing and research should be done to assess the continued feasibility of utilizing black soldier flies as a potential tool in Martian colonization and soil reclamation.

References

Anyega, A.O., Korir, N.K., Beesigamukama, D., Changeh, G.J., Nkoba, K., Subramanian, S., et al. (2021) Black soldier fly-composted organic fertilizer enhances growth, yield, and nutrient quality of three key vegetable crops in sub-saharan Africa. *Front Plant Sci* 12, 680312.

Bessa, L.W., Pieterse, E., Marais, J., and Hoffman, L.C. (2020) Why for feed and not for human consumption? The black soldier fly larvae. *Comprehensive Reviews in Food Science and Food Safety* **19**: 2747–2763.

Broadley, M.R., White, P.J., Hammond, J.P., Zelko, I., and Lux, A. (2007) Zinc in plants. *New Phytologist* **173**: 677–702.

Brown, P.H., Cakmak, I., and Zhang, Q. (1993) Form and Function of Zinc Plants. In *Zinc in*

Soils and Plants: Proceedings of the International Symposium on 'Zinc in Soils and Plants' held at The University of Western Australia, 27–28 September, 1993. Robson, A.D. (ed). Dordrecht: Springer Netherlands, pp. 93–106.

Bunchek, J.M., Curry, A.B., and Romeyn, M.W. (2021) Sustained Veggie: A Continuous Food Production Comparison. *International Conference of Environmental Systems*. ID 20210014997.

Burnell, J.N. (1988) The Biochemistry of Manganese in Plants. In *Manganese in Soils and Plants: Proceedings of the International Symposium on 'Manganese in Soils and Plants' held at the Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, South Australia, August 22–26, 1988 as an Australian Bicentennial Event*. Graham, R.D., Hannam, R.J., and Uren, N.C. (eds). Dordrecht: Springer Netherlands, pp. 125–137.

Cakmak, I. and Yazeie, A. (2010) Magnesium: A Forgotten Element in Crop Production. *Better crops, 94(2), 23-25.*

Chen, X., Yang, F., Bai, C., Shi, Q., Hu, S., Tang, X., et al. (2022) *Bacillus velezensis* strain gumt319 reshapes soil microbiome biodiversity and increases grape yields. *Biology* **11**: 1486.

Connor, D.J. and Mínguez, M.I. (2012) Evolution not revolution of farming systems will best feed and green the world. *Global Food Security* **1**: 106–113.

Douglas, G.L., Wheeler, R.M., and Fritsche, R.F. (2021) Sustaining Astronauts: resource limitations, technology needs, and parallels between spaceflight food systems and those on Earth. *Sustainability* **13**: 9424.

Douglas, G.L., Zwart, S.R., and Smith, S.M. (2020) Space Food for Thought: challenges and

considerations for food and nutrition on exploration missions. *The Journal of Nutrition* **150**: 2242–2244.

Giliad, Y., Politi, Y., and Alyagor, I. (2023) Cryopreserved insects and methods for producing same. PN 20230020006

Guo, W., Nazim, H., Liang, Z., and Yang, D. (2016) Magnesium deficiency in plants: An urgent problem. *The Crop Journal* **4**: 83–91.

Jiang, J., Zhang, M., Bhandari, B., and Cao, P. (2020) Current processing and packing technology for space foods: a review. *Critical Reviews in Food Science and Nutrition*. *60*(21), 3573-3588.

Johnston, R.S., Dietlein, L.F., and Berry, C.A. (1975) Biomedical Results of Apollo, Scientific and Technical Information Office, National Aeronautics and Space Administration.

Khatoon, Z., Huang, S., Rafique, M., Fakhar, A., Kamran, M.A., and Santoyo, G. (2020) Unlocking the potential of plant growth-promoting rhizobacteria on soil health and the sustainability of agricultural systems. *Journal of Environmental Management* **273**: 111118. ISSN 0301-4797

Klammsteiner, T., Turan, V., Fernández-Delgado Juárez, M., Oberegger, S., and Insam, H. (2020) Suitability of black soldier fly frass as soil amendment and implication for organic waste hygienization. *Agronomy* **10**: 1578.

Kleczkowski, L.A. and Igamberdiev, A.U. (2021) Magnesium signaling in plants. *International Journal of Molecular Sciences* **22**: 1159.

Kumar, S., Negi, S., Mandpe, A., Singh, R.V., and Hussain, A. (2018) Rapid composting techniques in Indian context and utilization of black soldier fly for enhanced decomposition of biodegradable wastes - A comprehensive review. *Journal of*

Environmental Management **227**: 189–199.

Lambers, H. (2022) Phosphorus acquisition and utilization in plants. *Annual Review of Plant Biology* **73**: 17–42.

Lee, S., Kim, J.-A., Song, J., Choe, S., Jang, G., and Kim, Y. (2024) Plant growth-promoting rhizobacterium *Bacillus megaterium* modulates the expression of antioxidant-related and drought-responsive genes to protect rice (*Oryza sativa* L.) from drought. *Front Microbiol* 15, 1430546.

Linck, E., Crane, K.W., Zuckerman, B.L., Corbin, B.A., Myers, R.M., Williams, S.R., et al. (2019) Evaluation of a human mission to Mars by 2033. *IDA Science and Technology Policy Institute*. IDA D-10510

Lindsay, W.L. (1972) Zinc in Soils and Plant Nutrition. In *Advances in Agronomy*. Brady, N.C. (ed). Academic Press, pp. 147–186.

Liu, T., Awasthi, M.K., Chen, H., Duan, Y., Awasthi, S.K., and Zhang, Z. (2019) Performance of black soldier fly larvae (*Diptera: Stratiomyidae*) for manure composting and production of cleaner compost. *Journal of Environmental Management* **251**: 109593.

Mahdi, I., Fahsi, N., Hafidi, M., Allaoui, A., and Biskri, L. (2020) plant growth enhancement using rhizospheric halotolerant phosphate solubilizing bacterium *Bacillus licheniformis* qa1 and *Enterobacter asburiae* qf11 isolated from *Chenopodium quinoa* willd. *Microorganisms* **8**: 948.

Marschner, H. (2011) Marschner's Mineral Nutrition of Higher Plants, Academic Press.

Nadeem, S.M., Ahmad, M., Zahir, Z.A., Javaid, A., and Ashraf, M. (2014) The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotechnology Advances* **32**: 429–448.

Nelson, L.M. (2004) Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. *Crop Management* **3**: 1–7.

Oluwafemi, F.A., De La Torre, A., Afolayan, E.M., Olalekan-Ajayi, B.M., Dhital, B., Mora-Almanza, J.G., et al. (2018) Space food and nutrition in a long term manned mission. *Adv Astronaut Sci Technol* **1**: 1–21.

Perchonok, M.H., Cooper, M.R., and Catauro, P.M. (2012) Mission to mars: food production and processing for the final frontier. *Annual Review of Food Science and Technology* **3**: 311–330.

Prajapati, K. (2012) The importance of potassium in plant growth – a review. *Indian Journal of Plant Sciences* **1**: 177–186.

Prasad, R., Kumar, M., and Varma, A. (2015) Role of PGPR in Soil Fertility and Plant Health. In *Plant-Growth-Promoting Rhizobacteria (PGPR) and Medicinal Plants*. Egamberdieva, D., Shrivastava, S., and Varma, A. (eds). Cham: Springer International Publishing, pp. 247–260.

Reid, J.M., Lutwak, L., and Whedon, G.D. (1968) Dietary control in the metabolic studies of Gemini-7 Space Flight1. *Journal of the American Dietetic Association* **53**: 342–347.

Schachtman, D.P., Reid, R.J., and Ayling, S.M. (1998) Phosphorus uptake by plants: from soil to cell. *Plant Physiology* **116**: 447–453.

Schmidt, S.B. and Husted, S. (2019) The biochemical properties of manganese in plants. *Plants* **8**: 381.

Shen, J., Yuan, L., Zhang, J., Li, H., Bai, Z., Chen, X., et al. (2011) Phosphorus dynamics: from soil to plant. *Plant Physiology* **156**: 997–1005.

da Silva, G.D.P. and Hesselberg, T. (2020) A Review of the use of black soldier fly larvae,

Hermetia illucens (*Diptera*: *Stratiomyidae*), to compost organic waste in tropical regions. *Neotrop Entomol* **49**: 151–162.

Smith, D.A. (2018) Space Launch System (SLS) Mission Planner's Guide. No. M17-6014.

Smith, S.M., Zwart, S.R., Block, G., Rice, B.L., and Davis-Street, J.E. (2005) The Nutritional Status of Astronauts Is Altered after Long-Term Space Flight Aboard the International Space Station1. *The Journal of Nutrition* **135**: 437–443.

SpaceX (2020) Starship Users Guide. https://www.spacex.com/media/starship_users_guide_v1.pdf

Toukabri, W., Ferchichi, N., Hlel, D., Jadlaoui, M., Kheriji, O., Mhamdi, R., and Trabelsi, D. (2021) Response of intercropped barley and fenugreek to mono- and co-inoculation with *Sinorhizobium meliloti* F42 and *Variovorax paradoxus* F310 under contrasting agroclimatic regions. *Arch Microbiol* **203**: 1657–1670.

Uri, J. (2020) Space Station 20th: Food on ISS. *NASA Johnson Space Center*.

Verbruggen, N. and Hermans, C. (2013) Physiological and molecular responses to magnesium nutritional imbalance in plants. *Plant Soil* **368**: 87–99.

Wagner, E.B., Charania, A.C., and Kornuta, D. (2021) From Concept to Reality: Research Opportunities on Blue Origin Space Platforms. 685–697.

Wang, Y.-S. and Shelomi, M. (2017) Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. *Foods* **6**: 91.

White, P.J. and Karley, A.J. (2010) Potassium. In *Cell Biology of Metals and Nutrients*. Hell, R. and Mendel, R.-R. (eds). Berlin, Heidelberg: Springer, pp. 199–224.

Wilkins, M. and Sowers, G. (2010) Atlas V Launch Services User's Guide. *United Launch Alliance*.

Chapter 4:

Optimizing Microinjection Technique of *Wolbachia* for *Alphitobius diaperinus*

Abstract

Alphitobius diaperinus are a significant pest in poultry production, presenting challenges related to feed efficiency, structural damage, and pathogen transmission. This study aimed to optimize microinjection techniques for the introduction of *Wolbachia* into *A. diaperinus* eggs as a precursor to establishing an infected colony for future research involving Incompatible Insect Technique (IIT) for population suppression. Initial screening confirmed the absence of natural *Wolbachia* infections in *A. diaperinus* in poultry houses, a prerequisite for IIT. Microinjection methodologies were utilized and refined, including assessments of dechorionation, halocarbon oil usage, and incubation conditions. *Wolbachia* injections via cytoplasmic transfer and *Wolbachia* purified SPG buffer were evaluated. The SPG buffer method was optimized for efficiency and *Wolbachia* concentration. PCR analyses confirmed successful *Wolbachia* presence in injected eggs and newly hatched larvae. This study establishes foundational techniques for creating *Wolbachia*-infected *A. diaperinus* lines, supporting future efforts to developing an infected colony for future IIT applications.

Introduction

Alphitobius diaperinus [Panzer], commonly known as the lesser mealworm or litter beetle, is an economically significant pest in poultry production systems. Native to sub/tropical regions, this beetle has proliferated globally (Geden and Hogsette 1994; Mozaffar et al. 2004). Primarily in broiler and turkey farms, *A. diaperinus* is associated with various economic and health-related challenges (Dinev 2013). The beetles' ability to proliferate in warm, humid environments with abundant organic material, such as poultry litter, makes it exceptionally suited

for poultry farms. Beetle infestations can reduce feed efficiency and cause nutritional imbalances leading to slower growth rates if consumed by poultry (Despins et al. 1994; Despins and Axtell 1995). Its capacity to serve as a vector for pathogens and contribute to structural damage further complicates its management and underscores the importance of developing integrated pest control strategies (Vaughan et al. 1984; Despins et al. 1987; Despins et al. 1994; Hazeleger et al. 2008).

A. diaperinus under favorable conditions, such as the warm and moist environments of poultry houses, can development from egg to adult in ~30 to 60 days, depending on environmental factors like temperature and humidity which can effect survivorship and development (Rueda and Axtell 1996; Zafeiriadis et al. 2023). Adult females deposit eggs in crevices, poultry litter, or manure, while larvae consume organic debris, spilled feed, and waste (Salin et al. 2000; Retamales et al. 2011). Along with ease of proliferation in poultry houses, the larvae of *A. diaperinus* are known to burrow into the insulation and wooden structures of poultry houses, causing infrastructure damage (Vaughan et al. 1984; Geden and Axtell 1987). The burrowing behavior is driven due to a search for a suitable pupation site. Damage to wood and insulation can lead to increased energy costs, as it reduces the thermal efficiency of poultry houses, requiring producers to use more energy for heating and cooling (Axtell and Arends 1990). One report in the 1990s claimed poultry houses damaged by *A. diaperinus* experienced 67% higher energy costs than uninfested ones (Geden and Hogsette 1994).

A. diaperinus's infestations serve as reservoirs for various pathogens, bacteria, viruses, and parasites. Studies have demonstrated that lesser mealworms can carry and transmit pathogens that are harmful to poultry, including *Salmonella* spp., *Escherichia coli*, and *Campylobacter jejuni*, which are associated with foodborne illnesses in humans (de las Casas et

al. 1968; McAllister et al. 1994; Skov et al. 2004; Strother et al. 2005; Smith et al. 2022). These pathogens could persist in beetles, and when ingested by poultry, may lead to infections in the birds (Despins and Axtell 1995; Beckmann et al. 2021). One study showed one day old chickens positive with *Salmonella* detection on cloacal swabs within 24 hours of consuming one infected *A. diaperinus* larvae (McAllister et al. 1994). Additionally, *A. diaperinus* have been implicated in the spread of viral diseases such as infectious bursal disease (IBD), and Newcastle disease, all of which can lead to mortality in poultry and reduced productivity (De Las Casas et al. 1976; McAllister 1993). *A. diaperinus* also possess the ability to carry *Eimeria* spp., a cause of intestinal coccidiosis, further exacerbates its role as a pest in poultry systems(Goodwin and Waltman 1996). Coccidiosis is a significant protozoan parasitic disease in poultry, leading to intestinal damage, reduced weight gain, and higher mortality rates, and beetles that harbor *Eimeria* can be damaging to poultry operations (Reyna et al. 1983; Gussem 2007).

Given the significant economic and health impacts of *A. diaperinus* infestations, effective management strategies are crucial for mitigating the risks posed by this pest. Regarding Integrated Pest Management (IPM) approaches, chemical control using insecticides remains one of the primary methods for reducing beetle populations in poultry houses. However, concern over insecticide resistance, environmental contamination, and the potential impact on non-target organisms have led to increased interest in alternative solutions (Smith et al. 2022). Chemical insecticide resistance has been demonstrated in populations of prolonged insecticide usage of the same mode of action (Sammarco et al. 2023). Mechanical control methods involve sanitation metrics such as litter or waste removal from poultry houses as infrequent cleanout can result in the development of large populations in manure (Vaughan 1982). Biological control agents, such as entomopathogenic fungi and nematodes, have shown promise in laboratory and field trials as

potential alternatives to chemical insecticides, however, are currently less viable (Santoro et al. 2008; Alves et al. 2012; Del Valle et al. 2016). Management strategies for *A. diaperinus* must be selected carefully, especially in the case of poultry houses as downstream human health concerns may apply. Sterile insect technique (SIT) or incompatible insect technique (IIT) may be a viable option for *A. diaperinus* if done correctly.

SIT is a control method which involves releasing sterilized male insects into wild populations to reduce the population size through failed reproduction (Knipling 1955; Knipling 1959). Traditionally, SIT has relied on irradiation to sterilize males; however, recent advancements in incompatible insect technique (IIT) incorporate the endosymbiotic bacterium *Wolbachia* to reduce targeted wild population numbers (Zhang et al. 2015a; Zhang et al. 2015b). *Wolbachia* are endosymbiotic gram negative bacteria which possess the ability to induce Cytoplasmic Incompatibility (CI) in noncompatible hosts resulting in sterilization through a deubiquitylating enzyme which causes lack of egg viability (Zhang et al. 2015c; Beckmann et al. 2017; Beckmann et al. 2019b; Zheng et al. 2021). CI occurs when *Wolbachia*-infected males mate with uninfected females or females carrying different *Wolbachia* strains (Laven 1967; Bordenstein et al. 2001; Shropshire et al. 2020). The mechanism acts as a toxin-antidote system by which cytoplasmic incompatibility factors (CIF) interact. *Wolbachia* infected males have sperm contain CifB which act as the toxin, which can only be rescued by infected females whose eggs contain CifA, the antidote (Beckmann et al. 2019a). This means an infected male will produce sperm which can only be made viable in conjunction with a *Wolbachia* infected female. Should an infected male mate with an uninfected female, the toxin located in the sperm will lead to failure in fertilization and no egg viability. IIT focuses around creating a laboratory *Wolbachia*

strain for insects which do not possess *Wolbachia* or a particular strain in the wild, then systematically releasing only males into the population for control.

Studies have demonstrated that Wolbachia-based IIT have been successful in controlling populations of *Aedes aegypti*, the primary vector of dengue, Zika, and chikungunya viruses, in field trials across several countries(Hoffmann et al. 2011; O'Neill et al. 2019; Nazni et al. 2019) (O'Neill et al. 2018). This technique offers a biological alternative to chemical pesticides which reduces the risks of insecticide resistance, off target impact, and environmental consequences inherent to synthetic chemical pesticides. *A. diaperinus* as illustrated above poses a serious threat to poultry production systems due to its ability to damage infrastructure, reduce feed efficiency, and transmit a wide range of pathogens. Effective management of this pest requires a comprehensive understanding of its biology and behavior, as well as the implementation of appropriate IPM strategies. Our research focused on developing microinjection methodologies for *A. diaperinus* for the purpose of generating a *Wolbachia* infected line for future usages in IIT.

Materials and Methods

Microinjection Equipment

A Kite Manual Micromanipulator (World Precision Instruments #5464) with a WPI M-3 Tilting base attachment was used as the microinjection implement. The micromanipulator is outfitted with a WPI microelectrode holder connected to a Clippard URH1-0804 air tubing. The air tubing is connected to a Clippard pressure regulator. An air tube leads to a larger RIDGID 250psi air compressor. Borosilicate glass needles are heated and pulled (Sutter Instrument Model P-97 Micropipette Puller at (Setting: P=500, Heat-590, Pull=115, Vel=15, Dell=90) with filament O.D. 1.0mm, I.D. 0.050mm with a length of 10cm.

Beetle Egg Lay Setup

Beetles aged 1–8 weeks were selected for egg laying. Petri plates (150 x 15 mm) were labeled with the corresponding beetle group. A 6 x 7 cm piece of 2 mm thick corrugated cardboard (Juvale brand packaging pads) was placed in the center of each plate, followed by 100 g of brown rice (Mahatma or Great Value). Approximately 200 beetles were added to each plate, ensuring that neither beetles nor rice were trapped beneath the cardboard. A small apple slice was placed in the center of the plate without obstructing the lid to prevent beetle escape. The plates were incubated at 40°C for 2 hrs in darkness, with the incubator door slightly ajar to prevent over humidifying. After 2 hrs, plates with at least 10 eggs were identified. The beetles and rice were gently transferred to the labeled plastic boxes using a spoon and brush ensuring the cardboard remained undisturbed as the eggs were located underneath, and the adult beetles were returned to the incubator. The number of eggs per plate was estimated and recorded then egg plates were transported to the microinjection station for further processing.

Field Check: A. diaperinus poultry farm check

To confirm that wild beetles are not infected with Wolbachia, we screened beetles from two poultry farms. Beetles were collected from two poultry farms at two different collection dates 2/12/2021 and 6/14/2021 from the same Farm1 and Farm 2. Wolbachia Surface Protein (WSP) primers were used to amplify Wolbachia specific genes and a DNA control gene Histone 2. The primers were: WSPf: GTCCAATARSTGATGARGAAAC, WSPr: CYGCACCAAYAGYRCTRTAAA and H3AF: ATGGCTCGTACCAAGCAGACVGC and H3AF: ATATCCTTRGGCATRATRGTGAC.

Assay 1: A. diaperinus Microinjection Optimization

Prior to microinjection, dechorionation and halocarbon oil (Halocarbon 700 Poly chlorotrifluoroethylene, 700 centistokes) bioassays were conducted. Dechorionation involves the removal of the chorion outer layer of an insect egg. This can allow for easier penetration. Halocarbon oil is a gas permeable oil which allows for oxygen to flow over coated eggs while preventing egg dehydration (Su et al. 2023). The purpose of this bioassay was to determine the optimal way to prepare a beetle egg prior to injection which would not decrease hatch rate. Beetle eggs were prepared as above and after ~24hr treated with, no-oil dechorionated (dechorionation = 10% bleach for 1 min submersion), oil dechorionated, no-oil dechorionated, or oil dechorionated. Eggs were left on the plate at 28°C for 72 hrs. The 4-day beetle hatch rate was then checked, and survivorship was recorded.

Following the initial oil/dechorionation hatch rate survival assay, PBS and initial *Wolbachia* injections were conducted to gauge *A. diaperinus* survivorship following injection. Needles were pulled and prepared as previously described. Beetle eggs were prepared as above and collected for injection following the 2hr egg lay period to ensure early developmental egg stage. Beetle eggs were injected on either the Petri dish they were laid on, or the cardboard (if they were attached to the cardboard following its removal). An additional group of *Wolbachia* injections in beetle eggs (cytoplasmic transfer) with 189+wMel infected fruit fly eggs was conducted. This was to compare the survivorship of injected beetle eggs with the addition of transgenic inter-order cytoplasm being added. Subsequently a temperature hatch rate assay was conducted to determine at what temperature, 23°C, 28°C, or 32°C injected eggs should be incubated at, and on which day either four or seven, to assess injected egg hatch rate.

Cytoplasmic Transfer

Cytoplasmic transfer was conducted by transferring *Wolbachia* from 189+wMel *Drosophila melanogaster* eggs to *A. diaperinus* eggs. Groups of ~100-200 *Drosophila* adults were introduced to apple juice plates (1 Liter: 100% apple juice 405mL, Dextrose 52.2g, Sucrose 26.06g, Agar 19.8g, 1.25M NaOH 24mL, dH2O 484mL) prepared with a dollop of yeast (used to sexually invigorate the flies) and deionized water (DIW) applied to the corner of apple juice plates for mating resulting in *Drosophila* egg lay. Eggs were collected at intervals of 20-60 min, depending on the robustness of the egg-laying for cytoplasmic transfer. Once *Drosophila* eggs and beetle eggs were collected, they were transferred to the microinjection station where needles were pulled to the above metrics. Double-sided sticky tape was placed on the beetle egg plates directly under the egg line following cardboard removal. *Drosophila* eggs from the apple juice plates were brushed onto the double-sided sticky tape, preparing them for microinjection. The tip of the needle was carefully broken with a fine razor to open it before inserting the needle into the Kite Manual Manipulator. The air compressor tank was turned on to reach approximately 100 psi while the air gauge was set to 10 psi. Injections began by piercing the *Drosophila* egg with the needle, allowing the cytoplasm to be drawn up into the needle via osmotic pressure differential. Once sufficient cytoplasm was collected, the needle was carefully withdrawn from the *Drosophila* egg and inserted into a beetle egg on the dorsal side. The injection air release trigger was gently tapped or pressed to push the cytoplasm into the beetle egg. Care was taken to avoid over-pressurizing the egg, which could result in cytoplasmic expulsion or egg rupture. This process was repeated with a minimal transfer of 10 eggs per plate, and needles were changed regularly if clogged.

Assay 2: *Wolbachia* SPG Methodology

Wolbachia SPG buffer loaded into needles was assessed as an alternative strategy for injection to cytoplasmic transfer. The SPG (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM l-glutamate, pH 7.2) buffer was initially made with *Wolbachia*, similar to (Xi and Dobson 2005), with first dechorionating *Wolbachia* infected *Drosophila* eggs (1-2 min 50% bleach) then egg rinse with distilled water following egg transfer to a 1.5 ml tube, then rinsed with 0.5 ml (SPG) buffer. Eggs are then transferred into 1 ml fresh SPG buffer in a Dounce tissue grinder (Fisher Scientific, Pittsburgh, PA) and homogenized (~10 strokes at room temperature with the tight-fitting B-type pestle). Homogenate is then transferred into a 1.5-ml tube and centrifuged at 300 × G for 5 min to remove large debris. Supernatant is then moved into a separate tube and centrifuged at 12,000 × G for 10 min to pellet the *Wolbachia* cells. The supernatant is then removed, leaving a pellet in ~50 µl, which is resuspended by pipetting. Finally, debris is cleared from the suspension by centrifuging at 300 × G for 3 min and the supernatant is transferred into a clean tube at 25°C until used for injection (<5 hr). *Wolbachia* SPG buffer is then withdrawn via MICROFIL 34-guage/67MM long MF34G-5 (World Precision Instrument) and loaded into the back of a pulled needle where it is then ready once inserted into the Kite Manual Manipulator for direct injection into *A. diaperinus* eggs.

Wolbachia SPG methodology screening was conducted to optimize the buffer. Screening was comprised of six assessments, egg fecundity of +wMel *Drosophila* egg lay numbers across 4 time periods, PCR screening of strength of *Wolbachia* in SPG buffer across different starting egg amounts, PCR tracking of Wolbachia across each stage of the *Wolbachia* SPG purification process, assessing modifications to the SPG process to increase *Wolbachia* concentration in the purified buffer, PCR check of duel *Wolbachia* strains in SPG buffer, and finally hatch rate assessment of *A. diaperinus* after injection of varying methods. Egg fecundity of +wMel

Drosophila egg was assessed in order to ascertain the number of *Drosophila* eggs which could be used to make the *Wolbachia* SPG buffer. For this assay, 189+wMel *Drosophila* flies were set on apple juice media plates with yeast where eggs were counted at 4 hr, 6 hr, 8 hr, and 16 hr (overnight). The PCR screening of strength of *Wolbachia* in SPG buffer across different egg amounts was done by PCR screening the finalized *Wolbachia* SPG buffer made from varying amounts of 189+wMel fly eggs either 50, 100, or 200. This was to assess how the number of eggs used would affect the strength of *Wolbachia* in the finalized buffer. PCR tracking of *Wolbachia* across each stage of the *Wolbachia* SPG purification process was done by removing 25µl from the ~100 189+wMel eggs SPG buffer after each key step in the *Wolbachia* SPG buffer protocol above for DNA extraction and PCR screening. The purpose of this was to ensure *Wolbachia* was present in the final buffer solution as well as to see if any *Wolbachia* was lost after any of the preparation steps. Varying *Wolbachia* SPG method alteration were assessed. Three modifications to the *Wolbachia* SPG protocol were made to assess concentration of *Wolbachia* within the buffer as well as for preparation efficiency. *Wolbachia* was screened using VirD4 primers, VirD4f CCTACAGGYTCKGGYAARGGTG, VirD4r GCCAAAARTCYTGYTCAGGC (~257bp).

Injected Egg & Larvae Wolbachia Screening

Proceeding *Wolbachia* SPG optimization, injected egg and hatched larval PCR screening was done. The purpose of this was to show if the microinjection process did result in successful injection of *Wolbachia* into eggs and subsequently could be detected in hatched larvae. For the egg check, *A. diaperinus* eggs were injected with *Wolbachia* via cytoplasmic transfer and *Wolbachia* SPG buffer comprised from wMel or wHa infected *Drosophila* fly lines. *Wolbachia* SPG buffer injected larvae were PCR screened for presence of *Wolbachia* in hatched larvae.

Software

Adobe Photoshop, Adobe illustrator, and GraphPad Prism 9.31 were used to create figures below. Statistics involving Statistics involving Ordinary One-Way ANOVA & Brown-Forsythe and Welch ANOVAs (alpha 0.05) were run and analyzed on GraphPad Prism.

Results & Discussion

Initial Feasibility Screening

Initial *Wolbachia* screening of *A. diaperinus* in poultry houses showed no natural infection status **Fig 1A**. At both farms across the 4-month separation in collection, *A. diaperinus* showed a negative infection status in all samples. A non-infected status is essential for utilizing *Wolbachia* to suppress *A. diaperinus* populations via CI as the target population must be uninfected (Zabalou et al. 2004; Werren et al. 2008; Beckmann et al. 2019a). It is of note that bidirectional CI is possible in infected populations if they are mated with individuals of separate *Wolbachia* strains, or superinfected strains, which carry more than one *Wolbachia* strain (Sinkins 1995; Sicard et al. 2014; Joubert et al. 2016). The confirmation of lack of *Wolbachia* in native populations of *A. diaperinus* beetles in poultry houses supports the premise that IIT through the inception of *Wolbachia* infected population may be able to suppress poultry house populations.

Fig 1B illustrates dechorionation and halocarbon oil hatch rate assessment. Dechorionation and halocarbon oil are commonly used in insect microinjection techniques (Yoshiyama et al. 2000; Xu et al. 2015; Bui et al. 2020; Harrell 2024; Pearce et al. 2024). Dechorionation is used to remove the chorion outer layer of the embryo to allow for easier penetration of the needle. Halocarbon is subsequently used to coat the egg to help prevent desiccation while allowing air permeability. Our assessment showed a statistical significance ($p = 0.0118$, $p = 0.0068$) difference in hatch rate between oil non dechorionated eggs and both oil

dechorionated and non-dechorionated eggs. From this we decided to proceed without future use of halocarbon oil. **Fig 1C** depicts hatch rate survival of PBS injected eggs both dechorionated and non-dechorionated on the Petri dish the eggs were laid on as well as control plates and initial wMel cytoplasm injected eggs. While there was no statistically significant change due to dechorionating in a Petri dish, or non-dechorionated eggs on cardboard, we chose to proceed forward with not dechorionating *A. diaperinus* eggs as the mean egg survival was higher, and not dechorionating eggs saves time and labor efforts. Of note initial wMel injected eggs yielded a hatch rate survival rate of ~5%. Additionally, we decided to proceed forward with injection being sealed in parafilm with half of a Kim wipe saturated in DIW (DI water). **Fig 1D** illustrates the hatch rate survival of PBS injected eggs at three separate temperatures checked at either four or seven days. Significance was shown between 23°C at four days PBS injection checked vs 28°C four and seven day control and 32°C four and seven day control checked eggs ($p = 0.0282$, $p = 0.0485$, $p = 0.0186$, $p = 0.0175$). With the lack of additional significance among treatments we decided to proceed with injections at 28°C checked at seven days. The justification for this is *Wolbachia* has demonstrated a higher density at 26°C in *Leptopilina heterotoma* when examined from 14-26°C (Mouton et al. 2006). The strain wHa in uninfected females has been shown to have preferential temperatures of 25.98°C ± 1.02°C (Hague et al. 2020). Additionally, one study found that wMel carrying *A. aegypti* females showed Wolbachia levels higher in controls raised between 20-30°C compared to heat treated females 30-40°C (Ulrich et al. 2016).

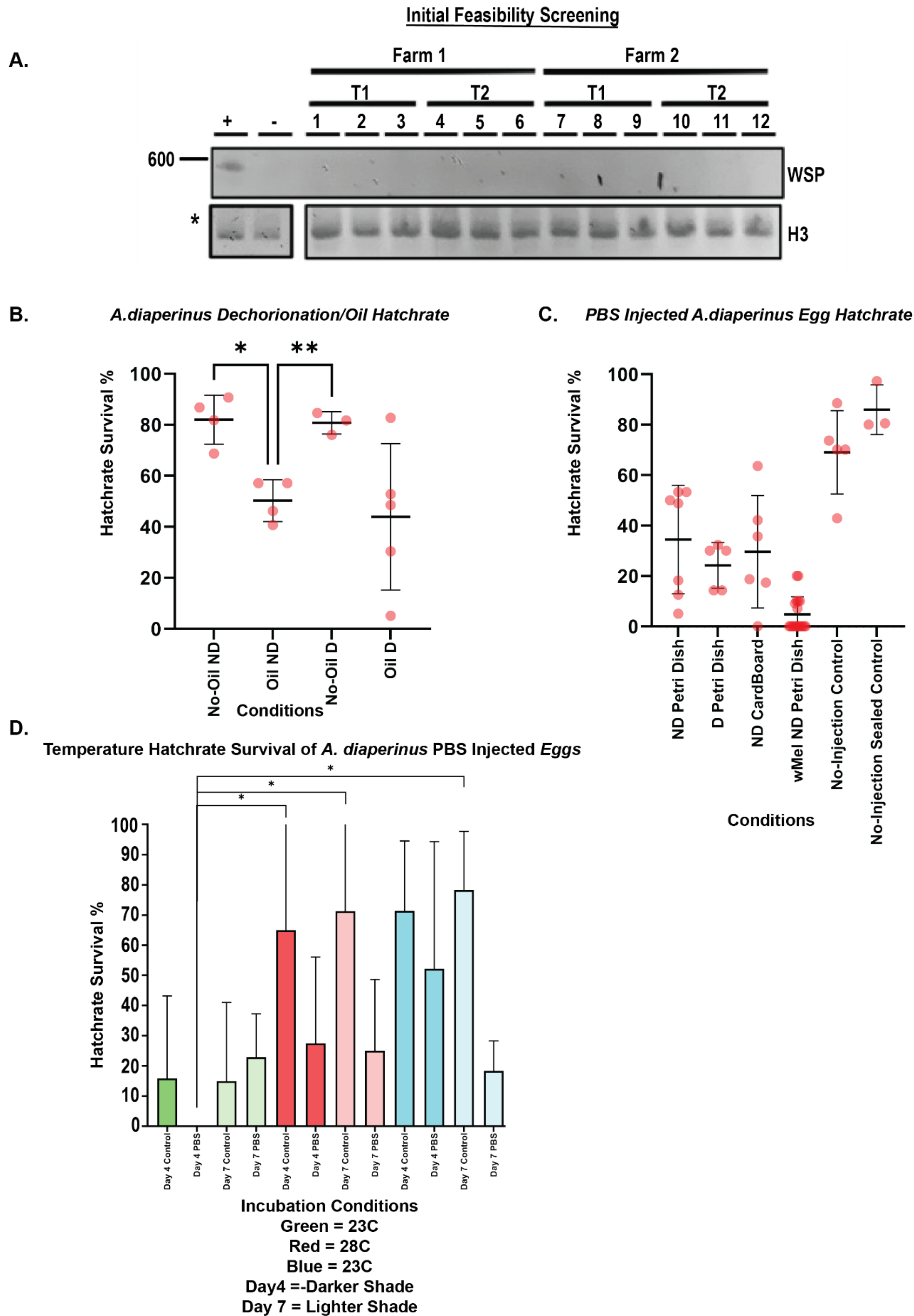


Fig 1. Initial Feasibility Screening. **A.** *Wolbachia* screening of *A. diaperinus* from two poultry farms at two-time intervals (T1/T2) 4 months apart. + lane = 189+wMel adults, - lane = negative

189- adults, 1-12 = *A. diaperinus* adults from farms. **B.** *A. diaperinus* dechorionation/oil hatch rate survival. *A. diaperinus* eggs either dechorionated or not or covered in Halocarbon oil or not. **C.** PBS Injected *A. diaperinus* egg hatch rate. *A. diaperinus* injected eggs were either dechorionated or not on Petri dish or on cardboard. Two controls as well as initial wMel cytoplasmic transfer *A. diaperinus* eggs injected on Petri dish. **D.** PBS injected eggs at three different temperatures 23°C, 28°C, 32°C at two recovery intervals 4-days or 7-days.

Cytoplasmic transfer injection has advantages and disadvantages. While direct *Wolbachia* infected cytoplasm can be transferred, the process is timely and can require numerous needles as cytoplasmic clogging can and does occur. To increase speed and the potential injected *Wolbachia* concentration we explored utilizing *Wolbachia* SPG buffer for injection. **Fig 2** depicts our *Wolbachia* SPG methodology assessments. **Fig 2A** shows eggs laid by 189+wMel flies over four time periods. A 16 hr egg lay duration was utilized for high-yield egg collection for the SPG buffer. **Fig 2B** demonstrates the presence of *Wolbachia* in SPG buffers made from 50, 100 and 200 eggs. 100 and 200 eggs showed brighter bands than that of 50 eggs. **Fig 2C** Depicts the tracking of *Wolbachia* throughout the steps of making the SPG buffer. PCR reveals some *Wolbachia* can be lost in the final step as well as after the first 300G spin. **Fig2 D** depicts the *Wolbachia* presence among the final steps of three method modification to our initial SPG protocol. Subsequently we decided to proceed with Method 1, which is faster to make than our initial protocol and omits two steps where *Wolbachia* can be lost. Using Method 1, ~16-hour eggs are dechorionated, homogenized in 1mL SPG, spun at 12000G for 10 minutes, then 950µl of SPG are removed where the pellet of *Wolbachia* is resuspended in the remaining 50µl of SPG and ready for injection. **Fig 2E** demonstrates this method can be utilized with a combined egg set of wMel and wHa eggs yielding *Wolbachia* presence. The inclusion of combined strains is to

increase the potential chance of a future transmission as one or the other may “take” within a potential injected egg. **Fig 2E** shows the hatch rate of over 4,600 injected *A. diaperinus* beetles across the varying *Wolbachia* techniques. Of note, the Cytoplasmic transfer of wHa had the lowest hatch rate which was statistically different from SPG injected eggs.

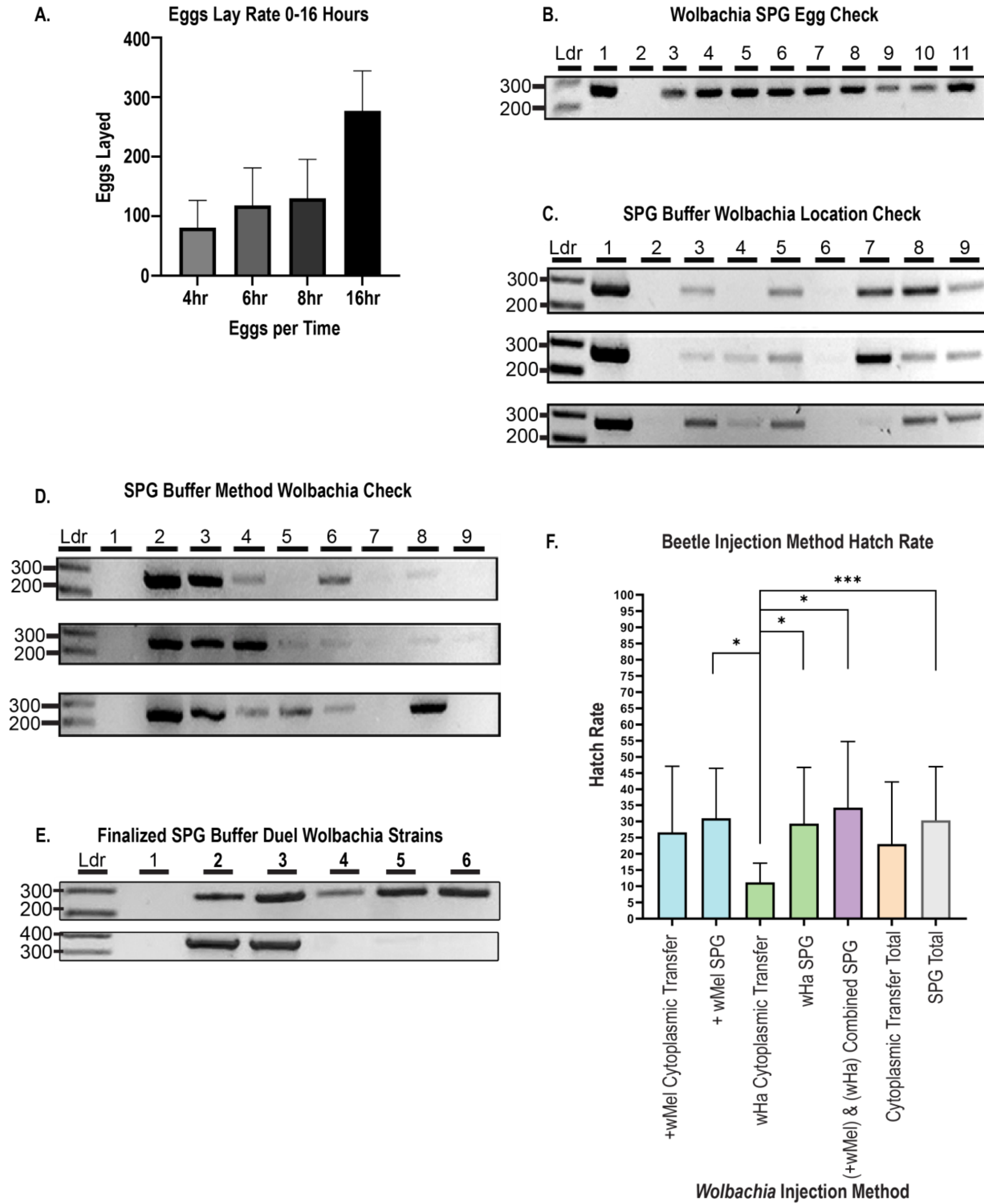


Fig 2. Wolbachia SPG Buffer Methodology Assessment. VirD4 primers were used for *Wolbachia* confirmation **A.** Egg Lay Rate 0-16hr. ~100-200 189+wMel *Drosophila* flies set on

apple juice plates with eggs checked at 4, 6, 8, 16 hours. **B.** Wolbachia SPG Egg Check. Three replicates of 50, 100, and 200 189+wMel. Lanes: 1=100bp ladder, 2=189+wMel Flies, 3=50 egg SPG buffer, 4=100 egg SPG buffer, 5=200 egg SPG buffer, 6=50 egg SPG buffer, 7=100 egg SPG buffer, 8=200 egg SPG buffer, 9=50 egg SPG buffer, 10=100 egg SPG buffer, 11=200 egg SPG buffer. **C.** SPG Buffer *Wolbachia* Location Check. This process was used to see if *Wolbachia* was present or lost in any part of the original buffer protocol. Lanes: 100bp ladder, 1= 189+wMel adult flies, 2= water, 3= after egg homogenization, 4= supernatant after 300G/5min spin, 5= pellet after 300G/5min spin, 6= supernatant after 12000G/10min spin, 7= pellet after 12000G/10min spin, 8= supernatant after 300G/3 min spin, 9= pellet after 300G/3min spin. **D.** SPG Buffer Method *Wolbachia* Check. This shows supernatants and pellets of the three buffer methods. Each method involves the usage of ~150 189+wMel fly eggs ~16hr overnight for usage. Method 1 involves dechorionating the egg, homogenization, and centrifuge 12000G for 10 min; Method 2 involves homogenization, push through 5-micron filter, and centrifuge 12000G for 10 min; Method 3 involves homogenization of eggs then centrifuge 12000G for 10 min. Lanes: 100bp ladder, 1= water, 2= 189+wMel adult flies, 3= 189+wMel eggs, 4= Method 1 pellet, 5= Method 1 supernatant, 6= Method 2 pellet, 7 = Method 2 supernatant, 8= Method 3 pellet, Method 9 supernatant. **E.** Finalized SPG Buffer Duel Wolbachia Strains check. Both VirD4 and His3 primers were used. SPG buffer is made from finalized method (Method 1) with wMel & wHa strains. Lanes: 100bp ladder, 1= ~50 wMel adult flies, 2= ~50 wHa adult flies, 4 = wMel + wHa Combined SPG, 5=Wmel + wHa Combined SPG, 6 = wMel + wHa Combined SPG. **F.** Beetle Injection Method Hatch Rate. *A. diaperinus* hatch rate percentage after injection by both cytoplasmic transfer, SPG buffer injection with either +wMel, wHa, or +wMel & wHa combined strains is displayed.

Following buffer optimization, we assessed if the microinjection process was indeed injecting *Wolbachia* into *A. diaperinus* eggs. **Fig 3** illustrates these *Wolbachia* tracking checks. **Fig 3A** shows *Wolbachia* presence in eggs immediately following injection. Of note, not all replicates of both cytoplasmic transfer and SPG buffers showed *Wolbachia* in one or multiple replicates. Additionally, the strength of *Wolbachia* band size varies both among treatment and replica. This may indicate that while *Wolbachia* is present in SPG buffer or initial infected egg, the amount transferred via microinjection to the beetles may vary from not detectible to detectible. **Fig 3B** goes a step farther by screening hatched wMel + wHa combined SPG injected larvae. Of the 70 larvae screened (in groups of five individuals), three groups of five individuals show *Wolbachi*a presence. One band appears strong while the other two appear less vibrant/weak. The visualization in these groups suggests between aa ~4% (3/70) to ~21% (15/70) percent chance of hatched larvae showing presence of *Wolbachia*. **Fig 3C** continues testing hatched wMel, wHa, and combined injected larvae. PCR results reveal ~3-4 bands out of the 8 replicates per injection type mostly faint scattered across the 3 strain comprised buffers. Of note, some samples showed VirD4 confirmation but no histone confirmation, and the wHa gel showed no VirD4 band in the positive control. The presence of only very faint bands suggests larvae may not be up taking concentrated amounts of *Wolbachia*. On the contrary, however, the presence of darker bands may indicate success as only a small number of larvae were screened per group, with larvae being ~1-2mm in size and having only received a minute volume of *Wolbachia* which came from a < ~1µl egg injection. Taking the ~3/8 *Wolbachia* PCR band visual confirmation, roughly 38% of hatched larvae may still contain *Wolbachia* post egg injection.

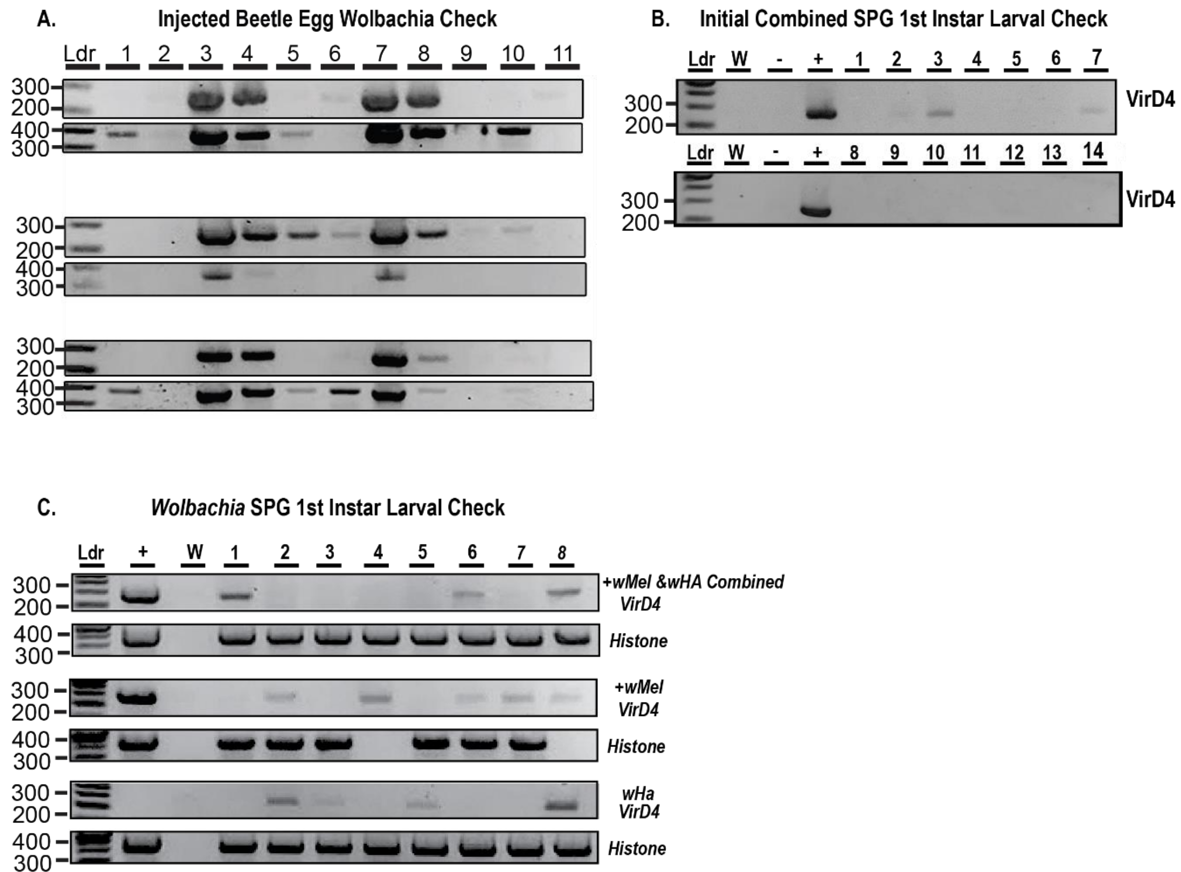


Fig 3. A. Injected Beetle Egg *Wolbachia Check*. Check of presence of *Wolbachia* in injected beetle eggs. VirD4 and His3 primers were used. Lanes: 100bp ladder, 1= 10 *A. diaperinus* eggs, 2= 10 PBS injected *A. diaperinus eggs*, 3= ~50 189+wMel adult flies, 4= +wMel SPG, 5= +wMel SPG injected *A. diaperinus* eggs (10), 6= +wMel cytoplasmic transfer *A. diaperinus* eggs (10), 7 = ~50 wHa adult flies, 8= wHa SPG, 9= wHa SPG injected *A. diaperinus* eggs (10), 10= wHa cytoplasmic transfer *A. diaperinus* eggs (10). **B.** Initial Combined SPG 1st Instar Larval Check. Checking the larvae of combined strain injected *A. diaperinus* for *Wolbachia*. Lanes: 100bp ladder, W= water, - = *A. diaperinus*, + = ~50 189+wMel adult flies, 1-14= 5 +wMel & wHa combined SPG injected 1st instar hatched larvae.

This research focused on optimizing *Wolbachia* microinjection into *A. diaperinus*. Our findings suggest that *A. diaperinus* could be a promising candidate for *Wolbachia* population control through Incompatible Insect Technique (IIT), as native poultry house populations have demonstrated a lack of natural *Wolbachia* infection. We established an efficient method for egg collection that facilitates microinjection and evaluated the viability of pre-treatment strategies, such as dechorionation and halocarbon oil application, to enhance egg stability prior to injection. Efficient incubation temperature conditions were identified to improve hatch rate and survivorship of injected eggs. We achieved successful *Wolbachia* injection into *A. diaperinus* eggs using both cytoplasmic transfer and *Wolbachia* purified SPG buffer. The SPG buffer-based method was further optimized for increased *Wolbachia* concentration, preparation efficiency, and loss mitigation to improve concentration of future injections. Hatch rates were assessed following injection with both cytoplasmic and SPG buffer transfer methods for wMel and wHa *Wolbachia* strains, including their combined strains in a single buffer. Through PCR, we confirmed *Wolbachia* presence in the SPG buffer, injected eggs, and larvae hatched from injected eggs, demonstrating our ability to track infection throughout initial development. Future steps may include large-scale injection of eggs with combined wMel and wHa SPG buffer, with the aim of rearing these larvae to adulthood for assessment of establishment of a stable *Wolbachia*-infected *A. diaperinus* colony as well as *Wolbachia* persistence through growth.

References

Alves Vs, Neves Pmdo, Alves Lf, Moino Jr A, Holz N (2012) Entomopathogenic nematodes (*Rhabditida: Heterorhabditidae* and *Steinernematidae*) screening for lesser mealworm *Alphitobius diaperinus* (*Coleoptera: Tenebrionidae*) control. *Revista Colombiana de Entomología* **38**:76–80

Axtell RC, Arends JJ (1990) Ecology and Management of Arthropod Pests of Poultry. *Annual Review of Entomology*. *35*(1), 101-126.

Beckmann JF, Bonneau M, Chen H, Hochstrasser M, Poinsot D, Merçot H, Weill M, Sicard M, Charlat S (2019a) The Toxin–Antidote Model of Cytoplasmic Incompatibility: Genetics and Evolutionary Implications. *Trends in Genetics* **35**:175–185. https://doi.org/10.1016/j.tig.2018.12.004

Beckmann JF, Dormitorio T, Oladipupo SO, Bethonico Terra MT, Lawrence K, Macklin KS, Hauck R (2021) *Heterakis gallinarum* and *Histomonas meleagridis* DNA persists in chicken houses years after depopulation. *Veterinary Parasitology* **298**:109536. https://doi.org/10.1016/j.vetpar.2021.109536

Beckmann JF, Ronau JA, Hochstrasser M (2017) A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nat Microbiol* **2**:1–7. https://doi.org/10.1038/nmicrobiol.2017.7

Beckmann JF, Sharma GD, Mendez L, Chen H, Hochstrasser M (2019b) The *Wolbachia* cytoplasmic incompatibility enzyme CidB targets nuclear import and protamine-histone exchange factors. *eLife* **8**:e50026. https://doi.org/10.7554/eLife.50026

Bordenstein SR, O'Hara FP, Werren JH (2001) *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in Nasonia. *Nature* **409**:707–710. https://doi.org/10.1038/35055543

Bui M, Li M, Raban R, Liu N (2020) Embryo microinjection techniques for efficient site-specific mutagenesis in *Culex quinquefasciatus*. *Journal of Visualized Experiments*. https://doi.org/10.3791/61375

De Las Casas E, Harein PK, Deshmukh DR, Pomeroy BS (1976) Relationship between the lesser mealworm, fowl pox, and Newcastle disease virus in poultry12. *Journal of Economic Entomology* **69**:775–779. https://doi.org/10.1093/jee/69.6.775

de las Casas E, Pomeroy BS, Harein PK (1968) Infection and quantitative recovery of *Salmonella typhimurium* and *Escherichia coli* from within the lesser mealworm, *Alphitobius diaperinus* (Panzer).1,2. *Poultry Science* **47**:1871–1875. https://doi.org/10.3382/ps.0471871

Del Valle EE, Frizzo LS, Malmierca M, Zbrun MV, Lax P, Doucet ME (2016) Biological control of *Alphitobius diaperinus* with *Steinernema rarum* CUL and *Heterorhabditis bacteriophora* SMC and feasibility of application in rice hull. *J Pest Sci* **89**:161–170. https://doi.org/10.1007/s10340-015-0669-8

Despins JL, Axtell RC (1995) Feeding behavior and growth of broiler chicks fed larvae of the darkling beetle, *Alphitobius diaperinus*. *Poultry Science* **74**:331–336. https://doi.org/10.3382/ps.0740331

Despins JL, Axtell RC, Rives DV, Guy JS, Ficken MD (1994) Transmission of enteric pathogens of turkeys by darkling beetle larva (*Alphitobius diaperinus*). *Journal of Applied Poultry Research* **3**:61–65. https://doi.org/10.1093/japr/3.1.61

Despins JL, Turner EC, Ruszler PL (1987) Construction profiles of high rise caged layer houses in association with insulation damage caused by the lesser mealworm, *Alphitobius diaperinus* (Panzer) in Virginia. *Poultry Science* **66**:243–250. https://doi.org/10.3382/ps.0660243

Dinev I (2013) The darkling beetle (*Alphitobius diaperinus*)—A health hazard for broiler chicken production. *Trakia J Sci* **1**:1–4

Geden CJ, Axtell RC (1987) Factors affecting climbing and tunneling behavior of the lesser mealworm (*Coleoptera: Tenebrionidae*). *Journal of Economic Entomology* **80**:1197–1204. https://doi.org/10.1093/jee/80.6.1197

Geden CJ, Hogsette J (1994) Research and extension needs for integrated pest management for arthropods of veterinary importance. Publications from USDA-ARS / UNL Faculty 1039

Goodwin MA, Waltman WD (1996) transmission of eimeria, viruses, and bacteria to chicks: darkling beetles (*Alphitobius diaperinus*) as vectors of pathogens. *Journal of Applied Poultry Research* **5**:51–55. https://doi.org/10.1093/japr/5.1.51

Gussem MD (2007) Coccidiosis in poultry: review on diagnosis, control, prevention and interaction with overall gut health. Proceedings of the 16th *European Symposium on Poultry Nutrition* 253–261

Hague MTJ, Caldwell CN, Cooper BS (2020) Pervasive effects of *Wolbachia* on host temperature preference. *mBio* **11**:10.1128/mbio.01768-20. https://doi.org/10.1128/mbio.01768-20

Harrell RA (2024) Mosquito embryo microinjection under halocarbon oil or in aqueous solution. Cold Spring Harb Protoc 2024.7, prot108203

Hazeleger WC, Bolder NM, Beumer RR, Jacobs-Reitsma WF (2008) Darkling beetles (*Alphitobius diaperinus*) and their larvae as potential vectors for the transfer of *Campylobacter jejuni* and *Salmonella enterica Serovar Paratyphi B* variant java between successive broiler flocks. *Applied and Environmental Microbiology* **74**:6887–6891. https://doi.org/10.1128/AEM.00451-08

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, O'Neill SL (2011) Successful establishment of *Wolbachia* in Aedes populations to suppress dengue transmission. *Nature* **476**:454–457. https://doi.org/10.1038/nature10356

Joubert DA, Walker T, Carrington LB, Bruyne JTD, Kien DHT, Hoang NLT, Chau NVV, Iturbe-Ormaetxe I, Simmons CP, O'Neill SL (2016) Establishment of a *Wolbachia* superinfection in *Aedes aegypti* mosquitoes as a potential approach for future resistance management. *PLOS Pathogens* **12**:e1005434. https://doi.org/10.1371/journal.ppat.1005434

Knipling EF (1955) Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology* **48**:459–462. https://doi.org/10.1093/jee/48.4.459

Knipling EF (1959) Sterile-male method of population control. *Science* **130**:902–904

Laven H (1967) A possible model for speciation by cytoplasmic isolation in the *Culex pipiens* complex. Bulletin of the *World Health Organization* **37**:263

McAllister JC (1993) Determination of the potential of lesser mealworms, *Alphitobius diaperinus* (*Coleoptera: Tenebrionidae*), to transmit poultry pathogens. Ph.D., University of Arkansas

McAllister JC, Steelman CD, Skeeles JK (1994) Reservoir competence of the lesser mealworm (*Coleoptera: Tenebrionidae*) for *Salmonella typhimurium* (*Eubacteriales: Enterobacteriaceae*). *Journal of Medical Entomology* **31**:369–372. https://doi.org/10.1093/jmedent/31.3.369

Mouton L, Henri H, Bouletreau M, Vavre F (2006) Effect of temperature on *Wolbachia* density and impact on cytoplasmic incompatibility. *Parasitology* **132**:49–56. https://doi.org/10.1017/S0031182005008723

Mozaffar H, Khan A, Hosen M (2004) Growth and development of the lesser mealworm, *Alphitobius diaperinus* (Panzer) (*Coleoptera: Tenebrionidae*) on cereal flours. *Pakistan Journal of Biological Sciences* 7. https://doi.org/10.3923/pjbs.2004.1505.1508

Nazni WA, Hoffmann AA, NoorAfizah A, Cheong YL, Mancini MV, Golding N, Kamarul GMR, Arif MAK, Thohir H, NurSyamimi H, ZatilAqmar MZ, NurRuqqayah M, NorSyazwani A, Faiz A, Irfan F-RMN, Rubaaini S, Nuradila N, Nizam NMN, Irwan SM, Endersby-Harshman NM, White VL, Ant TH, Herd CS, Hasnor AH, AbuBakar R, Hapsah DM, Khadijah K, Kamilan D, Lee SC, Paid YM, Fadzilah K, Topek O, Gill BS, Lee HL, Sinkins SP (2019) Establishment of *Wolbachia* strain wAlbB in Malaysian populations of

Aedes aegypti for Dengue control. *Current Biology* **29**:4241-4248.e5. https://doi.org/10.1016/j.cub.2019.11.007

O'Neill SL, Ryan PA, Turley AP, Wilson G, Retzki K, Iturbe-Ormaetxe I, Dong Y, Kenny N, Paton CJ, Ritchie SA, Brown-Kenyon J, Stanford D, Wittmeier N, Jewell NP, Tanamas SK, Anders KL, Simmons CP (2019) Scaled deployment of *Wolbachia* to protect the community from dengue and other *Aedes* transmitted arboviruses. *Gates Open Research* **2**:36. https://doi.org/10.12688/gatesopenres.12844.2

Pearce J, Housden A, Senior N, Champion O, Prior J, Titball R, Wakefield J (2024) A microinjection protocol for the greater waxworm moth, *Galleria mellonella*. *bioRxiv*, 2024-09.

Retamales J, Vivallo F, Robeson J (2011) Insects associated with chicken manure in a breeder poultry farm of Central Chile. *Arch med vet* **43**:79–83. https://doi.org/10.4067/S0301-732X2011000100011

Reyna PS, McDougald LR, Mathis GF (1983) Survival of coccidia in poultry litter and reservoirs of infection. *Avian Diseases 27*:464–473. https://doi.org/10.2307/1590172

Rueda L m., Axtell RC (1996) Temperature-dependent development and survival of the lesser mealworm, *Alphitobius diaperinus*. *Medical and Veterinary Entomology* **10**:80–86. https://doi.org/10.1111/j.1365-2915.1996.tb00085.x

Salin C, Delettre YR, Cannavacciuolo M, Vernon P (2000) Spatial distribution of *Alphitobius diaperinus* (Panzer) (*Coleoptera: Tenebrionidae*) in the soil of a poultry house along a

breeding cycle. *European Journal of Soil Biology* **36**:107–115. https://doi.org/10.1016/S1164-5563(00)01054-2

Sammarco BC, Hinkle NC, Crossley MS (2023) Biology and management of lesser mealworm *Alphitobius diaperinus* (*Coleoptera: Tenebrionidae*) in broiler houses. *Journal of Integrated Pest Management* **14**:2. https://doi.org/10.1093/jipm/pmad003

Santoro PH, Neves PMOJ, Alexandre TM, Sartori D, Alves LFA, Fungaro MHP (2008) Selection of *Beauveria bassiana* isolates to control *Alphitobius diaperinus*. *Journal of Invertebrate Pathology* **97**:83–90. https://doi.org/10.1016/j.jip.2007.07.009

Shropshire JD, Leigh B, Bordenstein SR (2020) Symbiont-mediated cytoplasmic incompatibility: What have we learned in 50 years? *eLife* **9**:e61989. https://doi.org/10.7554/eLife.61989

Sicard M, Bouchon D, Ceyrac L, Raimond R, Thierry M, Le Clec'h W, Marcadé I, Caubet Y, Grève P (2014) Bidirectional cytoplasmic incompatibility caused by *Wolbachia* in the terrestrial isopod *Porcellio dilatatus*. *Journal of Invertebrate Pathology* **121**:28–36. https://doi.org/10.1016/j.jip.2014.06.007

Sinkins S (1995) *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 261(1362), 325-330.

Skov MN, Spencer AG, Hald B, Petersen L, Nauerby B, Carstensen B, Madsen M (2004) The role of litter beetles as potential reservoir for *Salmonella enterica* and *Thermophilic Campylobacter spp.* between broiler flocks. *avdi* **48**:9–18. https://doi.org/10.1637/5698

Smith R, Hauck R, Macklin K, Price S, Dormitorio T, Wang C (2022) A review of the lesser mealworm beetle (*Alphitobius diaperinus*) as a reservoir for poultry bacterial pathogens and antimicrobial resistance. *World's Poultry Science Journal* **78**:197–214. https://doi.org/10.1080/00439339.2022.2003172

Strother KO, Steelman CD, Gbur EE (2005) Reservoir competence of lesser mealworm (*Coleoptera: Tenebrionidae*) for *Campylobacter jejuni (Campylobacterales: Campylobacteraceae*). *Journal of Medical Entomology* **42**:42–47. https://doi.org/10.1093/jmedent/42.1.42

Su Y, Lin H-C, Dale C (2023) Protocol to establish a genetically tractable synthetic symbiosis between *Sodalis praecaptivus* and grain weevils by insect egg microinjection. *STAR Protocols* **4**:102156. https://doi.org/10.1016/j.xpro.2023.102156

Ulrich JN, Beier JC, Devine GJ, Hugo LE (2016) Heat Sensitivity of wMel Wolbachia during *Aedes aegypti* Development. *PLOS Neglected Tropical Diseases* **10**:e0004873. https://doi.org/10.1371/journal.pntd.0004873

Vaughan JA (1982) Biology and control of the lesser mealworm. Dissertation. Virginia Polytechnic Institute and State University

Vaughan JA, Turner EC, Ruszler PL (1984) Infestation and damage of poultry house insulation by the lesser mealworm, *Alphitobius diaperinus* (Panzer)1,2. *Poultry Science* **63**:1094–1100. https://doi.org/10.3382/ps.0631094

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: Master manipulators of invertebrate biology. *Nat Rev Microbiol* **6**:741–751. https://doi.org/10.1038/nrmicro1969

Xi Z, Dobson SL (2005) Characterization of *Wolbachia* transfection efficiency by using microinjection of embryonic cytoplasm and embryo homogenate. *Applied and Environmental Microbiology* **71**:3199–3204. https://doi.org/10.1128/AEM.71.6.3199-3204.2005

Xu Q, Guerrero FD, Palavesam A, León AAP de (2015) Use of electroporation as an option to transform the horn fly, *Haematobia irritans*: a species recalcitrant to microinjection. *Insect Science*, *23*(4), 621-629

Yoshiyama M, Kimura K, Honda H (2000) Technique for microinjection into eggs of the housefly, *Musca domestica* (*Diptera: Muscidae*). *Applied Entomology and Zoology* **35**:27–30. https://doi.org/10.1303/aez.2000.27

Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, Bourtzis K (2004) *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences* **101**:15042–15045. https://doi.org/10.1073/pnas.0403853101

Zafeiriadis S, Baliota GV, Athanassiou CG (2023) The effect of temperature and moisture content on population growth of *Alphitobius diaperinus* (Panzer) (*Coleoptera: Tenebrionidae*). *Agronomy* **13**:2535. https://doi.org/10.3390/agronomy13102535

Zhang D, Lees RS, Xi Z, Gilles JRL, Bourtzis K (2015a) Combining the sterile insect technique with *Wolbachia*-based approaches: II- a safer approach to *Aedes albopictus* population suppression programmes, designed to minimize the consequences of inadvertent female release. *PLOS ONE* **10**:e0135194. https://doi.org/10.1371/journal.pone.0135194

Zhang D, Zheng X, Xi Z, Bourtzis K, Gilles JRL (2015b) Combining the sterile insect technique with the incompatible insect technique: I-Impact of *Wolbachia* infection on the fitness of triple- and double-infected strains of *Aedes albopictus*. *PLOS ONE* **10**:e0121126. https://doi.org/10.1371/journal.pone.0121126

Zheng B, Yu J, Li J (2021) Modeling and analysis of the implementation of the *wolbachia* incompatible and sterile insect technique for mosquito population suppression. *SIAM J Appl Math* **81**:718–740. https://doi.org/10.1137/20M1368367

Chapter 5:

Using Baker's Yeast to Determine Functions of Novel *Wolbachia* (and other prokaryotic) Effectors.

Abstract

Yeast are single-celled eukaryotic organisms classified as fungi, mostly in the phylum Ascomycota. Of about 1500 named species, *Saccharomyces cerevisiae*, also known as baker's yeast, domesticated by humans in the context of cooking and brewing, is a profound genetic tool for exploring functions of novel effector proteins from *Wolbachia* and prokaryotes in general. *Wolbachia* is a Gram-negative alpha-proteobacterium that infects up to ~75% of all insects as an obligate intracellular microbe (Jeyaprakash 2000). *Wolbachia's* lifestyle presents unique challenges for researchers. *Wolbachia* cannot be axenically cultured and has never been genetically manipulated. Furthermore, many *Wolbachia* genes have no known function or well-annotated orthologs in other genomes. Yet given the effects of *Wolbachia* on host phenotypes, which have considerable practical applications for pest control, they undoubtedly involve secreted effector proteins that interact with host gene products. Studying these effectors is challenging with *Wolbachia's* current genetic limitations. However, some of the constraints to working with *Wolbachia* can be overcome by expressing candidate proteins in *S. cerevisiae*. This approach capitalizes on yeast's small genome (~6500 genes), typical eukaryotic cellular organization, and the sophisticated suite of genetic tools available for its manipulation in culture. Thus, yeast can serve as a powerful mock eukaryotic host background to study *Wolbachia* effector function. Specifically, yeast is used for recombinant protein expression, drug discovery, protein localization studies, protein interaction mapping (yeast two-hybrid system), modelling chromosomal evolution, and has been used for examining interactions between proteins responsible for complex

phenotypes in less tractable prokaryotic systems. As an example the paired genes responsible for *Wolbachia*-mediated cytoplasmic incompatibility (CI) encode novel proteins with limited homology to other known proteins, and no obvious function. This article details how *S. cerevisiae* was used as an initial staging ground to explore the molecular basis of one of *Wolbachia's* trademark phenotypes (CI).

1. Introduction

Egyptians and Sumerians brewed beer with *S. cerevisiae* more than 8 thousand years ago (Feldmann 2011) and strains of this versatile fungus are widely used throughout the world to make wines, ale, sake, and leavened breads (Greig 2009). In 1857 the famous microbiologist, Louis Pasteur, characterized the fermentation abilities of yeast (Pasteur 1858), and in the 1930's the Carlsberg Laboratory was first to manipulate budding yeast for better brewing traits (Barnett 2007). Budding refers to *S. cerevisiae's* mode of reproduction, where a daughter cell buds off the mother cell prior to separation by cell division (Hartwell 1974). Other yeasts, known as fission yeasts, are rod-shaped, and divide by medial fission. This chapter focuses exclusively on *S. cerevisiae*.

S. cerevisiae is the first eukaryote to have its entire genome sequenced (Goffeau 1996). Most of its ~6500 genes have well-described functions, have been mapped to yeast chromosomes, and are referenced in public repositories like the *Saccharomyces* Genome Database (SGD; https://www.yeastgenome.org/). *S. cerevisiae* has comparatively fewer genes than other eukaryotes and many of them are highly conserved and involved in essential eukaryotic cellular processes. When proteins of unknown function exhibit interactions in yeast, these interactions are often conserved in higher eukaryotes and translate to other organismal contexts. Genetic tools have been constructed such that complementation of a yeast deficiency, or alteration of a yeast

phenotype by expression of a gene of unknown function from another species can provide insight into properties of the unknown gene. Furthermore, yeast undergo meiosis, have haploid and diploid phases, and sexual reproduction; thus they enable the study of crossing over and other complex eukaryotic phenomena (Feldmann 2001).

S. cerevisiae used in the laboratory have been specifically engineered for scientific experimentation. The S288c *S. cerevisiae* strain has been adopted by the scientific community as a main reference isolate and is maintained in a stable haploid state, which facilitates detection of mutations (Mortimer 1986). Since 1996, multiple strain libraries have been made from S288c, including libraries with varying deletions, tagged reporter genes, overexpression constructs, and mutants (Jones et al. 2008, Liti 2015, Giaever 2014, Howson 2005). A physical library typically consists of 96-well plates or cryotubes in which each well/vial contains a unique (frozen) clonal isolate. Deletion libraries contain clones with individual deletions of non-essential genes, and/or temperature sensitive mutants for essential genes (Giaever 2014); overexpression libraries contain strains with nearly all genes cloned into high copy plasmids (Jones 2008); and GFP libraries contain individual genes fused to GFP fluorophores that facilitate localization experiments (Howson 2005). In addition to these tools, yeast also exhibits highly efficient recombination which has been used to assemble plasmids *in vivo* (Joska 2014). Another advantage of the yeast model is speed: the S288c strain doubling time can be manipulated but is typically ~90 minutes (Thompson 1980). Yeast clones can be cryopreserved for long periods of time and still grow rapidly from a single individual isolate years later (Greig 2009). Research can move quickly in yeast because many genetic toolkits and reagents are already built. Considering these tools, researchers working on organisms where genetics are less tractable (like obligate intracellular prokaryotes that live in

eukaryotic cells) have employed yeast systems as a valuable resource to circumnavigate technical genetic problems (Beckmann et al. 2019, 2021, Berk 2022).

Here we describe use of yeast to investigate effector proteins involved in the *Wolbachia*-associated phenotype called CI (Beckmann 2021, Werren 2008, Chen 2020, Zheng 2019, Moreira 2009). CI is a unique form of reproductive parasitism (Gillespie 2018) that causes embryonic death when *Wolbachia*-modified sperm fertilizes uninfected eggs (Presgraves 2000). Understanding the molecular details of CI and of *Wolbachia* effector proteins in general would facilitate downstream applications of *Wolbachia*-based phenotypes for insect control. Because many prokaryotic effector proteins have no known function, a first step toward functional understanding can be cloning the unknown effector into a yeast plasmid (Beckmann 2017, 2019, 2021, Chen et al. 2019, Sun et al. 2022, Sheehan et al. 2016, Rice 2017, Xiao et al. 2021). The reasoning underlying this approach with respect to *Wolbachia* is based on its obligate intracellular lifestyle within a eukaryotic host. To affect host reproduction, *Wolbachia* must secrete effectors that interact with eukaryotic host proteins (Rice 2017, Whitaker et al. 2016). Thus, testing functions in yeast have provided important preliminary data (Beckmann 2017, Rice 2017). This paradigm is not only applicable to *Wolbachia*, but universally applies to any prokaryotic organism or virus protein that must interact with a eukaryotic context. In this article we will outline examples of yeast studies that provided new, novel insights into *Wolbachia*-mediated CI and discuss details of how to perform basic analysis using yeast for the novice intent on exploring these tools.

2. Yeast Overexpression Studies Reveal Genetic Features of Wolbachia CI Loci

Proteins involved in CI were uncovered by examining protein extracts from mosquito spermathecae. These sperm storage organs would be expected to contain *Wolbachia*-modified sperm, but not *Wolbachia* itself, which is eliminated during sperm maturation in the male. When

identified, the genes encoding CI loci were uncharacterized and had minimal homology to anything outside *Wolbachia* (Beckmann 2013). Today, CI controlling genes dubbed CI factors (*cifs*) are numerous and form a family of clearly related homologs/orthologs/paralogs. Biochemically, only three functions have been confirmed for *cifs.* These activities include deubiquitylating functions in CI inducing deubiquitylases (*cids*) and DNA nuclease activity in CI inducing nucleases (*cins*) (see **Fig 1**) (Beckmann 2017, 2021, Chen et al. 2019).

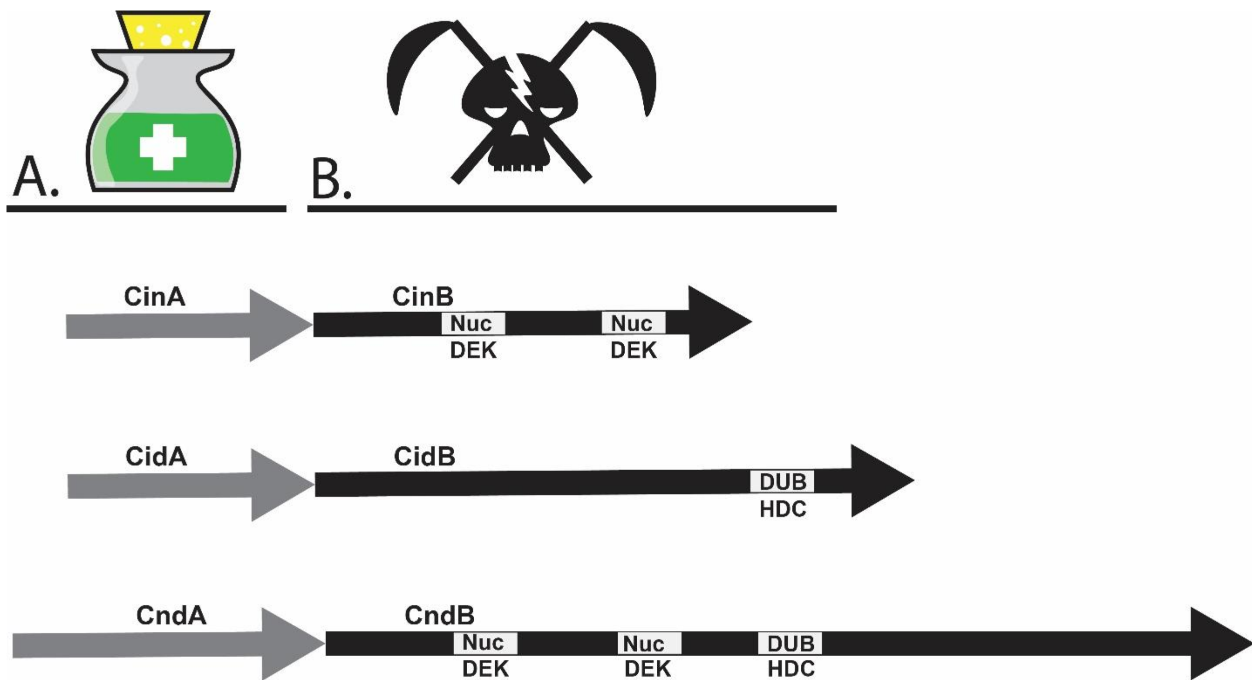


Fig 1. Schematic representation of CI operons. Three operons are shown, with tandem antidote (A) and toxin (B) genes. The A proteins are poorly understood, and are named according to motifs in the cognate toxin protein: CinA/B operons (top) encode a nuclease domain (Nuc) with a catalytic (DEK) triad; CidA/B operons encode a deubiquitylase (DUB) with a catalytic (HDC)

triad, and Cnd operons encode both motifs in the toxin protein. The (+) vial represents the antidotes (A genes), while the skull and scythes represent the toxins (B genes).

When yeast studies were initiated, it was known *cifs* were organized as two-gene operons, that Cif proteins were enriched in mature sperm, and that presence/absence of *cif* genes correlated with CI phenotype (Beckmann 2013). Thus, the challenge was how to begin functional characterization of two completely novel genes with potential DUB and/or nuclease functions. Initial insights emerged from cloning multiple *cifs* from *Wolbachia* genomes into various yeast overexpression plasmids (Beckmann 2017). In short, a first strategy to understand *cif* gene function was to simply overexpress the genes in yeast and look for phenotypes.

Overexpression studies seek to induce accumulation of a target protein to measure downstream side effects. Overexpression relies on two key features of yeast plasmids. Plasmids that overexpress cloned DNA are engineered to contain high copy origins of replication. In yeast, the high copy replication origin (2μ origin) comes from the 2-micron endogenous yeast plasmid (Futcher 1988, Strope et al. 2015). The presence of this origin raises plasmid copy number to about 60 copies per yeast cell (Chan et al. 2013). Additionally, overexpression plasmids typically utilize strong inducible promoters like GAL1P upstream of a multi-cloning site. The GAL1P promoter turns on expression of target genes when the medium is supplemented with the sugar galactose and represses expression in the presence of glucose (Mumberg 1994, Mumberg 1995). Notably, eukaryotic promoters in yeast are flexible in terms of start codon positioning and need only be proximal to the 5' upstream open reading frame to induce strong expression. This is a stark contrast when compared to prokaryotic recombinant expression plasmids where exact positioning of the ATG start codon is absolutely required for successful transcription. In summary, the abundance of

mRNA derived from plasmid inserts reflects two independent mechanisms: high copy number and strong promoters.

One of the first phenotypes that can easily be observed in yeast overexpression experiments is an effect on cell growth. Cell growth differences are qualitatively observed by plating cells (see **Fig 2**). In serial dilutions, researchers standardize cultures to an initial optical density (OD) reading of 0.5 at 600 nanometers, then plate drops of cells in at 5-fold decreasing concentrations. Yeast serial dilutions are highly sensitive to minute growth differences. While not quantitative per se, they are reliable, sensitive, and discerning in a qualitative sense.

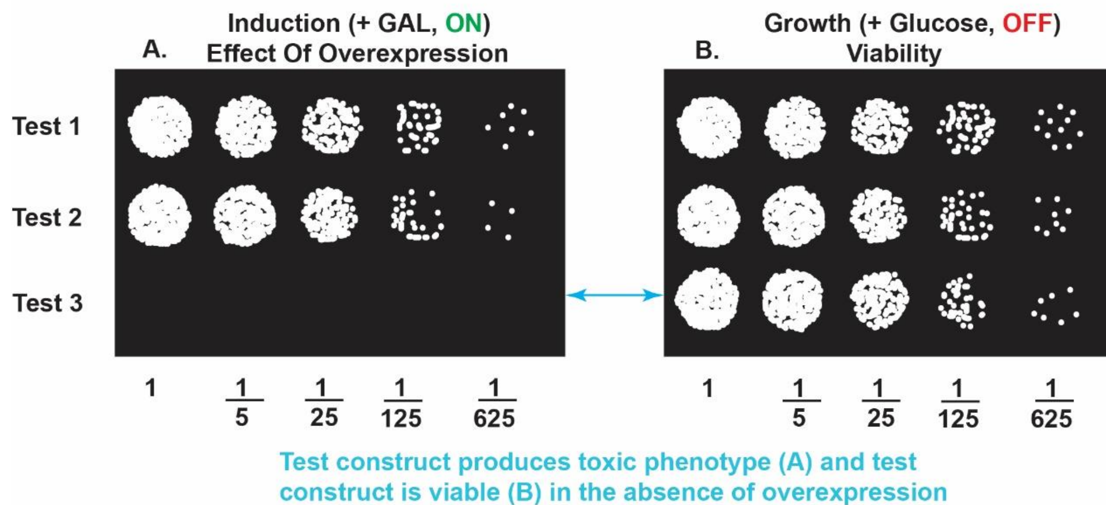


Fig 2. A conceptual serial dilution constructed in Adobe Illustrator demonstrating how to interpret and analyze typical yeast serial dilution data. Yeast growth is represented by clones (circular white dots). **A.** Shows mock cells plated in five dilutions (1x-1/625x) under inducing medium [ON] with three theoretical overexpression constructs (Test 1-3 etc.). Comparing relative growth of different genotypes on the same plate can confirm phenotypic differences. In this theoretical plate, test 3 is

not growing due to some toxic phenotype. **B.** A mock negative control serial dilution of yeast grown under repressive promoter conditions where the transgene is [OFF] in the presence of glucose. Panel **B** can also be considered a positive control for yeast growth. Comparing the same genotype in panel A to panel B demonstrates that any negative effects on growth are directly due to overexpression of a given transgene.

In an initial study, overexpression analysis and serial dilutions such as those depicted in **Fig 2** provided accurate insights on the functions of *cifs* and the genetic architecture of CI (Beckmann 2017). Because the CI phenotype results in death of fertilized embryos an initial hypothesis was that the inducer of CI would also exhibit toxic effects on yeast cells. To test this hypothesis, *cifB* paralogs (*cinB*$^{w\mathrm{Pip}}$ and *cidB*$^{w\mathrm{Pip}}$*)* and corresponding *cifA* paralogs (*cinA*$^{w\mathrm{Pip}}$ and *cidA*$^{w\mathrm{Pip}}$*)* were overexpressed independently in yeast. Overexpression of the two *cifB* paralogs each resulted in toxic effects on yeast growth while overexpression of *cifA* paralogs did nothing, thus validating that *cifB* genes were sufficient to induce toxic phenotypes in eukaryotes. This constituted the first evidence that cifB alone functions as a toxin in yeast, and supported the sufficiency of *cifB*s as inducers of CI. These data confirmed and agreed with concurrent genomic comparisons suggesting the same, that presence of unique cifB genes correlated with CI induction phenotypes and cifA genes did not (Beckmann 2017, Bonneau et al. 2018, Beckmann et al. 2019).

Once an overexpression phenotype is observed a researcher can dissect the biochemical basis of that phenotype by performing site directed mutagenesis of the transgenes. For example, the *cidB* gene contains a deubiquitylating (DUB) domain and *cinB* a nuclease (Nuc) domain. The mutagenesis of key catalytic amino acids in DUB and nuclease domains domains confirmed that the source of toxicity in yeast was attributable to these enzymatic components (Beckmann 2017,

2021, Chen et al. 2019). Thus, yeast models provided the first evidence for the basic enzymatic activities of *cifs*. One tactical observation worth mentioning regarding site directed mutagenesis in yeast is that the large size of yeast plasmids makes PCR-based mutagenesis less efficient with yeast plasmids as opposed to prokaryotic plasmids. Common mutagenesis methodologies relying on PCR necessitate that a polymerase circumnavigate the entire plasmid without error. In our hands, the most efficient strategy has always been to perform PCR based mutagenesis strategies inside of small prokaryotic plasmids like pBluescript (~3000 bp) then subclone successfully mutated transgenes into destination yeast plasmids via restriction enzyme cloning.

3. Yeast Plasmids Rely on Auxotrophic Selection.

More complicated yeast genetics and follow-up experiments pertaining to CI involved multiple selectable markers and auxotrophic selection. Auxotrophic yeast strains have mutations in key biosynthetic enzymes, primarily those required for amino acid biosynthesis (Baker et al. 1998). Thus, in auxotrophic selection, inability to synthesize a vital biomolecule, by deletion mutants, is restored by providing the wild type gene on a plasmid, making the yeast strain dependent on the plasmid for survival so long as the culture medium lacks the required precursor (i.e., drop-out media).

One example of auxotrophic selection popularized since the 1980s involves mutants that lack the capacity for uracil production (Boeke 1984, Sikorski 1989). In yeast, the URA3 gene is necessary for biosynthesis of pyrimidine nucleobases (Lacroute 1968). Therefore, a yeast bearing a mutation in the URA3 gene requires uracil in the medium. In practice, a specialized plasmid containing a complementing URA3 cassette can then be introduced to the yeast, permitting growth on uracil drop-out media. Thus, auxotrophic selection identifies and confirms insertion of a plasmid complementing the yeast mutation (see **Fig 3**).

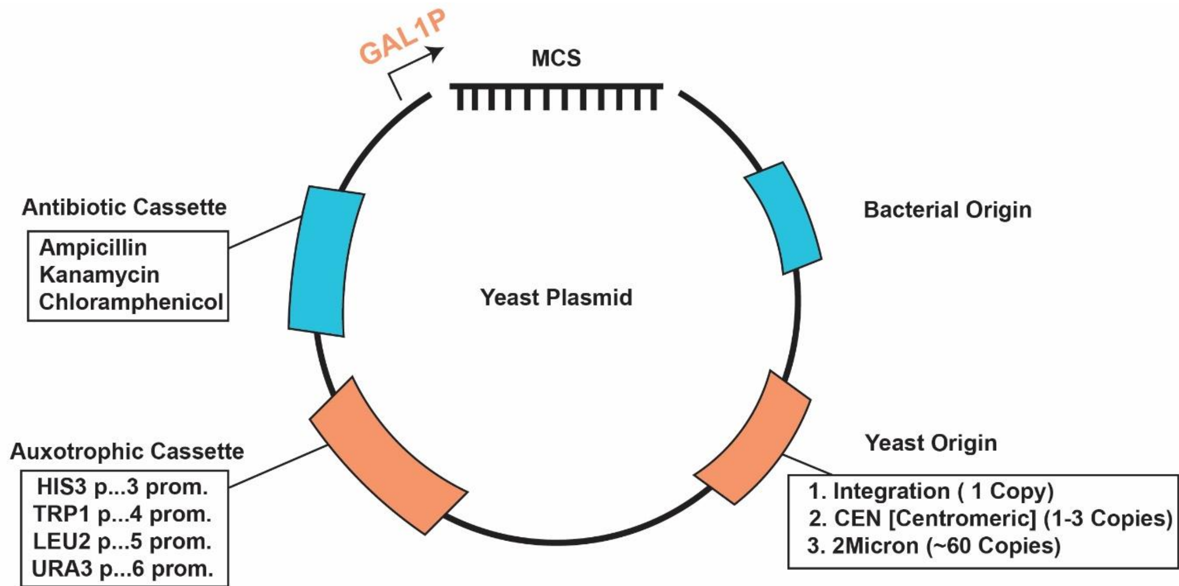


Fig 3. Sample yeast plasmid. Plasmid must first be grown in bacteria, so a bacterial origin and antibiotic selective cassette (Blue rectangles) are required. A yeast origin is also added with the key function of encoding for plasmid copy number, and an auxotrophic selectable cassette is required and specific to a yeast strain selected for growth (orange rectangles). Finally, a yeast promoter such as GAL1P and a multicloning site with restriction enzyme sites for target insertion are included. Yeast plasmid names often contain numerical coding for cassettes and origins. For example, the code "6" designates a URA3 auxotrophic marker. For details see Mumberg and Funk 1994.

In addition to URA3, a plethora of useful yeast auxotrophic markers have been developed, including LYS2, LEU2, TRP1, HIS3, and ADE2. The first four encode essential enzymes in the biosynthetic pathways of lysine, leucine, tryptophan, and histidine (Chattoo et al. 1979, Barnes 1986, Sikorski 1991, Hinnen 1978, Hope 1985, Orr-Weaver 1981). The ADE2 gene encodes a carboxylase required for biosynthesis of adenine. Many of the modern engineered research strains

contain mutations in all these markers (Baker et al. 1998). Importantly, not all research strains have identical mutations. Some markers are generated by full gene deletions and others are generated by point mutations. Importantly, auxotrophic markers that have been generated by point mutations are susceptible to "reverting-mutations" where a spontaneous mutation can regenerate the wild-type sequence thereby eliminating the efficiency of auxotrophic selection. Similarly, various engineered mutations and backgrounds confer additional phenotypes. For example, HIS3 yeast auxotrophs are sensitive to copper, nickel and cobalt salts (Pearce 1999). When using yeast as a research model it is important to diligently research the respective strain genotype to understand its cellular behaviors. For example, Both the BY4741 strain and W303 yeast strain are of interest because in *Wolbachia* effector studies, they showed phenotypic responses to CifB enzymes (Beckmann et al. 2019). However, the BY4741 strain is susceptible to reverting TRP1 mutations because the deletion does not encompass the complete gene (Baker et al. 1998). If possible, reverting marker mutations can be avoided by not using those markers in experiments. Most yeast strains have published genotypes on the Saccharomyces Genome Database. Recipes for common yeast media, dropout mixes, and standard lithium-based transformation protocols are available in yeast methodology textbooks and will not be covered here (Feldmann 2011, Amberg 2005).

4. Yeast Co-expression Studies Evidenced Toxin-Antidote Function of cif Operons.

The toxin-antidote (TA) hypothesis suggested that the genetic architecture underlying CI would be governed by two genes where one gene encodes the toxin and the other, the antidote (Poinsot 2003). Direct evidence that the *cifs* behaved in this pattern was first shown in yeast (Beckmann 2017). To test the toxin-antidote hypothesis *cifB* and *cifA* genes were co-expressed simultaneously. Co-expression studies necessitate a few technical considerations. First plasmid

replication origins need to be carefully chosen to avoid origin competition (Yamaguchi 1982). In origin competition, if two distinct plasmids share the same origin, they will compete for replication factors, and subsequent generations of yeast will lose one or the other plasmid. Thus, coexpression of two transgenes from two plasmids requires compatible replication origins in the individual plasmids. In the early studies with CI effectors, researchers expressed *cifA* antidotes on high copy 2μ plasmids and *cifB* toxins on plasmids with centromeric (CEN) plasmids (Beckmann 2017). CEN plasmids contain an autonomous replication origin from centromeric DNA and cause the plasmid to replicate as a mini chromosome (Marczynski 1985). CEN plasmids occur as ~1-3 copies per cell and are considered low copy plasmids. Importantly CEN plasmids do not compete with 2μ origins and thus facilitate dual co-expression experiments (see **Fig 3** and **Fig 4**). The second technical consideration is that independent plasmids should not utilize the same auxotrophic selectable marker. In early CI studies and in later suppressor screens, researchers successfully utilized URA3 and LEU2 markers on dual CEN and 2μ plasmids to co-express various *cifBs* and *cifAs* (Beckmann 2017, Beckmann et al. 2019). In these studies, CifA proteins were expressed from 2-micron plasmids and CifB from CEN plasmids, so there is more antitoxin than toxin, based on copy numbers

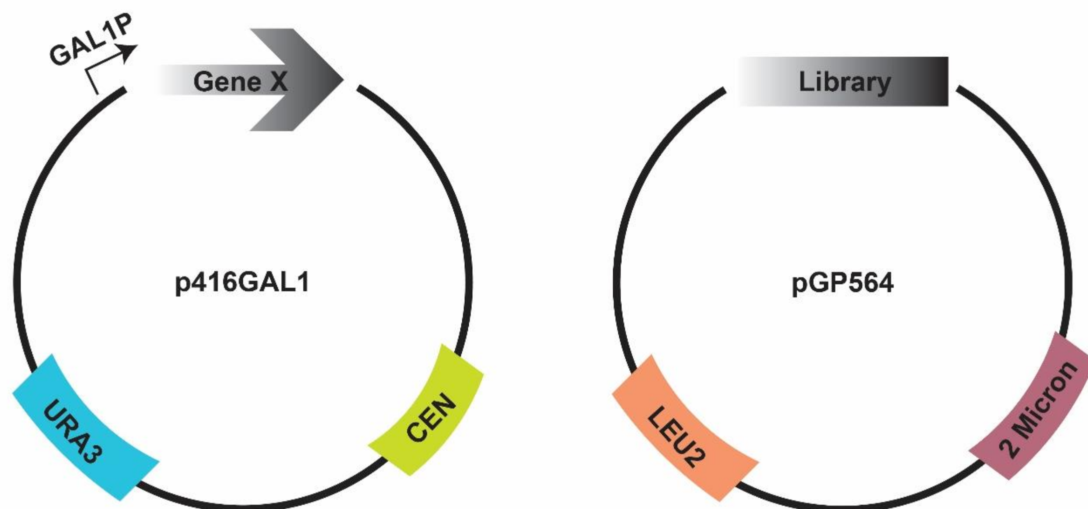


Fig 4. Sample diagram of two compatible co-expression plasmids. The first plasmid (p416GAL1) has CEN (low copy) origin, and URA3 auxotrophic marker, followed by a Gal1P promoter and multicloning site containing target Gene X. The second plasmid (pGP564) has a 2μ origin (high copy number) and a different (LEU2) auxotrophic marker. These two plasmids are compatible and will not compete. In this example, the pGP564 plasmid is from a library and contains a library insert (rather than an individual gene) inserted in multicloning site. The combination of these specific plasmids was used to screen for yeast suppressors of CidB (described below and in Beckmann et al. 2019). The concept is simply that co-expression requires strategic choice of plasmids in each unique case.

Co-expression experiments demonstrated that *cifA* proteins expressed from a 2 micron plasmid rescued the toxic effects of *cifB* expressed from a CEN plasmid. Rescue only occurred when toxins matched their cognate antidote thereby demonstrating toxin-antidote functional specificity of multiple *cif* operons (Beckmann 2017, Chen et al. 2019, sun et al. 2022). Cognate binding specificity also correlated with the rescue phenotype (Beckmann 2017, Chen et al. 2019, Xiao et all 2021). These yeast results provided a framework explaining bi-directional incompatibility at a molecular level. The yeast data indicated that cognate binding preferences of CifA and CifB proteins determine compatibility/incompatibility for any given *Wolbachia* strain. Since the initial study, cognate specific rescue of the yeast CI phenotype has been consistently demonstrated in $cid^{w\mathrm{Pip}}$, $cin^{w\mathrm{Pip}}$, $cin^{w\mathrm{No}}$, and $cid^{w\mathrm{Ha}}$ systems and provides substantial empirical evidence that *cif* operons behave as TA systems (Beckmann 2017, 2021, Chen et al. 2019, Sun et al. 2022, Xiao et al 2021).

5. Yeast Suppressor Screens Identify Conserved Eukaryotic Interactions of Cifs.

If a phenotype can be observed in the yeast model, downstream genetic assays can be employed to determine the relevant disrupted cellular pathways. One such assay is a suppressor screen. A suppressor screen relies on a yeast overexpression library (Jones et al 2008). The purpose of a suppressor screen is to find proteins capable of suppressing the specific phenotype, thereby determining key genetic interactions. Yeast plasmid libraries are stably stored as frozen *E.coli* clones. For functional experiments, plasmids are recovered with *E.coli* minipreps, then used to transform yeast cells for phenotypic testing. Physically, the overexpression library is constructed by inserting large segments of the yeast genome into high copy overexpression 2μ plasmids via cloning (Jones et al. 2008). The *E.coli* clones which house the yeast library plasmids are organized in a series of 96-well plates, frozen in glycerol/media. Thus, each well stores an *E.coli* clone holding a unique yeast 2μ plasmid with a unique chunk of the yeast genome inserted therein. Each clone's contents are carefully annotated and defined in a corresponding excel spreadsheet. To prepare the entire library, approximately 17, 96-well plates containing the frozen *E.coli* clones are thawed and pinned with a 96-pin replicator onto solid LB agar plates containing a selective antibiotic. Thereafter, bacteria grow as 96 distinct colonies on the plates under standard growth conditions. Bacteria from each of the plates is then pooled and the corresponding pools of plasmids are miniprepped from the pooled cultures. In this manner, plasmids encoding nearly the entire yeast genome are purified for downstream transformation into yeast.

Toxicity, cell death, or retarded yeast growth are easily analyzed via suppression screens. Toxic phenotypes themselves act as a selection regime that will only permit growth of yeast colonies successfully transformed with suppressing library plasmids. To perform an actual suppression screen, yeast cells are first transformed with a CEN plasmid containing an inducible toxin like *cifB* in a repressed [OFF] state. These cells are then transformed again with the 17

individual pools of miniprepped library plasmids which overexpress large chunks of the yeast genome. Once dually transformed, cells are plated on dual selective media that selects for both toxin and suppressor plasmids. The media also contains an inducing agent like galactose to induce toxin expression. Thus, in theory, the only clones that grow are yeast colonies that contain an overexpressed repressor of *cifB* toxin.

The identify of the suppressor can then be ascertained to provide insights into pathways targeted by *cifB*, or a given toxin. Once a positive suppressor clone has been recovered, it should be immediately regrown on the selective media a second time to confirm a strong and consistent suppression of the phenotype. If suppression is maintained, the plasmid should then be identified. To identify the plasmid the yeast clones are grown to high density in yeast peptone dextrose agar (YPD) and their DNA is extracted (Beckmann et al. 2019). Plasmids can then be recovered and stored in *E.coli* by highly efficient electroporation. Their identity and encoded ORFs are determined by Sanger sequencing. Thus, this strategy necessitates plasmids that are dually compatible in both *E.coli* and yeast systems and the actual process requires efficient and frequent transfer between both systems.

The suppressor screen technique has been utilized to identify karyopherin nuclear importers as key interactors targeted by Cifs (Beckmann 2019). Because overexpression of *cidB*$^{w\mathrm{Pip}}$ induces strong yeast toxicity, this inducible phenotype was dissected with a suppressor screen. As a first step, the researchers ensured that the library plasmids would not compete by cloning *cidB*$^{w\mathrm{Pip}}$ into a CEN plasmid with a URA3 cassette (see **Fig 4**). The library backbone was a plasmid with the 2µ origin and a LEU2 cassette (pGP564; see **Fig 4**). Therefore, the suppressor screen could be carried out on dual selective leucine/uracil dropout media containing 2% galactose.

After performing the screen, seven plasmids capable of suppressing the toxicity of *cidB*$^{w\mathrm{Pip}}$ were initially identified. However, in a suppressor screen, identifying suppressing library plasmids is only an initial step. Any suppressor screen will have some rate of false positive discovery. A good heuristic is not to trust any initially identified suppressor until it has been replicated and independently recovered in distinct clones at least 2-3 times. This will lower the burden of downstream analysis, which requires significant labor. Because the library plasmids contain multiple yeast ORFs, a researcher cannot initially conclude which ORF is the true suppressor. To identify the true suppressor, the ORFs on the suppressing plasmid must be subcloned individually onto into an empty 2µ plasmid (pGP564) and re-checked for suppression against the corresponding phenotype when in isolation. Only after subcloning and re-checking in triplicate can a positive suppressor be confirmed. The full process of clearly identifying real suppressors is outlined in Beckmann et al., 2019.

6. Overlooked Considerations and Key Controls.

When planning a large project in yeast key considerations are often overlooked. For example, the choice of any given strain can mean the success or failure of a given experiment. Due to genotypic differences in host backgrounds a given phenotype might be more or less perceptible. With respect to the study of *cifs*, researchers noted significantly higher sensitivity to CidB toxins in a W303 strain of yeast when compared to BY4741, despite the strains expressing the exact same plasmid constructs (Beckmann et al. 2019). Sometimes genotypic information is insufficient to make decisions on the most optimal strain. In this case, strains should be empirically tested and compared as a first step to any advanced project.

Another important consideration for many models including yeast is codon optimization of recombinant DNA constructs. Importing wild type DNA sequences from niche dwelling

prokaryotes into yeast does not mean that these sequences will replicate and express well. In one case, a DUB from *Orientia tsutsugamushi* (the scrub typhus pathogen) showed far stronger phenotypes after codon optimization (Berk et al. 2022). In this manner, weak phenotypes can often be optimized for scientific study.

Varying growth conditions, temperature, and the addition of stressors and/or chemicals can often be used to bring out or exacerbate a previously unobservable phenotype. When studying *cifB* orthologs, researchers raise temperature of the yeast culture to ranges from 35-37ºC (Beckmann 2017). The various strains exhibit differing tolerances to these temperatures. All these factors must be carefully considered, empirically tested, and standardized before conclusions about any phenotype can be reached.

A final point is that data from any overexpression analysis should always be analyzed in the context of SDS-PAGE and/or Western blots to validate protein expression. With forethought, these downstream controls can be facilitated via the insertions of epitope tags (i.e., FLAG, 3x-FLAG, His6, etc) (Beckmann 2017). Liquid cultures of yeast can then be grown, induced, lysed via a sodium hydroxide lysis step, boiled, and run on gels. Often mutations, tags, and codon optimized ORFs will show demonstratively different protein translation and stability levels. These experiments should be conducted to make sure phenotypic differences from expressed proteins cannot be attributed to altered protein stability or translation.

7. Constructive Criticisms of the Yeast Model.

George Box (a British mathematician and professor of statistics at the University of Wisconsin) said, *"All models are wrong, but some are useful."*

The yeast system, while impressive for its ability to dissect phenotypes and genotypes at the molecular level, is not without criticism. The yeast model excels at uncovering conserved

phenomena at a cellular level. On the other hand, more complex phenotypes involving tissues, organ systems, and organisms, might not be translatable. Where CI falls on that spectrum is up for debate. One perspective envisions CI as the result of cellular actions determined by the core TA system (Beckmann et al. 2019). Another perspective envisions CI as a more complex developmental phenotype involving spermatogenesis, fertilization, and embryonic development. Both are probably correct on some level. In summary, how to extrapolate *cif* phenotypic data from yeast models in the context of insect CI is a challenge.

However, yeast data has often provided initial insights into CI that were later supported by empirical data in flies. For example, overexpression of *cifA*s in yeast rescued toxicity of matching *cifB*s (Beckmann 2017). These data immediately suggested that *cifA* was sufficient to recue CI, and supported a toxin-antidote CI model when fly rescue data at the time were less clear. Specifically, *Drosophila* data (at that point in time) indicated that *cifA* was not the rescue factor and thus the yeast data were called into question by some (Lepage et al 2017). Only later, with increased expression of *cifA* in flies, did the fly data come into concordance with the yeast data and validate the original results (Shropshire 2019). Now all empirical data supports the sufficiency of *cifA* in rescue of CI (Beckmann et al. 2019, Chen et al. 2019, Xiao et al 2021, Shropshire 2019, Meany et al. 2019). Another example where yeast data was perfectly correlated to orthogonal datasets was in the suppression of CidB by yeast karyopherin (SRP1). In this case, overexpression of *Drosophila* karyopherins likewise suppressed actual CI phenotypes and CidB was demonstrated to physically interact with *Drosophila* karyopherins in multiple species (*Drosophila and Aedes*) (Beckmann et al. 2019, Oladipupo 2023).

A debate which remains unresolved is the role (if any) of *cifA* in induction of CI (Beckman et al. 2019). Two main models have emerged. A strict TA model suggests that the genetic

architecture of CI is organized as a discrete toxin with a paired antidote; ~~and~~ thus induction relies solely on the toxin and rescue relies solely on the antidote (Poinsot 2003, Beckmann et al. 2019). In the "2x1" model, pronounced two by one, the antidote has dual functions as a sole rescue factor but also as a distinct secondary toxin (Shropshire 2019). This model was initially proposed when researchers observed in *Drosophila* that transgenic CI from the *cid*$^{w\mathrm{Mel}}$ system could not be produced unless both A and B proteins were co-expressed (LePage et al. 2017). In contrast, yeast data never supported this hypothesis. In all studies to date, overexpression of five orthologous *cifAs* (*cidA*$^{w\mathrm{Pip}}$, *cinA*$^{w\mathrm{Pip}}$, *cndA*$^{w\mathrm{Str}}$, *cidA*$^{w\mathrm{Ha}}$, and *cinA*$^{w\mathrm{No}}$) has never resulted in any toxic phenotype in yeast and thus never supported a role for CifA proteins in enzymatic induction of CI (Beckmann 2017, Beckmann et al. 2019, Chen et al. 2019, Sun et al. 2022). Subsequent studies in *Drosophila* confirmed strict TA functionality for multiple systems, yet the *cid*$^{w\mathrm{Mel}}$ system still remains enigmatic as a supporter of the 2x1 framework (LePage 2017, Shropshire 2019). Thus, in this debate, yeast data consistently argue for a general TA framework, while the *Drosophila* data leaves some room for nuance and specialization within specific insect host cases and specific symbionts, like *w*Mel. We should not expect all CI systems to behave exactly the same and traditionally there has been clear evidence that even within *Drosophila* there is consistent variation with respect to CI rates amongst strains such as *w*Yak, *w*San, and *w*Tei (Cooper et al. 2017, Cooper et al. 2019, Zabalou et al. 2008).

A final point of debate that remains to be resolved is the role of DUB and nuclease domains in CI. From the beginning yeast data supported an induction role for the DUB and nuclease domains in *cid* and *cin* respectively. Yeast toxicity was ablated when these enzymatic domains were disabled by catalytic mutations (LePage 2017). However, in a recent publication, Horard et al. (Howard et al. 2022) found that inactivation of the CidB DUB did not completely remove its

ability to induce CI in *Drosophila*. In contrast, a study utilizing both yeast and *Drosophila* models demonstrated that DUB enzyme efficiency perfectly correlates with CI penetrance (Beckmann et al. 2021). Notably researchers found that mutations reducing toxicity in yeast (specifically within the DUB domain), also reduced CI penetrance in flies in near perfect correlation. The amino acid change on the DUB domain helped describe in part why natural *Wolbachia* strains such as *w*Yak, *w*San, and *w*Tei, may experience varying CI phenotypic strengths. In this case, the specific valine to leucine mutation was not only tested in yeast first but concurrently tested in the fly strains showing convergent results; again validating the yeast model. More data will need to be collected to resolve these remaining discrepancies.

In conclusion, while *Drosophila* models are indeed insect models (and thus pertinent to CI), they should not be used to discount the data provided by yeast models when the two disagree. Notably, transgenic *Drosophila* is itself a model with its own set of problems. For example, generating sufficient expression of transgenes has been problematic and contributed to false negative data from multiple groups where *cifA* wouldn't rescue in the earliest *Drosophila* models (Beckmann 2017, LePage et al. 2017). Yeast data should be seen for what it is, a basal underlying cellular model that is often conserved in higher eukaryotes at the cellular level. Organismal factors might complicate or modify that basal level thereby generating nuanced phenotypes; but these observations need not necessarily invalidate what is observed in yeast at a cellular level. In the best studies, yeast models can be used to quickly screen for significant characteristic phenotypes which should then be confirmed downstream by fly data. Thus, the two work together to help point toward scientific fact where observations converge.

REFERENCES

Amberg DC (2005) Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual.

Baker Brachmann C, Davies A, Cost GJ, et al (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**:115–132

Barnett JA (2007) A history of research on yeasts 10: foundations of yeast genetics1. *Yeast* **24**:799–846

Barnes DA, Thorner J (1986) Genetic manipulation of *Saccharomyces cerevisiae* by use of the LYS2 gene. *Mol Cell Biol* **6**:2828–2838

Beckmann JF, Bonneau M, Chen H, et al (2019) Caution does not preclude predictive and testable models of cytoplasmic incompatibility: a reply to Shropshire et al. *Trends Genet* **35**:399–400

Beckmann JF, Bonneau M, Chen H, et al (2019) The toxin–antidote model of cytoplasmic incompatibility: genetics and evolutionary implications. *Trends Genet* **35**:175–185

Beckmann JF, Fallon AM (2013) Detection of the *Wolbachia* protein WPIP0282 in mosquito spermathecae: implications for cytoplasmic incompatibility. *Insect Biochem Mol Biol* **43**:867–878

Beckmann JF, Ronau JA, Hochstrasser M (2017) A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nat Microbiol* **2**:1–7. https://doi.org/10.1038/nmicrobiol.2017.7

Beckmann JF, Sharma GD, Mendez L, et al (2019) The *Wolbachia* cytoplasmic incompatibility enzyme CidB targets nuclear import and protamine-histone exchange factors. *Elife* **8**:e50026

Beckmann JF, Van Vaerenberghe K, Akwa DE, Cooper BS (2021) A single mutation weakens symbiont-induced reproductive manipulation through reductions in deubiquitylation efficiency. *Proc Natl Acad Sci* **118**:e2113271118

Berk JM, Lee MJ, Zhang M, et al (2022) OtDUB from the human pathogen *Orientia tsutsugamushi* modulates host membrane trafficking by multiple mechanisms. *Mol Cell Biol* **42**:e00071-22

Boeke JD, La Croute F, Fink GR (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet MGG* **197**:345–346

Bonneau M, Atyame C, Beji M, et al (2018) *Culex pipiens* crossing type diversity is governed by an amplified and polymorphic operon of *Wolbachia*. *Nat Commun* **9**:1–10

Bonneau M, Caputo B, Ligier A, et al (2019) Variation in *Wolbachia* cidB gene, but not cidA, is associated with cytoplasmic incompatibility mod phenotype diversity in *Culex pipiens*. *Mol Ecol* **28**:4725–4736

Chan K-M, Liu Y-T, Ma C-H, et al (2013) The 2 micron plasmid of *Saccharomyces cerevisiae*: a miniaturized selfish genome with optimized functional competence. *Plasmid* **70**:2–17

Chattoo BB, Sherman F, Azubalis DA, et al (1979) Selection of lys2 mutants of the yeast *Saccharomyces cerevisiae* by the utilization of α-aminoadipate. *Genetics* **93**:51–65

Chen H, Ronau JA, Beckmann JF, Hochstrasser M (2019) A *Wolbachia* nuclease and its binding partner provide a distinct mechanism for cytoplasmic incompatibility. *Proc Natl Acad Sci* **116**:22314–22321

Chen H, Zhang M, Hochstrasser M (2020) The biochemistry of cytoplasmic incompatibility caused by endosymbiotic bacteria. *Genes* **11**:852

Cooper BS, Ginsberg PS, Turelli M, Matute DR (2017) *Wolbachia* in the *Drosophila yakuba* complex: pervasive frequency variation and weak cytoplasmic incompatibility, but no apparent effect on reproductive isolation. *Genetics* **205**:333–351

Cooper BS, Vanderpool D, Conner WR, et al (2019) *Wolbachia* acquisition by *Drosophila yakuba*-clade hosts and transfer of incompatibility loci between distantly related *Wolbachia*. *Genetics* **212**:1399–1419. https://doi.org/10.1534/genetics.119.302349

Feldmann H (2011) Yeast: molecular and cell biology. John Wiley & Sons

Futcher AB (1988) The 2 µm circle plasmid of *Saccharomyces cerevisiae*. *Yeast* **4**:27–40

Giaever G, Nislow C (2014) The yeast deletion collection: a decade of functional genomics. *Genetics* **197**:451–465

Gillespie JJ, Driscoll TP, Verhoeve VI, et al (2018) A tangled web: origins of reproductive parasitism. *Genome Biol Evol* **10**:2292–2309

Goffeau A, Barrell BG, Bussey H, et al (1996) Life with 6000 genes. *Science* **274**:546–567

Greig D, Leu J-Y (2009) Natural history of budding yeast. *Curr Biol* **19**:R886–R890

Hartwell LH (1974) *Saccharomyces cerevisiae* cell cycle. *Bacteriol Rev* **38**:164–198

Hinnen A, Hicks JB, Fink GR (1978) Transformation of yeast. *Proc Natl Acad Sci* **75**:1929–1933

Hope IA, Struhl K (1985) GCN4 protein, synthesize in vitro, binds HIS3 regulatory sequences: implications for general control of amino acid biosynthetic genes in yeast. *Cell* **43**:177–188

Horard B, Terretaz K, Gosselin-Grenet A-S, et al (2022) Paternal transmission of the *Wolbachia* CidB toxin underlies cytoplasmic incompatibility. *Curr Biol* **32**:1319–1331

Howson R, Huh W-K, Ghaemmaghami S, et al (2005) Construction, verification and experimental use of two epitope-tagged collections of budding yeast strains. *Comp Funct Genomics* **6**:2–16

Jeyaprakash A, Hoy MA (2000) Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* **9**:393–405

Jones GM, Stalker J, Humphray S, et al (2008) A systematic library for comprehensive overexpression screens in *Saccharomyces cerevisiae*. *Nat Methods* **5**:239–241

Joska TM, Mashruwala A, Boyd JM, Belden WJ (2014) A universal cloning method based on yeast homologous recombination that is simple, efficient, and versatile. *J Microbiol Methods* **100**:46–51

Lacroute F (1968) Regulation of pyrimidine biosynthesis in *Saccharomyces cerevisiae*. *J Bacteriol* **95**:824–832

LePage DP, Metcalf JA, Bordenstein SR, et al (2017) Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature* **543**:243–247

Liti G (2015) The Natural History of Model Organisms: The fascinating and secret wild life of the budding yeast *S. cerevisiae*. *Elife* **4**:e05835

Marczynski GT, Jaehning JA (1985) A transcription map of a yeast centromere plasmid: unexpected transcripts and altered gene expression. *Nucleic Acids Res* **13**:8487–8506

Martinez J, Ok S, Smith S, et al (2015) Should symbionts be nice or selfish? Antiviral effects of *Wolbachia* are costly but reproductive parasitism is not. *PLoS Pathog* **11**:e1005021

Meany MK, Conner WR, Richter SV, et al (2019) Loss of cytoplasmic incompatibility and minimal fecundity effects explain relatively low *Wolbachia* frequencies in *Drosophila* mauritiana. *Evolution* **73**:1278–1295

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, et al (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell* **139**:1268–1278

Mortimer RK, Johnston JR (1986) Genealogy of principal strains of the yeast genetic stock center. *Genetics* **113**:35–43

Mumberg D, Müller R, Funk M (1994) Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* **22**:5767

Mumberg D, Müller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156:119–122

Oladipupo SO, Carroll JD, Beckmann JF (2023) Convergent Aedes and *Drosophila* CidB interactomes suggest cytoplasmic incompatibility targets are conserved. Insect Biochem *Mol Biol* **155**:103931. https://doi.org/10.1016/j.ibmb.2023.103931

Orr-Weaver TL, Szostak JW, Rothstein RJ (1981) Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci* **78**:6354–6358

Pasteur L (1858) Nouveaux faits concernant l'histoire de la fermentation alcoolique. *Comptes Rendus Chim* **47**:1011–1013

Pearce DA, Sherman F (1999) Toxicity of copper, cobalt, and nickel salts is dependent on histidine metabolism in the yeast *Saccharomyces cerevisiae*. *J Bacteriol* **181**:4774–4779

Poinsot D, Charlat S, Mercot H (2003) On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: Confronting the models with the facts. *Bioessays* **25**:259–265

Presgraves DC (2000) A genetic test of the mechanism of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila*. *Genetics* **154**:771–776

Rice DW, Sheehan KB, Newton IL (2017) Large-scale identification of *Wolbachia pipientis* effectors. *Genome Biol Evol* **9**:1925–1937

Sheehan KB, Martin M, Lesser CF, et al (2016) Identification and characterization of a candidate *Wolbachia pipientis* type IV effector that interacts with the actin cytoskeleton. *MBio* **7**:e00622-16

Shropshire JD, Bordenstein SR (2019) Two-By-One model of cytoplasmic incompatibility: Synthetic recapitulation by transgenic expression of cifA and cifB in *Drosophila*. *PLOS Genet* **15**:e1008221. https://doi.org/10.1371/journal.pgen.1008221

Sikorski RS, Boeke JD (1991) [20] In vitro mutagenesis and plasmid shuffling: From cloned gene to mutant yeast. In: *Methods in enzymology. Elsevier*, pp 302–318

Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27

Strope PK, Kozmin SG, Skelly DA, et al (2015) 2µ plasmid in *Saccharomyces* species and in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **15**:fov090

Sun G, Zhang M, Chen H, Hochstrasser M (2022) The CinB Nuclease from w No *Wolbachia* Is Sufficient for Induction of Cytoplasmic Incompatibility in *Drosophila*. *Mbio* **13**:e03177-21

Thompson PW, Wheals AE (1980) Asymmetrical division of *Saccharomyces cerevisiae* in glucose-limited chemostat culture. *Microbiology* **121**:401–409

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6**:741–751

Whitaker N, Berry TM, Rosenthal N, et al (2016) Chimeric coupling proteins mediate transfer of heterologous type IV effectors through the *Escherichia coli* pKM101-encoded conjugation machine. *J Bacteriol* **198**:2701–2718

Xiao Y, Chen H, Wang H, et al (2021) Structural and mechanistic insights into the complexes formed by *Wolbachia* cytoplasmic incompatibility factors. *Proc Natl Acad Sci* **118**:e2107699118. https://doi.org/10.1073/pnas.2107699118

Yamaguchi K, Yamaguchi M, Tomizawa J (1982) Incompatibility of plasmids containing the replication origin of the *Escherichia coli* chromosome. *Proc Natl Acad Sci* **79**:5347–5351

Zabalou S, Apostolaki A, Pattas S, et al (2008) Multiple rescue factors within a *Wolbachia* strain. *Genetics* **178**:2145–2160

Zheng X, Zhang D, Li Y, et al (2019) Incompatible and sterile insect techniques combined eliminate mosquitoes. *Nature* **572**:56–61