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Investigating Microbe-Plant Symbioses in Space

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by

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Abstract

Title: Investigating Microbe-Plant Symbioses in Space

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As life has evolved on Earth, gravity has been a constant around which biological processes have developed, rather than a pressure that has been adapted to. As humanity ventures into space, we remove this constant and can observe a variety of changes in biological systems, such as bone loss in humans, stunted plant growth, or increased virulence in pathogens. The growing body of data shows that life adapts to spaceflight and microgravity in unique ways that, due to our limited understanding, can be difficult to predict. A particularly under-studied area is that of host-microbe interactions. Despite efforts to limit their introduction, microbes have been found nearly everywhere on the International Space Station (ISS). This is because virtually all macroscopic life relies on a diverse microbiome to support it, such as in humans, we rely on gut microbes for healthy digestion and nutrient uptake. Analogously, plants rely on a diverse microbiome in their tissues and around their roots, for nutrient uptake, pathogen mediation, and even phytohormone regulation.

All materials launched to the ISS are required to be sterilized or sanitized as thoroughly as possible. This includes the materials for hydroponic plant growth systems, even the seeds to be grown. Despite these efforts, microbial analysis of plants grown in the

Vegetable Production System (Veggie) have revealed an active microbiome. This begs the question, how is spaceflight affecting the interactions in these holobiont communities?

The spaceflight environment causes a variety of issues for plant growth; abiotic stressors such as waterlogging due to unique fluid dynamics in microgravity, the lack of convective currents causing the buildup of oxygen and ethylene, which leave plants vulnerable to biotic stressors such as opportunistic fungi causing plant loss. The plants that survive these stressors are returned to Earth for analysis, allowing access to this population of microbes made unique by their spaceflight history. By studying these samples, I aim to improve our understanding of the microbe-plant interactions occurring in the microgravity environment and identify bacteria that could improve plant growth.

In order to better understand this population of microbes, which has been made unique by its presence in space, I have devised an experimental pipeline to elucidate their plant growth promoting (PGP) potential. By screening these microbes for plant growth promoting properties such as indole production, 1-aminocyclopropane-1-carboxylic acid deaminase activity, siderophore production, and phosphate solubilization, I have identified candidate isolates that may have contributed to the health and quality of the plants harvested.

However, many bacteria experience altered phenotypic expression. Simulated microgravity (SM) can be used to determine how these behaviors may have been altered in spaceflight, giving insight to advantages these microbes may have had over others. Most studies on bacteria use fluid suspended cultures in low fluid-shear modeled SM. However,

I am interested in the interface between these isolates and their host plants, where symbionts inhabit the surface and interior of host tissues. As such, I have designed experiments to use solid media to test these phenotypes. In testing this initial group of bacteria, I have noted that these bacteria do experience changes in simulated microgravity, though they are unique to each other, with each species responding to SM differently, implicating highly dynamic interactions when in conjunction with hosts in SF. This work is the first effort, to my knowledge, to investigate these plant-growth promoting phenotypes under simulated microgravity on surfaces that mimic the host-microbial interface as it occurs naturally.

Finally, these isolates must be tested as inoculants to determine definitively whether they are friend, foe, or neutral to host plants, as many of these phenotypes are shared between bacterial pathogens and mutualists. This may also give insight into the opportunistic infections afflicting stressed plants grown on the space station. Of the bacteria investigated here, I have observed both beneficial and harmful effects on inoculated plants dependent on the species. This indicates that opportunistic phytopathogens are among this population, yet the good health of the originating host indicates that the beneficial members are able to maintain host health.

In these studies, I have identified numerous microbes capable of PGP activities. SM investigations into samples most likely to be PGP have yielded promising results for their ability to retain these phenotypes in microgravity. In most cases, phenotype expression remained unchanged. In cases where the phenotype expression was changed, I have observed an upregulation of expression in all but one case where the indole

production of one strain was downregulated. Inoculation experiments have revealed that these ISS derived isolates contain not only PGP strains, as is the case with *Curtobacterium flaccumfaciens* and *Pantoea agglomerans*, but also contain potentially problematic microbes, as the strain of *Burkholderia pyrrocinia* was observed to inhibit plant growth.

The information gained from these studies will improve the collective understanding of host-microbe interactions in spaceflight and provide insight to how these interactions may be leveraged to benefit humanity's continued exploration of space. As humanity continues to expand deeper into space, exploration and settlement efforts will become increasingly dependent on robust and sustainable bioregenerative life support systems (BLSS). A key feature to these BLSS will be plant growth systems for air revitalization, waste management, and most importantly, food production. Beneficial microbes whose behaviors in spaceflight can support host resistance to the stressors faced in spaceflight will be key in ensuring the systems continuous production in support of the crew.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A_{550}- Absorbance at 550 nm

ACC- 1-aminocyclopropane-1-carboxylic acid

ACCd- 1-aminocyclopropane-1-carboxylic acid deaminase

BLSS- Bioregenerative life support systems

Bp- Burkholderia pyrrocinia

CAS- Chrome azurol S

Cf- Curtobacterium flaccumfaciens

CFU- Colony forming unit

DAP- Days after planting

DFS- Dworkin and Foster salts

GC- Gravity control

GY/TP- Glucose and yeast extract / tricalcium phosphate

IAA- Indole-3-acetic acid

IMA- Inhibitory mold agar

ISRU- *in situ* resource utilization

ISS- International Space Station

ISSd- Internation Space Station derived

KBC- King B with CAS

KBT- King B with tryptophan

KSC- Kennedy Space Center

LFS- Low fluid-shear

OD_{600}- Optical density at 600 nm

OSDR- Open Science Data Repository

Pa- Pantoea agglomerans

PGP- Plant growth promoting

PGPB- Plant growth promoting bacteria

QS- quorum sensing

RPM- Random position machine

SF- Spaceflight

SM- Simulated microgravity

TSA- Tryptic soy agar

Veggie- Vegetable production system

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Chapter 1
Gravity- An abiotic constant in the evolution of life on Earth.

1.1 Introduction

As long-term space settlements become increasingly more likely, numerous questions remain regarding how life adapts to challenges on other planets as well as within the spaceflight (SF) environment. One of the many differences between Earth and future off-world settlements such as the moon, Mars, or orbiting satellites such as the International Space Station (ISS), is gravity. On Earth, gravity is both spatially and temporally constant, varying <1% across the surface of the planet and has been so for at least 3.75 billion years. Indeed, gravity may be one of the few abiotic environmental factors that has remained a constant throughout the history of life on our planet. As a result, gravity has not served as a selective pressure in evolution, but rather a constant around which various processes have developed.

However, beyond Earth, gravity can vary significantly, ranging from the relative absence of gravity in environments like the ISS (microgravity), to hypogravity (< 1 G) on immediate targets like the moon (10%) or Mars (40%) or even hypergravity (> 1 G) as on some potentially habitable exoplanets [1]. Understanding how biology adapts to these environments is critical to identifying the challenges of settling beyond Earth as well as the development of solutions to potential challenges. Indeed, numerous examples of potentially gravity-related

challenges have been identified across various kingdoms of life. For future human settlers, examples include concerns on immune system functioning, loss of bone density, fetal development, and even the act of childbirth [2]. Among plants, variations to plant growth occur which could directly impact their potential for food production or as components to bioregenerative life support systems (BLSS). Understanding the roles that gravity plays in fundamental aspects of biology can provide valuable insight into strategies that were instrumental in the origins and persistence of life.

On Earth, microgravity simulations have provided considerable insight into the roles that gravity plays in both prokaryotic and eukaryotic biology [2,3]. However, since its completion, the ISS has been the primary center for research aimed at identifying biological adaptations to microgravity. These studies provide fundamental insight into the role of gravity on biology on Earth as well as how living systems adapt to the SF environment. After over a decade of ISS research, it has become clear that microgravity and increased radiation exposures in the SF environment can significantly alter prokaryotic and eukaryotic gene expression and resulting phenotypes [3–5]. When coupled to ground experiments, these efforts can decouple gravity and radiation to better understand this fundamental abiotic factor. Here I will briefly review plant and microbial responses to the spaceflight environment.

1.2 Plant Responses to the Spaceflight Environment

1.2.1 Osmotic Stress

Plants grown in SF exhibit differences at the cellular and gene expression levels, especially at early developmental stages, yet are often comparable to ground controls in terms of biomass, seed production, etc., likely due to compensatory growth strategies [6–12]. However, initial flight tests of the Veggie system reported the loss of multiple SF plants to fungi, significantly reducing total yield [13]. The behavior of water in microgravity is another major source of plant stress. The altered fluid dynamics can cause the nutrient solution to envelop plant tissues, creating hypoxic conditions in roots. Similarly, limited convective air currents can allow for the buildup of metabolic by-products that under these specific conditions are deleterious [14]. For instance, the accumulation of oxygen derived from photosynthesis can increase photorespiration frequency thereby inhibiting carbon fixation [15,16]. Meanwhile, the buildup of ethylene, the primary stress response hormone, can induce inappropriate immune responses, stunted growth, and other deleterious effects [8,17]. New SF plant growth systems include fans and/or ethylene scrubbers to limit these effects [18,19].

To compensate for the behavior of water, plant growth systems now use root modules containing porous clay-based substrates such as balkanine or arcilite which distribute water throughout the root module [13,20–22]. However,

mechanical systems, such as fans placed to provide air currents, can fail, resulting in the buildup of water and ethylene around the leaves, leaving plants vulnerable to opportunistic plant pathogens such as *Fusarium oxysporum* [23].

1.2.2 Tropic Responses

The primary change plants experience in SF is the lack of a gravitropic response. Gravitropism is the ability of plants to orient their growth based on the perceived direction of gravity. The process occurs primarily in the root tips where the plastid organelle is specialized into a starch-heavy amyloplast, also referred to as statoliths. These statoliths settle to the bottom of the cell where contact triggers relocation of PIN proteins responsible for directing auxin transport, resulting in cell elongation [24].

In shoots, phototropism, the response to light stimuli that directs plant growth, is dominant. Phototropism is driven by photoreceptors and the reorganization of PIN protein localization based on the direction of the light [25]. This protein reorganization causes an asymmetric transport of auxin. The asymmetric buildup of auxin accelerates elongation on one side, resulting in bending in the desired direction [26]. Though it is common for gravitropism to direct shoots in the absence of light, such as a buried seedling moving to breach the surface of the soil, the absence of gravity is only possible in space. Experiments on the ISS have revealed that roots do experience phototropism that is normally

overshadowed by gravitropic responses at 1g, and as low as 0.1g [27]. Other tropisms involved in the seeking of water and nutrients may also play a larger role in the absence of gravity.

1.2.3 Molecular Changes

On the cellular and molecular levels, changes in cell structure and gene expression have been observed in early stages of plant development, however these alterations do not seem to carry into mature plants [12]. These effects on plants occur at the cellular level, affecting cell wall development, cell division rate, photosynthetic capacity, and amyloplast sedimentation [28]. The observation that many of these changes do not persist as vegetative growth continues, such as the comparable biomass of various plants relative to ground controls, suggest that plants possess the ability to substitute gravity with other cues [6–11]. This is likely due to the fact that plants utilize multiple cues including light intensity, day:night cycles, temperature, moisture, and more to modulate their body plan throughout their life. The absence of gravity may simply require a period of adjustment to rely on these alternative cues.

Gene expression studies have shown that SF causes upregulation of stress related genes independent of those stressors, which may cause inappropriate responses to active stressors [9,29]. Stress-related genes, such as those associated with phytohormone production, can impact host-microbial associations [30,31].

Gene expression analysis in *Arabidopsis thaliana* revealed 182 genes were differentially expressed between flight and ground by more than 4-fold. Genes that were differentially expressed by more than 10 fold include genes related to chlorophyll-binding, ethylene signaling, and senescence [5]. Work by Barker et al. used the NASA GeneLab database to collate multiple *A. thaliana* SF experiments across multiple genotypes, which revealed even more differentially regulated genes, including 6 genes that were differentially expressed in all genotypes, indicating a common response for the species [32].

1.3 Microbial behaviors in spaceflight

Due to the concerns over astronaut health, most microgravity studies on bacteria focus on pathogen behavior in SF, with early experiments identifying phenotypic changes such as growth rates, antibiotic resistance, and biofilm formation, dependent on culturing method [33–36]. SF may even influence antibiotic resistance at the genetic level, such as the case of *Salmonella enteritidis* being more resistant to amikacin after being returned from a month long flight [37]. Virulence models in *S. typhimurium* reveal hypervirulence in SF, which can be exacerbated by ion composition in the media [38]. Other studies in *Candida, Listeria, Enterococcus, and MRSA* have shown the opposite; hypovirulence in SF experiments [39]. Bacterial biofilms have been observed forming unique architectures in SF, which corresponds to a significant increase in biomass

accumulation, though the phenotype is dependent on flagellar-driven motility [36]. In many of these cases, motility is implicated as a factor in a bacterium's susceptibility to overall behavioral changes in microgravity.

It has been observed that in fluid culture medium, motility of the organism impacts how it responds and adapts to microgravity. Microgravity creates a low fluid-shear (LFS) environment where the movement of dissolved molecules throughout the fluid medium is low. Studies comparing motile and non-motile strains of *E. coli* have shown no difference between flight and ground for motile strains, while non-motile strains appear to have a significantly shorter lag phase. It has been posited that this is due to flagellar movement causing fluid mixing, compensating for the LFS environment, while non-motile cells experience a quick depletion of nutrients and buildup of secondary metabolites which may alter genotypic and phenotypic expression [40,41] (Figure 1.1).

Experiments investigating gentamicin resistance in *E. coli* suggest that increased antibiotic resistance may be an artifact of these fluid dynamics, as *E. coli* grown on agar plates in SF were just as susceptible as ground controls [35]. This emphasizes the need to design *in vitro* experiments that appropriately address *in vivo* environmental factors. There are host environments where the LFS of liquid culture is applicable, such as in certain areas of the gastrointestinal tract, and other fluid-filled parts of the human body [42]. However, in many cases, microbes will

be on or inside of host tissues, such as the skin of an astronaut, or the root of a plant, which would be more accurately represented by a gel matrix rather than a fluid medium.

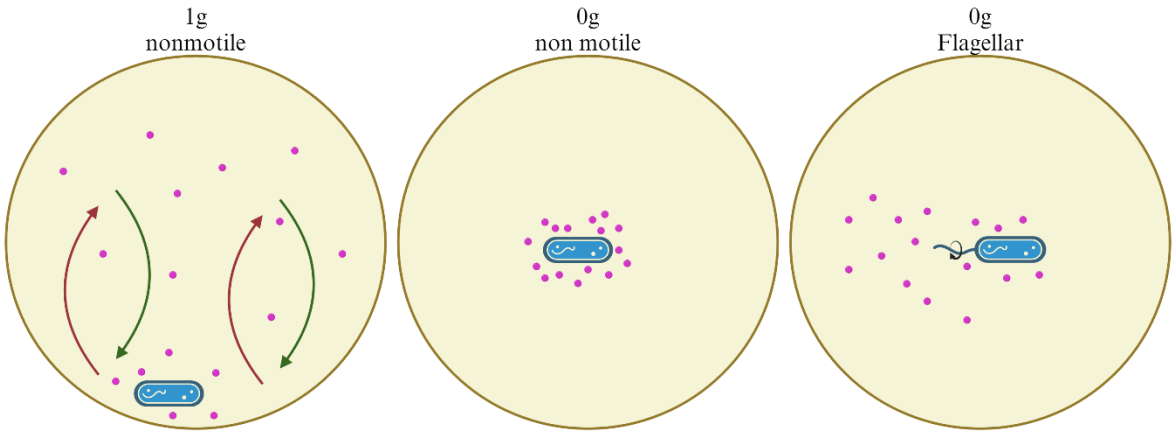

Figure 1.1: Fluid mixing is impacted by microgravity and cellular motility. (Left) cells sediment due to gravity (1 g) while buoyancy-driven convective currents disperse soluble metabolites. (Center) lack of convective currents in microgravity (0 g) causes buildup of metabolites around the cell. (Right) flagellar motion creates mechanical mixing, dispersing metabolites in microgravity.

Studies focusing on metabolites have noted alterations in carbohydrate and amino acid catabolism, with responses varying between species and culture conditions [43]. The results of many behaviors in SF vary significantly by species, suggesting that there are additional factors influencing these phenotypes and that there is likely not a universal response to SF conditions [44]. Early transcriptomic studies implicated common responses in certain regulatory pathways to SF,

however more recent comprehensive comparisons between unrelated taxa have shown a lack of common response to SF conditions [3,44]. Even less is known about host-microbial associations in the SF environment, underscoring the need to develop strategies to investigate these interactions.

1.4 Implications of Spaceflight Biology on Microbe-Plant Interactions

One consistent finding of these studies is that plants experience heightened stress in SF. As stated above, these abiotic stress factors can lead to stunted growth, malformed reproductive organs, and opportunistic infection[17,23,45]. However, as crewed missions move further from Earth, they will be increasingly reliant on robust and sustainable food production systems. It is therefore crucial that solutions to the challenges of yield and survival be resolved.

Numerous plant growth systems have been utilized in space, the first being the Oasis series developed by the Soviet Union for use on the Salyut 1 space station. Building from decades of experience from nearly two dozen systems, the most recent iterations of plant growth systems used for food production are the Vegetable Production System (Veggie) and Advanced Plant Habitat, as well as the Advanced Biological Research System for smaller experimental plant growth [8,13,46]. Though significant improvements have been made to these systems, such

as ethylene scrubbers and fans to facilitate air currents, outside factors such as abiotic stress and opportunistic pathogens remain a limiting factor [23].

With regards to prokaryotes, multiple phenotypes are clearly altered in SF, with emphasis on human pathogen-related behaviors such as virulence and antibiotic resistance [3,47]. These changes vary from species to species, especially in microorganisms, where the same gene or phenotype will often be differentially expressed due to microgravity [3,43]. Phenotypes associated with mutualistic symbiosis are virtually unexplored in spaceflight, despite their integral importance to host health. This is largely due to an abundance of concern for crew health. To prevent the introduction of potential pathogens, NASA policies include strict sterilization and sanitization requirements for all materials sent into space. This has been coupled with a hyperfocus on human pathogen related behaviors and reduced focus on microbial research in other areas due to limited experimental opportunities in SF. Yet, the study of microbial symbionts, especially plant growth promoting (PGP) microbes, will be crucial in the development of BLSS.

One key aspect of BLSS will be the inclusion of agricultural systems. Plants will be integral to gas exchange, food production, and waste management in BLSS, but they rely heavily on robust and diverse microbiomes. Microbes can provide a variety of benefits to host plants, including biocontrol, nutrient uptake, and abiotic stress reduction. An important aspect for BLSS, which has largely been ignored,

will be symbiotic microbes, which virtually all macroorganisms rely on to some degree. Plants are especially reliant on microbes for nutrient uptake, regulation of growth-related phytohormones, and pathogen mediation. The controlled addition of a consortium of PGP microbes may be vital in solving issues that lead to plant loss. However, because the responses of microorganisms in microgravity are so varied, it is important to verify that key phenotypes are not downregulated.

To this end, methodologies must be developed to identify candidates for this microbial consortium to ensure retention of PGP phenotypes, and that these microbes can effectively mitigate the issues the host plants face. To inform these methodologies, we must consider how plants respond to microgravity and the stresses it induces, how microbes respond to these same parameters, what PGP functions microbes may provide, and what technologies can be leveraged toward these investigations.

1.5 Roles of Plant Growth Promoting Bacteria

Bacteria can provide a variety of benefits to host plants, such as improving nutrient uptake, reducing abiotic stress effects, and improving pathogen resistance. The mature plant host effectively has two habitat ranges for associated microbes: the aboveground portion referred to as the phyllosphere, and the rhizosphere which encompasses the roots and surrounding soil [48]. The rhizosphere hosts an especially diverse microbial community which provides a wide variety of beneficial

functions. Microbial diversity is often reported by taxonomic grouping from the species to class level, and the rhizosphere can contain from 10^1 to 10^3 of distinct taxa [49]. Plants rely heavily on beneficial microbes from these diverse populations; to ignore the benefits of PGP microbes in the development of BLSS would be ignoring millions of years of co-evolution between plants and their symbionts [50].

The roles microbes can play in plant health are highly diverse, suggesting that if there is an issue a plant faces, the right microbe may be key to the plant's adaptation to the problem and subsequent survival. This is why plants have extremely diverse microbiomes, and why the makeup of these microbial communities are dynamic across the plants' life, and in response to environmental stressors [51,52]. This also means that knowledge of the stressors plants may face allows for the tailoring of artificial microbiomes towards combating these stressors.

1.5.1 Microbial Effects on Nutrient Uptake

Associations with soil microbes are especially important in plants for gathering nutrients with limited solubility, such as phosphorous and heavy metal micronutrients like iron, magnesium, and manganese. One of the common phenotypes associated with nutrient gathering is siderophore production. Siderophores are a diverse group of metabolites primarily defined by the ability to chelate iron, making it available for uptake by microbes and plants [53]. Only a

small portion of phosphorous in soil is actually bioavailable to plants due to limited solubility, however many PGP bacteria are able to solubilize unavailable phosphorous through a variety of means such as acid production and enzyme secretion [54]. Additionally, atmospheric nitrogen fixation is often regarded as one of the most important functions performed by microbes, as it is a necessary macronutrient for plants [55,56].

1.5.2 Microbial Manipulation of Phytohormones

Plants rely on a variety of phytohormones to regulate processes such as growth, cell differentiation, reproduction, and senescence. The interactions between these signals are complex, with many present in any given process, sometimes working in tandem, and other times working antagonistically to regulate each other. Overall growth and development are controlled by cytokinins, auxins, and gibberellic acid, while ethylene, abscisic acid, and salicylic acid act as indirect growth regulators [57–65]. Senescence is largely controlled by ethylene and gibberellic acid [61,62,66]. Seed dormancy is primarily controlled by gibberellic acid and abscisic acid [66,67]. Ethylene is involved in most processes to some degree, but its primary role is in stress response. It acts as a regulator to many other phytohormones in order to control growth and development in response to a variety of biotic and abiotic stresses [61–64].

Symbiotic bacteria can influence plant behavior through phytohormone manipulation. This is done through bacterial biosynthesis of phytohormones such as auxin or cytokinin to induce a response, or to interfere with plant biosynthesis of phytohormones such as ethylene to restrict a response [68,69]. The presence of microbe-associated molecular patterns trigger the plant immune response, primarily controlled by ethylene and salicylic acid [65,70]. PGP bacteria must circumvent these responses, a common method being the inhibition of ethylene production by breaking down the ethylene precursor molecule 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC deaminase [56,68,69]. Many PGP bacteria also produce auxin and/or gibberellic acid in order to stimulate growth, increasing the bacterial habitat within and on the surface of the plant, and increasing the amount of root exudates used by rhizobacteria [56,71].

1.5.3 Beneficial Microbes as Biocontrol Agents

Pathogenic bacteria can also manipulate plant hormones for various purposes, the first of which is infection. A common route is via stomatal openings, where exogenous auxin and cytokinins can cause pore opening, or interference in abscisic acid and salicylic acid biosynthesis to prevent closure [72,73]. While PGP bacteria may infect hosts using similar methods, once established as part of the microbiome, they can use these phytohormone manipulations to assist the plant defense response, acting as biocontrol [74,75]. In their roles of biocontrol, many

PGP species also produce antibiotics to inhibit potential pathogens, as well as competitive microbes [56]. Siderophore production is also relevant to biocontrol, as chelation of environmental iron limits a potential pathogen's access to the essential nutrient [53].

1.6 The Use of Simulated Microgravity to Investigate Biological Responses

Due to logistical hurdles and limited opportunity for space-based microgravity research, many studies employ the use of simulated microgravity (SM) achieved by machines, such as rotating wall vessels, clinostats, and random positioning machines (RPM), which rotate biological samples along one or more axes (Figure 1.2) [40]. This rotation achieves SM by constantly altering the organisms orientation, preventing sedimentation and confusing gravity sensing mechanisms [76]. Studies of bacterial behavior in SM show that changes in growth, chemotaxis, and metabolism are similar to those observed in SF, and have been identified in model organisms and species isolated from SF [43,77,78]. Plants grown in SM have been found to exhibit phenotypes comparable to those in SF, such as plastid sedimentation, tissue cell density, and growth development [76,79–83].

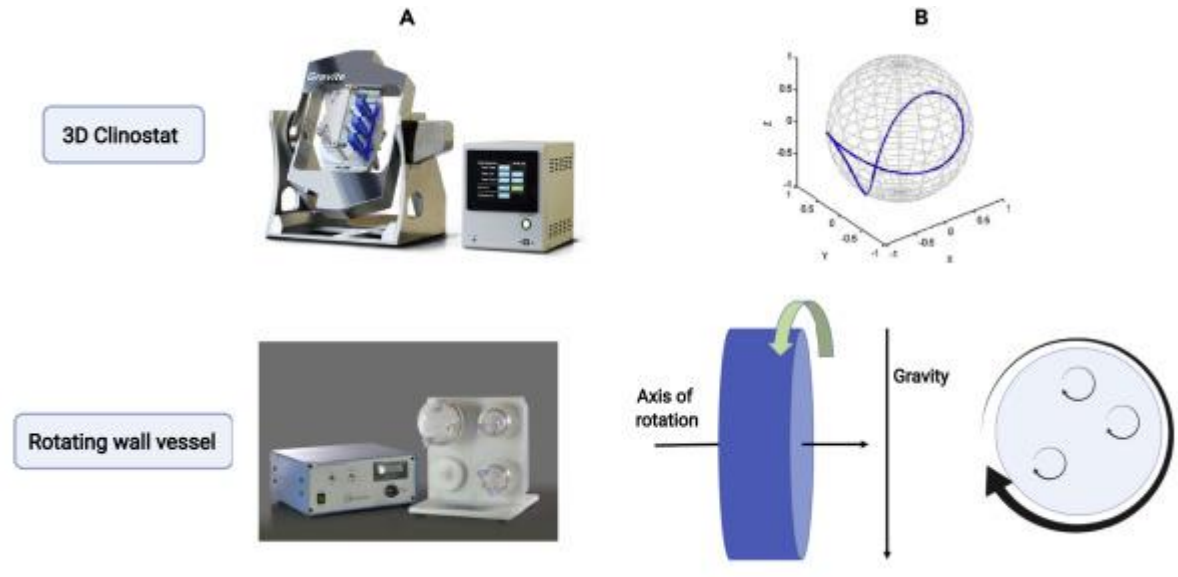

Figure 1.2. (A) Examples of ground based microgravity simulation machines and (B) their corresponding modes of microgravity simulation. 3D clinostat and rotating wall vessel images taken from As One International, Inc. and Synthecon Inc. respectively. Figure adapted from Bijlani et al. 2021.

Studies of bacterial behavior in SM have shown that changes in growth, chemotaxis, and metabolism can vary in similar ways to SF [43,77]. Some findings comparing liquid media SM to SF have been used to argue that the low-shear condition found in both environments creates unique phenotypes due to nutrient and metabolite gradients surrounding the bacteria, which is a secondary effect of SF caused by the unique fluid dynamics in microgravity, and not a direct response to microgravity [78]. These low-shear effects on growth have also been observed in the clinorotation of bacterial samples returned from the Mir space station [84].

Growth of *Paraburkholderia phymatum* in a fluid vessel on an RPM showed a reduction in siderophore production as compared to 1g [85].

However, work done on solid agar media has established that some metabolic processes, such as antibiotic resistance, remain the same in simulated microgravity, space flight, and ground controls. Meanwhile, the production of secondary metabolites has shown variable responses between SF and ground controls [34,35,43]. This is in contrast with SM experiments in low-shear simulation fluid environments. These discrepancies indicate that alterations to microbial phenotypes may be more related to the low-shear fluid environment than to microgravity itself.

Plants grown in SM have been found to exhibit phenotypes comparable to those observed in SF [76]. Experiments comparing *Arabidopsis thaliana* development in clinorotation vs SF have found them to cause similar effects to plastid sedimentation in columella cells, cell density in root meristems, alter germination rate, and seedling development [79,80]. Clinorotation has also been shown to impact growth in *Solanum lycopersicum* (tomato), *Medicago truncatula*, and *Oryza sativa* in ways unique to each species [81–83]. SM effects on stomatal openings have also been observed in various plants, likely related to alterations in IAA content in the shoots of the plants [86,87].

These effects on the stomata have been shown to leave the plant more susceptible to infection, though the addition of PGP bacteria was effective in negating the increased susceptibility [87]. However, in a less controlled system, this effect requires the presence of PGP bacteria, which may not be guaranteed as SM, as well as SF, has been shown to decrease or alter microbiome diversity, likely due to alteration of metabolite profiles [88,89]. SM also affects phenotypes related to microbial symbioses in *M. truncatula*, altering root nodule architecture and overall root structure [82] (Figure 1.3).

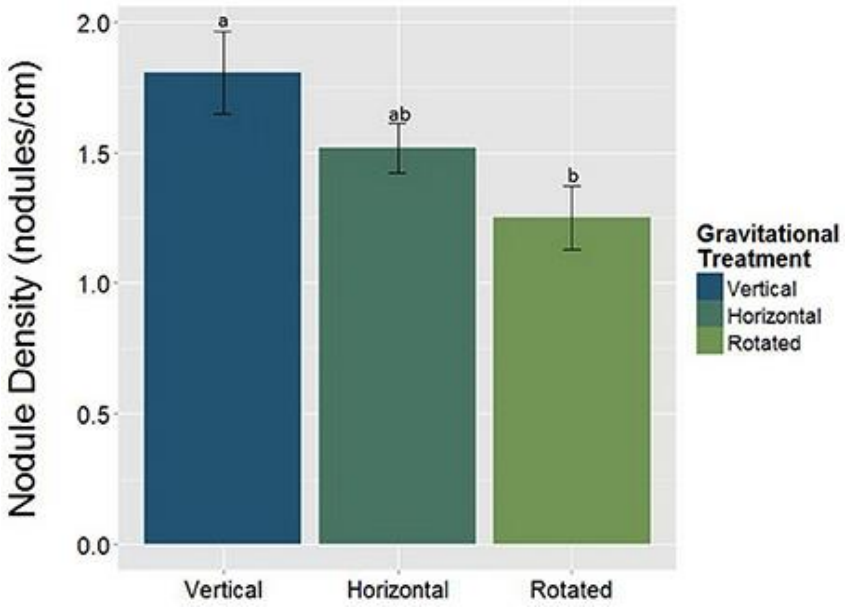

Figure 1.3. Root nodule density in M. truncatula is impacted when grown in SM (2D clinostat) compared to stationary controls positioned horizontally (plants oriented sideways) or vertically (plants oriented upright). Letters above error bars denote statistical differences between groups. Data adapted from Dauzart et al., 2016.

1.7 The Microbiome of the ISS

The sterile approach currently applied to the SF environment comes at the expense of millions of years of co-evolution between plants and microorganisms with the potential to improve the success of crops in space. Despite best efforts to maintain a sterile environment, a significant population of microorganisms persists onboard the ISS. These microbes are likely hitchhikers from one of two sources; human-associated microbes brought by astronauts, and seed-borne endophytes that survived the sanitizing processes [10,89,90].

Microbial analysis of plants grown in Veggie, a crop production system aboard the ISS, revealed heterogeneous populations of culturable bacteria between SF and ground, as well as between different experimental cycles, such as multiple genera appearing in a single group across all experiments. Community sequencing revealed microbial populations with some common taxa across experiments, but with differing ratios of these taxa, indicating that the SF environment influences the composition of these microbiomes [10,89]. For example, among the most common genera identified across 3 Veggie experiments, *Ralstonia* is more common in flight samples, *Burkholderia* is less common, and *Novosphingobium* only appears in ground control samples (Figure 1.4). This population of bacteria has been present in SF and been exposed to astronauts with no recorded cases of infection. By identifying strains with PGP phenotypes, crop production systems can be uniformly

inoculated with a microbiome designed to reduce stress response, improve nutrient uptake, and resist opportunistic infection.

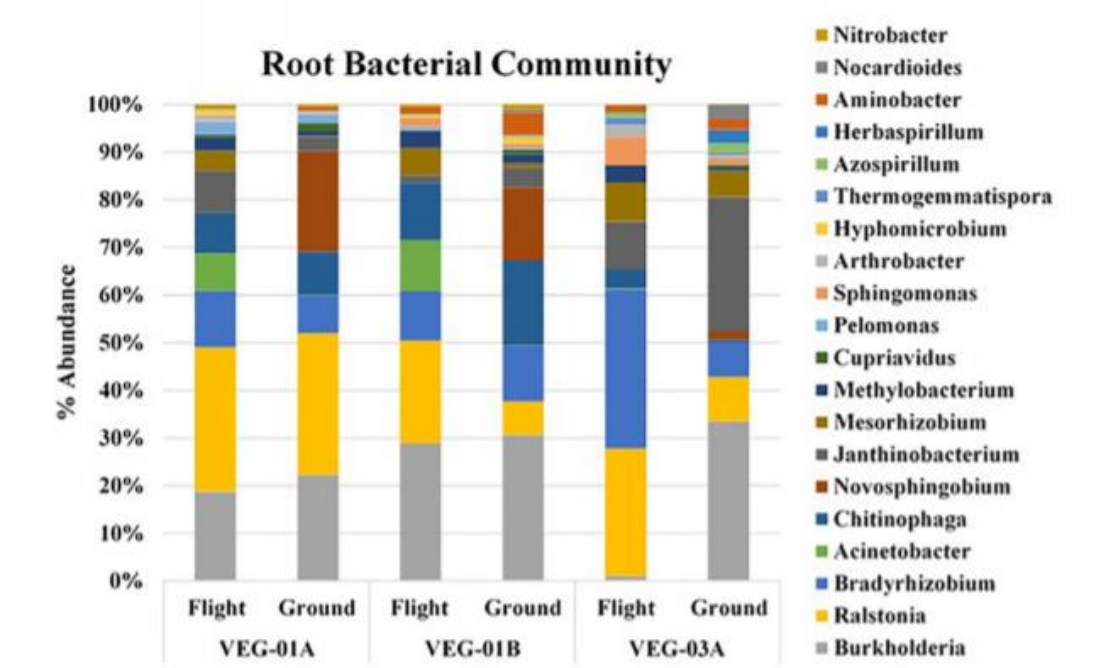

Figure 1.4. Twenty most common root associated bacterial genera identified in Veggie experiments using next generation sequence on the Illumina MiSeq. Less common genera are not included in calculation of % abundance. Data adapted from Khodadad et al. 2020.

Research toward identifying the properties of these microbes is limited, as the primary focus is ensuring that human pathogenic microbes are limited or non-existent. Previous work has used differential culturing methods to successfully identify PGP phenotypes among isolates from plants returned from the Veggie flight samples [91]. Genetic analysis on *Sphingomonas* species returned from the

ISS identified genes related to auxin production, phosphate solubilization, and various stress resistances [92]. Even with this limited sample size, the prospect of identifying helpful microbes from this environment is promising. By using beneficial microbes isolated from this environment, we can increase the confidence that relevant PGP behaviors will be conserved.

1.8 Efforts Toward Improving Microgravity Investigations on PGP Bacteria in this Dissertation Project

Alterations to the plant microbiome due to SF likely alters which microbes are retained through different stages of plant development. These differences in microbiome composition may explain why some host plants show improved tolerance to potential pathogens and abiotic stressors. Due to removal protocols, the microbial makeup of culled plants is unknown; we only know that stressors and opportunistic fungi forced astronauts to remove them [23]. It is therefore reasonable to hypothesize that microbial symbionts of the surviving plants may have been responsible for attenuating the stress responses and preventing fungal infection. Based on this model, it is reasonable to propose that the selective introduction of PGP bacteria isolated from plants grown in the spaceflight environment could improve yield and survivorship in space agriculture.

The collected work of this dissertation attempts to answer four major questions related to plant growth promoting bacteria, their potential activity in microgravity, and their interactions with prospective hosts. In order to answer the questions below, I have developed a research 'pipeline' for the screening of potential plant growth promoting bacteria for use in BLSS (Figure 1.5).

- Question 1: Do bacteria with plant growth promoting phenotypes persist in the microbiome of plants grown on the International Space Station? (CHAPTER 2)
- Question 2: Do plant growth promoting phenotypes occur under microgravity conditions? (CHAPTER 3)
- Question 3: Do bacteria with plant growth promoting phenotypes interact under microgravity? (CHAPTER 3)
- Question 4: Are bacteria with plant growth promoting phenotypes able to improve plant growth (CHAPTER 4)

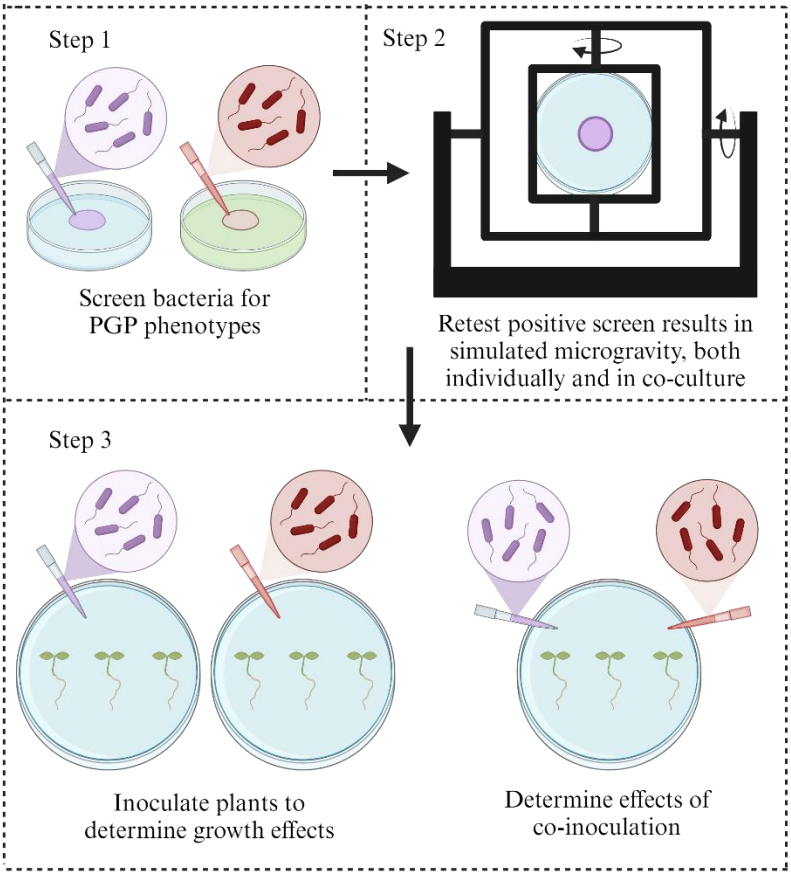

Figure 1.5. Proposed testing pipeline for identifying and verifying PGP properties of bacterial isolates from Veggie Flight samples. Figure generated using Biorender.

Simulated microgravity will be important in these investigations, as SF experiments are limited and expensive. When designing these experiments, the type of the medium is important to consider given the differences observed between liquid culture vessels and agar plates, both in SF and SM. These media types reflect a difference in the microenvironment, and the *in vivo* environment needs to be

considered when designing the *in vitro* test. Liquid culture vessels replicate the LFS of liquids in microgravity, however, many of these microbes are plant endophytes which will inhabit the intercellular space of the host plant tissues. This is more accurately represented by the gel matrix of an agar plate than by a fluid vessel. The need for this distinction is important, as a large majority of microbial work in SM is performed in liquid culture, but work discussed above has shown that many phenotypic changes may be a result of the LFS environment rather than microgravity itself.

1.9 Significance

The testing pipeline developed throughout this dissertation is intended to facilitate the identification of PGP microorganisms as well as provide insight into host-microbial associations in the microgravity environment. Studying host-microbe interactions is important for BLSS research as microbes will play important roles in maintaining the stability of these systems. The lack of information regarding how these interactions will be affected by SF creates a level of uncertainty in their implementation in BLSS. Because biological research, especially plant research, can take months to years to complete, it is important to perform these investigations sooner rather than later as NASA plans to establish permanent stations in orbit and on the surface of the Moon by the end of the decade [93].

Chapter 2
Identification of Plant Growth Promoting Bacteria Within Space Crop Production Systems

This chapter was adapted from a previous publication, cited here as Handy, et al. 2021 [91].

2.1 Introduction

To ensure the longevity of human space exploration it is imperative to develop safe and sustainable methods of life support on the surface of a planetary body, as well as on deep space transports. Bioregenerative life support systems (BLSS) use biological processes to support a human crew by recycling air and water, and in more advanced systems provide food. Food production is an extremely important aspect of BLSS for deep space missions such as establishing a Mars settlement. As humanity establishes settlements further from Earth the logistics of resupply missions will become increasingly complicated, limiting their frequency. Relying on resupply missions to provide food for an off-world settlement will not only be infeasible, but failed or delayed resupplies would be detrimental, potentially lethal, to astronauts.

Current food production systems in space are limited to modified hydroponic systems in microgravity such as the Veggie growth system or the Advanced Plant Habitat aboard the International Space Station (ISS). Crop selection for these

systems focuses on relatively fast-growing, pick-and-eat foods (*i.e.*, selections that require no processing/cooking to be eaten) that can supplement the astronaut diet. These selections provide nutrition lacking in prepackaged food such as potassium, or may not be stable over long duration missions, such as vitamin C [94], but are not a significant source of caloric intake. Food production systems for the moon and Mars will likely have the same considerations for early phase crop selection, retaining these nutritious accessory crops, while also moving toward the production of staple crops with higher caloric density.

The other benefit of these systems that has been noted is a psychological benefit, both to eating fresh food, and tending to the plants [95]. These benefits to the emotional and psychological health of crew members have been reported not only in space, but in the Neumayer Station in Antarctica [96]. These findings underscore the importance that plant growth systems will play in overall crew health in deep space missions, as well as in settlements on the moon and Mars.

The Veggie system is a modified hydroponic plant growth system that uses a "root pillow" containing an arcillite growth substrate which helps to carry water and suspended nutrients to the roots in microgravity [13]. Initial flight tests of the Veggie system, supported that the average fresh mass of surviving plants was higher in space flight samples compared to ground controls, albeit with large variability. However, the total number of ground control plants which survived

over the duration of the study was significantly higher than those in microgravity [13]. Many of the plants in the flight system exhibited guttation, stunted growth, leaf curling, and chlorosis, all hallmarks of different stress responses, and ultimately resulted in the death of many of these plants. One of the major sources of stress in these systems is the behavior of water in the microgravity environment which can limit nutrient uptake, suffocate roots, and allow potentially pathogenic microorganisms, such as fungi, better access to host tissues as water builds up on leaves and around roots [13]. Strategies to improve plant growth, relieve stress, and improve disease resistance are in high demand for these environments.

On Earth, plants are assisted by a multitude of microorganisms, generically classified as plant growth promoting bacteria (PGPB) which provide a variety of functions to their hosts, typically in exchange for a variety of carbon sources contained within root exudates. These include functions such as: (i) the production and manipulation of plant growth hormones [97], (ii) interference of ethylene production by deamination of 1-aminocyclopropane-1-carboxylate (ACC) [68], (iii) fixation of atmospheric nitrogen [98], (iv) scavenging nutrients from the environment [99,100], or (v) provide resistance to potential pathogens [101]. We propose that PGPB could perform a similar function aboard the ISS and other off-world sites to ameliorate the stressors associated with these environments.

In order to be sustainable, settlements on the moon and other planets will need to practice i*n situ* resource utilization (ISRU), or using resources available at the site of the settlement to ensure sustainable operations. ISRU efforts toward food production often look at using the regolith available on site as a plant growth substrate, though studies using regolith simulants have shown the need for applied fertilizers [102,103]. Bacteria capable of scavenging nutrients from regolith, or simply facilitating nutrient uptake from fertilizer solutions would reduce the demand for those nutrients from Earth. Many PGPB can solubilize the key nutrient, phosphate, which may be present in regolith minerals, or may precipitate out of solution when interacting with other geologic material such as calcium, iron, or aluminum [100,104].

The general potential for PGPB to limit the growth of potential pathogens, either by producing antimicrobials or through effective competition, is of great interest for future BLSS [101], as it has been proven difficult to completely eradicate potential plant pathogens on the ISS via sterilization and sanitization. Some PGPB bacteria can produce organic compounds known as siderophores as biocontrol agents for potential pathogens. These compounds frequently work by chelating iron and there is wide diversity among siderophores produced by different organisms, along with a variety of transport proteins. This allows an organism to compete for resources by making the nutrient unavailable to competitors [99]. Such

siderophores may also have the potential to scavenge iron from the rich iron-oxide deposits in Martian regolith making this crucial nutrient more available to plants in this environment [105].

While the biological control of potential pathogens is an important role for PGPB, the most common functions of PGPB are those that improve overall plant growth. One common mechanism for improving plant growth is through manipulation of plant hormones, such as the production of indole-3-acetic acid (IAA), or the interference of ethylene production. IAA is the most common form of auxin, the plant growth hormone. Stimulating auxin responses can encourage root and shoot elongation in most plants, while facilitating nodulation in legumes [97]. Ethylene is a volatile hormone commonly used by plants in abiotic stress responses, as well as in defense against pathogens, and is associated with a reduction in overall growth. Some PGPB, as well as some pathogens, can inhibit ethylene production by metabolizing the ethylene precursor 1-Aminocyclopropane-1-carboxylic acid (ACC) as a nitrogen source. This allows the plant to become more resistant to abiotic stress, by ignoring these effects at low concentrations. Reduced ethylene production is frequently associated with increased plant growth and nodulation given the inhibitory effect this hormone can have on auxin responses [68]. In summary, there are a variety of ways in which PGPB may ameliorate the

challenges plants face in both the spaceflight environment as well as on an off-world settlement.

However, for the protection of the crews who work with the plants, just like all the other items sent to space, everything in these systems is either sterilized or sanitized in the case of the seeds used [106], and these practices are likely to continue. Yet despite these efforts to remove microbes from these systems, the ISS has a fairly diverse microbiome both in the plant growth systems [10], and on the general space station surfaces [90]. The microbiome on the ISS is predominantly derived from the human microbiome [107], which is then spread to the plants via astronauts during watering, pruning, and multi-round harvesting.

Seed-borne endophytic microbes which were unaffected by the surface sanitization of the seeds are likely to contribute to the plant microbiome as well [108,109]. It is from this group of seed-borne endophytes that there may be a subset of plant growth promoting bacteria (PGPB) growing in the plant growth systems of the ISS. Yet, bacterial populations in Veggie are fairly heterogenous between various flight experiments, and while certain genera are consistently present, there are seemingly random differences in their distributions [10]. It is therefore prudent to consider the development of a curated microbiome enriched with specific PGPB with which seeds could be inoculated.

The routine use of such a PGPB ‘probiotic’ can improve current and future space food production systems, enhancing the sustainability of these systems on the moon and Mars. We hypothesized that previous isolates from the Veggie system, were an ideal starting point for the identification of potential PGPB as they already have spaceflight history, indicating their ability to survive the launch process as well as grow in the spaceflight environment. [10]. These bacterial isolates from the Veggie production system aboard the ISS were provided by NASA Kennedy Space Center (KSC) for study and a subset were screened for the presence of PGPB. Specifically, we have investigated these microorganisms for potential PGP functions including siderophore production, phosphate solubilization, indole production, ACC deaminase activity, and fungal growth inhibition. Our efforts confirm the existence of multiple PGPB strains as candidates for further study for their ability to improve yield and reduce stress on plants aboard the ISS. We discuss our findings in the context of their short-term benefits aboard the ISS along with the long-term potential to the development of bioregenerative life support systems in off-world sites.

2.2 Materials and Methods

2.2.1 Maintenance of ISS Isolates

Parameters of the Veggie system are described in [13]. The isolation and identification of the bacterial and fungal species used in this study were previously

reported as well [10]. From this study a subset of bacteria and fungi were selected for their evaluation as PGPB. Bacterial species used in the present study include: *Acinetobacter genomospecies 3, Bacillus altitudinis, Bacillus amyloliquefaciens, Bacillus pseudomycoides, Bacillus pumilus, Bacillus subtilis, Burkholderia pyrocinnia, Cupriavidus pauculus, Curtobacterium flaccumfaciens, Curtobacterium pusillum, Leifsonia aquatica, Methylobacterium rhodinium, Microbacterium marytipicum, Paenibacillus macerans, Paenibacillus pabuli, Pantoea agglomerans, Paracoccus yeeii, Pseudomonas fulva, Ralstonia picketii, Sphingobacterium multivorum,* and *Stenotrophomonas rhizophila*. Fungal species used in this study include: *Fusarium anthophilum, Aspergillus sydowii, Aspergillus ustus,* and *Emericella parvathecia*.

Bacterial samples were taken from freezer stocks made using 30% glycerol media maintained at -80°C. Fresh cultures were maintained on 1.5% solid agar King B media, containing 1.5 % [v/v] glycerol, 20 g/L peptone, 1.15 g/L K_2HPO_4, and 1.50 g/L $MgSO_4 \cdot 7H_2O$, as according to established protocols [104]. Fungal samples were maintained on Inhibitory Mold Agar (IMA) (BBL, Difco, Beckton Dickenson, Franklin Lakes, NJ). All isolates used were cultured from microbial screens of Veggie system flight samples. Stocks were streaked every two weeks to maintain fresh cultures.

2.2.2 Siderophore Production

Methods for detection of bacterial siderophores were adapted from Ambrosini and Passaglia (2017). Chrome azurol S dye solution (CAS) was made by combining, an iron(III) chloride solution, a chrome azurol S solution, and cetrimonium bromide solution were combined with a piperazine/HCl buffer, filter sterilized, then added to 0.2X King B molten agar in a 100 mL/L ratio.

21 Bacterial isolates were cultured in King B liquid media for 48-72 hours at 30°C, then pipetted onto four spots on solid assay plates containing 0.2X King B with CAS. Assay plates were incubated for 7 days at 30°C, then the orange halos were measured. Isolates that produced particularly large halos were redone with one central spot per plate in triplicate.

2.2.3 Phosphate Solubilization

Assessment methods for phosphate solubilization were adapted from Ambrosini and Passaglia (2017) using GY/tricalcium phosphate solid medium. GY solid growth medium was prepared using glucose, yeast extract, and 1.5% agar, then while molten sterile solutions of K_2HPO_4 and $CaCl_2$ were added just before pouring. The addition of these two solutions causes insoluble calcium phosphate to precipitate, making the medium cloudy.

21 Bacterial isolates were cultured in King B liquid media for 48-72 hours 30°C, then pipetted onto four spots on GY/tricalcium phosphate solid medium.

Plates were incubated for 7 days at 30°C then observed for growth and zones of clearing.

2.2.4 Indole Production

Twenty-one bacterial isolates were grown in King B liquid media supplemented with 2.5 mM tryptophan. IAA production is determined by a spectrophotometric described in Ambrosini and Passaglia (2017), which uses Salkowski's reagent, containing 0.012 g/mL $FeCL_3$ and 7.75 M H_2SO_4 to react with indoles present in the culture solution.

Liquid cultures were incubated for 48-72 hours at 30°C. Aliquots were taken to measure the OD_{600} of the culture, then centrifuged for 10 minutes at 5400 × g. The supernatant was pipetted off, mixed with Salkowski's reagent, then allowed to incubate at room temperature in the dark for 30 minutes. Samples were run in triplicate through a spectrophotometer at 550 nm with a set of IAA standards ranging from 0 to 250 µg/mL. Sample IAA concentrations calculated from the standard curve were normalized to the measured OD_{600} of the sample to express the amount of indole produced relative to the final concentration of bacteria.

2.2.5 ACC Deaminase Activity

The ACC deaminase activity assay uses an indirect method described in Ambrosini and Passaglia (2017). Bacteria are grown on Dworkin and Foster (DF) salts solid media, containing 2% agar, 2 g/L glucose, 2 g/L gluconic acid, 4 g/L

K_2HPO_4, 6 g/L Na_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L citric acid, 1 mg/L $FeSO_4 \cdot 7H_2O$, 0.01 mg/L H_3BO_4, 0.011 mg/L $MnSO_4 \cdot H_2O$, 0.12 mg/L $ZnSO_4 \cdot 7H_2O$, and 0.008 mg/L $CuSO_4 \cdot 5H_2O$. Each isolate is grown on two plates side by side, on one, an ACC solution is spread over the plate providing a 1 mM concentration of ACC, providing the only source of nitrogen. The other plate is left as a nitrogen free control.

21 Bacterial isolates were cultured in King B liquid media for 48-72 hours 30°C. Aliquots were centrifuged at 5400 × *g* for 10 minutes, the spent media pipetted off, and the bacteria resuspended in 0.85% saline. This rinse step was repeated 3 times, then the final resuspension pipetted onto three spots on each plate containing DF salts solid medium with or without ACC. Plates were placed in an incubator at 30°C for 7 days.

2.2.6 Fungal Biocontrol

Fungal biocontrol assays were performed on tryptic soy agar (TSA) (BBL,Difco, Beckton Dickenson, Franklin Lakes, NJ) plates. Bacterial isolates were cultured in King B liquid media for 48-72 hours before inoculating assay plates. Fungal samples were prepared by suspending stock isolates into sterile water until an optical density at 600 nm (OD_{600}) of 0.1 was reached. A sterile swab was used to streak lawns of fungal suspension on TSA plates, then bacterial samples were pipetted onto 4 spots on the plate. Plates were allowed to grow for 7

days then observed for interactions. 15 bacterial isolates were assayed for biocontrol capability against 4 fungal isolates.

2.3 Results

From the previously isolated microbiome samples from the Veggie crop production system [10], 21 bacteria were identified (see materials and methods for full list) that were readily culturable under general conditions (30°C on King B solid media) and remained viable in freezer stocks. Also selected from these previous microbiome studies were 4 fungal species to be used in the antifungal assessment. These samples were isolated from leaf tissue, root tissue, or from the root substrate of the spaceflight Veggie system and are therefore likely candidates to have derived from seed-borne endophytic populations.

2.3.1 Siderophore Production

Siderophores are heavily involved in iron uptake and in doing so can provide effective competition for nutrient resources. Excess iron taken up by PGPB can then be passed off to host plants to improve plant performance. CAS was used to determine the presence of siderphores in culture. This dye solution forms a blue dye complex consisting of chrome azurol S, iron(III), and cetrimonium bromide, which turns orange when a strong chelator, such as a siderophore, removes the iron [104]. 9 out of 20 tested isolates were able to grow on the media and produce orange halos including; *Acinetobacter genomospecies* 3, *Burkholderia pyrocinnia*,

Cupriavidus pauculus, Curtobacterium flaccumfaciens, Paenibacillus macerans, Paenibacillus pabuli, Pantoea agglomerans, Pseudomonas fulva, and *Ralstonia picketii* (Table 2.1**)**. Of these, the 5 isolates *B. pyrocinnia*, *C. flaccumfaciens*, *P. macerans*, *P. agglomerans*, and *R. picketii* exhibit strong siderophore production, producing large halos with diameters ranging from 4.47 to 5.87 cm (Figure 2.1A), compared to the other 4 isolates whose diameters ranged from 0.88 to 1.20 cm (Figure 2.1B) (Table 2.1).

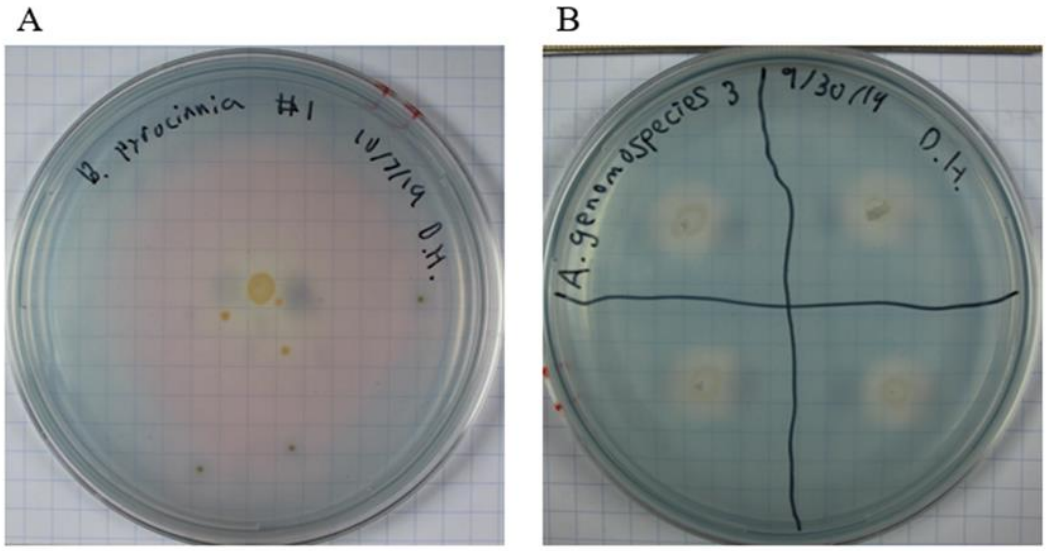

Figure 2.1. Growth of bacterial isolates on 0.2X King B/CAS assay plates showing examples of (A) a "strong" siderophore producer, *Burkholderia pyrrocinia,* and (B) a "weak" siderophore producer, *Acinetobacter genomospecies* 3. Orange/yellow zones indicate removal of iron from the CAS dye complex.

2.3.2 Phosphate Solubilization

Phosphate is a crucial nutrient for plants and bacteria that can easily precipitate out of solution depending on the environmental pH and geochemical interactions. Certain PGPB are able to solubilize phosphate to assist with uptake by both the bacteria and host plant. In this assay bacteria incapable of solubilizing the calcium phosphate precipitate will be unable to grow, bacteria that are particularly efficient will develop clear halos around the spot[104]. Out of 20 isolates, 5 were able to grow including; *Bacillus pumilus*, *B. pyrocinnia*, *C. flaccumfaciens*, *P. macerans*, and *P. agglomerans* (Table 2.1). Clear halos developed around spots of *B. pyrocinnia*, *P. macerans,* and *P. agglomerans* (Figure 2.2A) while the media around *B. pumilus* and *C. flaccumfaciens* remained opaque with no halo (Figure 2.2B) (Table 2.1**)**.

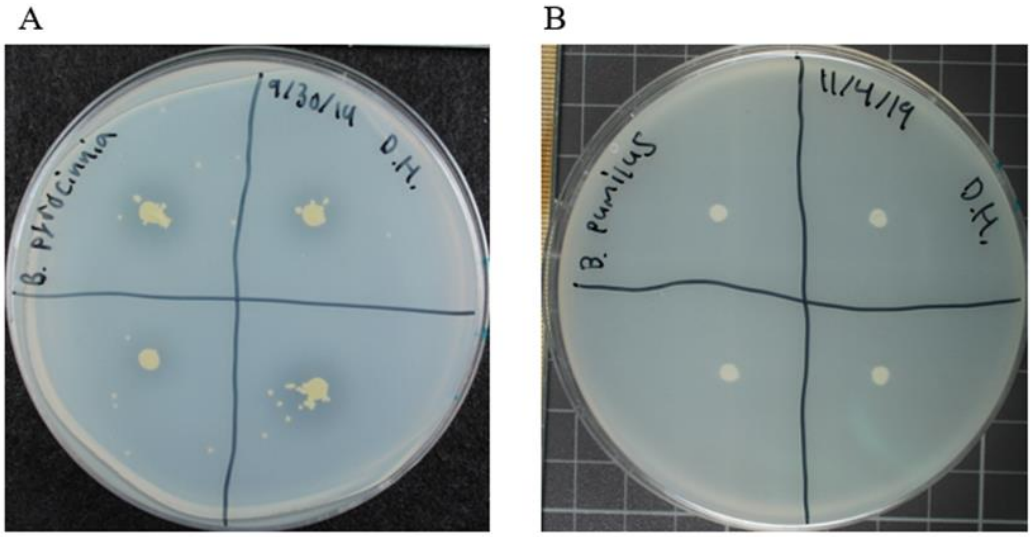

Figure 2.2. Growth of bacterial isolates on GY/tricalcium phosphate assay plates showing (A) *Burkholderia pyrrocinia*, capable of developing clear zones around spots, and (B) *Bacillus pumilus*, capable of growing on insoluble phosphate but unable to develop clear zones. Growth and/or development of clear zones indicate ability to solubilize calcium phosphate

2.3.3 Indole Production

Bacterial production of IAA, the most common auxin, can improve plant growth by stimulating root and stem elongation. A spectrophotometric assay [104] determined that most bacterial isolates produced some amount of indole, typically in the 10-25 μg/mL range, though *P. pabuli* produced a particularly low amount of indole that may be false due to instrument sensitivity (Table 2.1). Others produced a particularly high amount of indole, such as *P. agglomaerans*. To account for cultures with low cell density at time of analysis, calculated indole measurements

were normalized to the OD_{600} of the culture, creating ratio of indole concentration to relative cell abundance. This revealed that *C. flaccumfaciens*, *P. fulva*, *Sphingobacterium multivorum*, and *P. macerans* produce more significant amounts of indole than the raw data would indicate. This normalization also confirms that other bacteria, such as *P. pabuli and R. picketii*, do not produce notable amounts of indole.

2.3.4 ACC Deaminase Activity

The plant hormone ethylene is heavily involved in plant stress response and is responsible for many of the resulting phenotypes of stress conditions. The precursor molecule to ethylene, ACC, can be used as a nitrogen source by PGPB, and in doing so, the bacteria limits the amount of ethylene produced. An indirect assay was used to detect ACC deaminase based on growth patterns [104]. Isolates were rated based on differential growth between plates with and without ACC on a scale of 1-3, with 3 indicating improved growth on plates with ACC (Figure 2.3A), 2 indicating mildly improved growth (Figure 2.3B), and a 1 indicating poor growth on both plates (Figure 2.3C) Bacteria that produce ACC deaminase were able to grow on the plate with ACC and poorly on the plate without ACC, whereas bacteria that do not produce ACC deaminase grew poorly on both. Bacteria that showed growth unlike these, such as growing well on both, or better on the ACC free plate, are described as indeterminant. These bacteria are likely capable of nitrogen

fixation and therefore do not depend on nitrogen in the growth media. Of the 21 isolates examined, 9 species were given a rating of 3, 1 species was given a rating of 2, 4 species a rating of 1, and the remaining isolates were classified as indeterminant (Table 2.1).

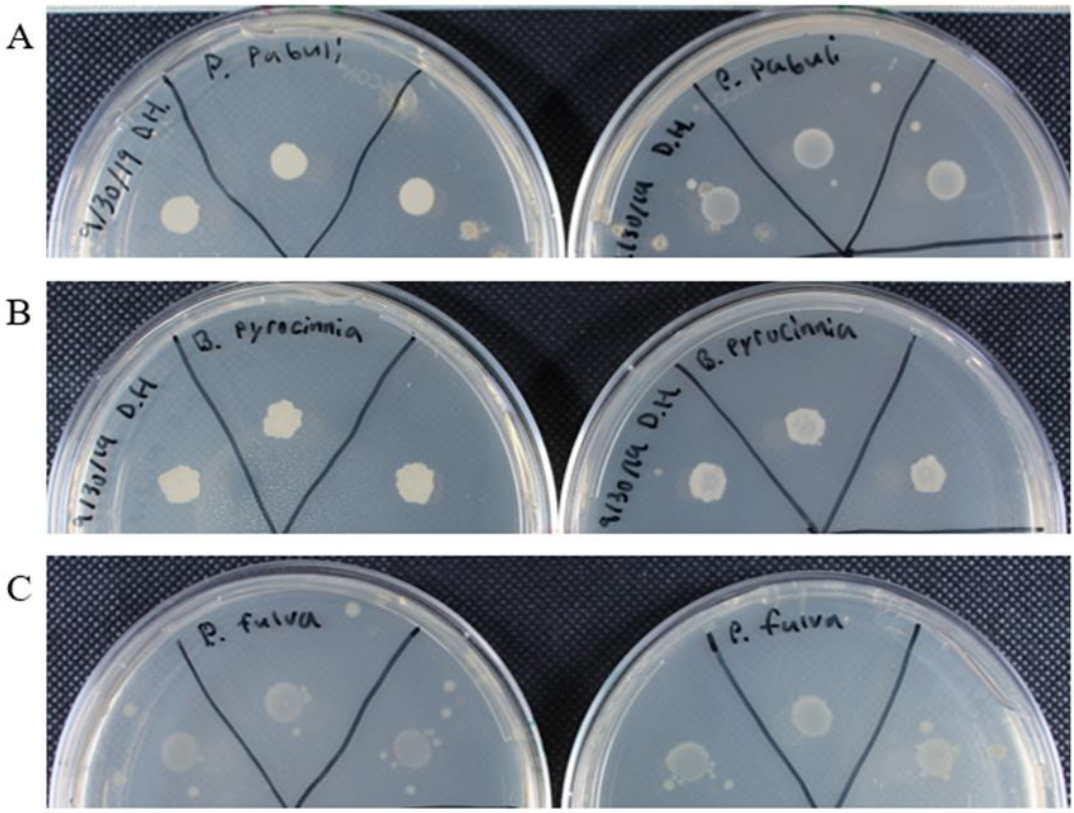

Figure 2.3. Growth of bacteria on DF salts medium with 1 mM ACC (left) and nitrogen free (right). (A) Example of rating 3 growth, *P. pabuli*, showing improved growth in the presence of ACC. (B) Example of rating 2 growth, *B. pyrocinnia*, showing mildly improved growth in the presence of ACC. (C) example of rating 1 growth, *P. fulva*, showing no improved growth in the presence of ACC.

Table 2.1. Siderophore production; growth and resulting halo diameter on 0.2X King B/CAS media after one week of growth. N = 4 for halos under 1.5 cm, N = 3 for halos over 1.5 cm. Phosphate Solubilization; growth and resulting halo diameter on GY/tricalcium phosphate media after one week of growth. For samples with growth but no surrounding halo, spot diameter was treated as halo diameter. N=4 for all samples. Indole production; results of spectrophotometric assay for indole production. Concentration of IAA is calculated based on a set of standards, expressed in µg/mL. IAA concentration was normalized to the final OD_{600} of the culture to account for slow growing bacteria producing low but significant amounts of indole. ACC deaminase; ACC deaminase indirect assay rankings. 1 indicates no ACC deaminase activity, 2 indicates mild ACC deaminase activity, 3 indicates strong ACC deaminase activity. A result of X indicates indeterminant based equivalent, unimpeded growth on both plates.

Species	Siderophore Production	Phosphate Solubilization			Indole Production		ACC Deaminase
	Growth (+/-); Halo Diameter (cm)	Growth (+/-)	Halo (+/-)	Halo Diameter (cm)	[IAA] (µg/mL)	[IAA]/OD_{600}	Ranking
A. genomospecies 3	+; 1.20 ± 0.07	-	-		18.46 ± 0.13	36.55 ± 0.25	3
B. altitudinis	-	-	-		13.08 ± 0.42	27.47 ± 0.88	3
B. amyloliquefaciens	-	-	-		15.85 ± 0.58	53.92 ± 1.98	3
B. pseudomycoides	-	+	-	0.30 ± 0.00	25.09 ± 0.13	53.85 ± 0.27	X
B. pumilus	-	-	-		11.10 ± 0.25	31.71 ± 0.72	X
B. subtilis	-	-	-		26.66 ± 0.75	84.64 ± 2.39	3
B. pyrocinnia	+; 5.87 ± 0.45	+	+	1.08 ± 0.19	10.74 ± 0.38	12.82 ± 0.45	2
C. pauculus	+; 0.88 ± 0.08	-	-		15.62 ± 0.56	20.68 ± 0.75	3
C. flaccumfaciens	+; 4.47 ± 0.24	+	-	0.38 ± 0.04	64.61 ± 0.56	203.82 ±1.78	1
C. pusillum	-	-	-		17.65 ± 0.63	52.84 ± 1.90	1
L. aquatica	-	-	-		15.50 ± 0.55	22.96 ± 0.82	1
M. rhodinium	-	-	-		10.65 ± 0.13	64.55 ± 0.77	1
M. marytipicum	-	-	-		21.86 ± 0.13	31.97 ± 0.19	1

P. macerans	+; 4.60 ± 0.47	+	+	0.53 ± 0.08	22.40 ± 0.46	263.56 ± 5.38	1
P. pabuli	+; 0.90 ± 0.07	-	-		2.58 ± 0.25	2.98 ± 0.29	3
P. agglomerans	+; 4.83 ± 0.48	+	+	0.85 ± 0.09	118.13 ± 14.85	309.23 ± 38.96	3
P. yeeii	-	-	-		9.75 ± 0.46	19.31 ± 0.91	X
P. fulva	+; 1.05 ± 0.18	-	-		35.41 ± 0.91	319.02 ± 8.24	1
R. picketii	+; 5.80 ± 0.08	-	-		11.64 ± 0.34	12.47 ± 0.36	3
S. multivorum	-	-	-		45.92 ± 0.56	110.64 ± 1.36	3
S. rhizophila	-	-	-		17.83 ± 0.83	44.13 ± 2.06	X

2.3.5 Fungal Biocontrol

PGPB can play a crucial role in plant pathogen defense by providing competition or producing antimicrobial compounds [101]. Assessments were made using a co-culturing assay examining simultaneous bacterial and fungal growth. Bacterial antifungal properties were classified on a rating scale from 1 to 4, with 1 being ineffective and 4 being most effective (Figure 2.4). Bacteria rating a 4 developed clear zones around the spot, i.e., no fungal growth was observed (Figure 2.4A) while those with a rating of 3 developed with a zone of reduced fungal growth around the spot (Figure 2.4B). In samples with a rating of 2, bacteria developed with no fungal growth on the spot but with no effects on the surrounding fungal growth (Figure 2.4C). Finally, bacteria with a rating of 1 showed no observable impacts on fungal growth on the plate or the spot itself (Figure 2.4D).

The assessment for each combination of bacteria and fungi is described in Table 2.2. More than half of the combinations (38/60) were given a rating of 2 against the fungal species *Fusarium anthophilum*, *Aspergillus sydowii*, *Aspergillus ustus*, and *Emericella parvathecia*. Only 5/60 combinations were given rating 1 against the fungi, three of them paired against *F. anthophilum*; *Leifsonia aquatica*, *Methylobacterium rhodinum*, and *P. agglomerans*, as well as *M. rhodinum* paired against *A. sydowii* and *E. parvathecia*. 13/60 combinations were given rating 3, most notable being *Bacillus amyloliquefaciens* causing decreased growth effects on all 4 fungal species, and *B. pumilus* causing decreased growth effects on all except

F. anthophilum. Other rating 3 combinations include: *Curtobacterium pusillum* against *A. sydowii* and *E. parvathecia*; *B. pyrocinnia*, *P. fulva*, and *Stenotrophomonas rhizophila* against *A. ustus*; and *Bacillus pseudomycoides* against *A. sydowii.* The 4 remaining combinations were given a rating of 4; *B. pyrocinnia* exhibited cleared zones with *A. ustus* and *E. parvathecia*, and *B. pseudomycoides* exhibited cleared zones on *A. sydowii* and *E. parvathecia.*

Table 2.2. Antifungal assessment results of bacterial isolates on four fungal isolates. Rating 4 exhibits zones of no fungal growth around the bacterial spot. Rating 3 bacteria have zones of reduced fungal growth around the spot. Rating 2 bacteria only prevent fungal growth within the bacterial spot. Rating 1 bacteria are unable to prevent fungal growth in or around the bacterial spot.

Species	*F. anthophilum*	*A. sydowii*	*A. ustus*	*E. parvathecia*
A. genomospecies 3	2	2	2	2
B. amyloliquefaciens	3	3	3	3
B. pseudomycoides	2	3	4	4
B. pumilis	2	3	3	3
B. pyrocinnia	2	4	3	4
C. pusillum	2	3	2	3
L. aquatica	1	2	2	2
M. rhodinum	1	1	2	1
M. marytipicum	2	2	2	2
P. pabuli	2	2	2	2
P. agglomerans	1	2	2	2
P. yeeii	2	2	2	2
P. fulva	2	2	3	2
R. picketii	2	2	2	2
S. rhizophila	2	2	3	2

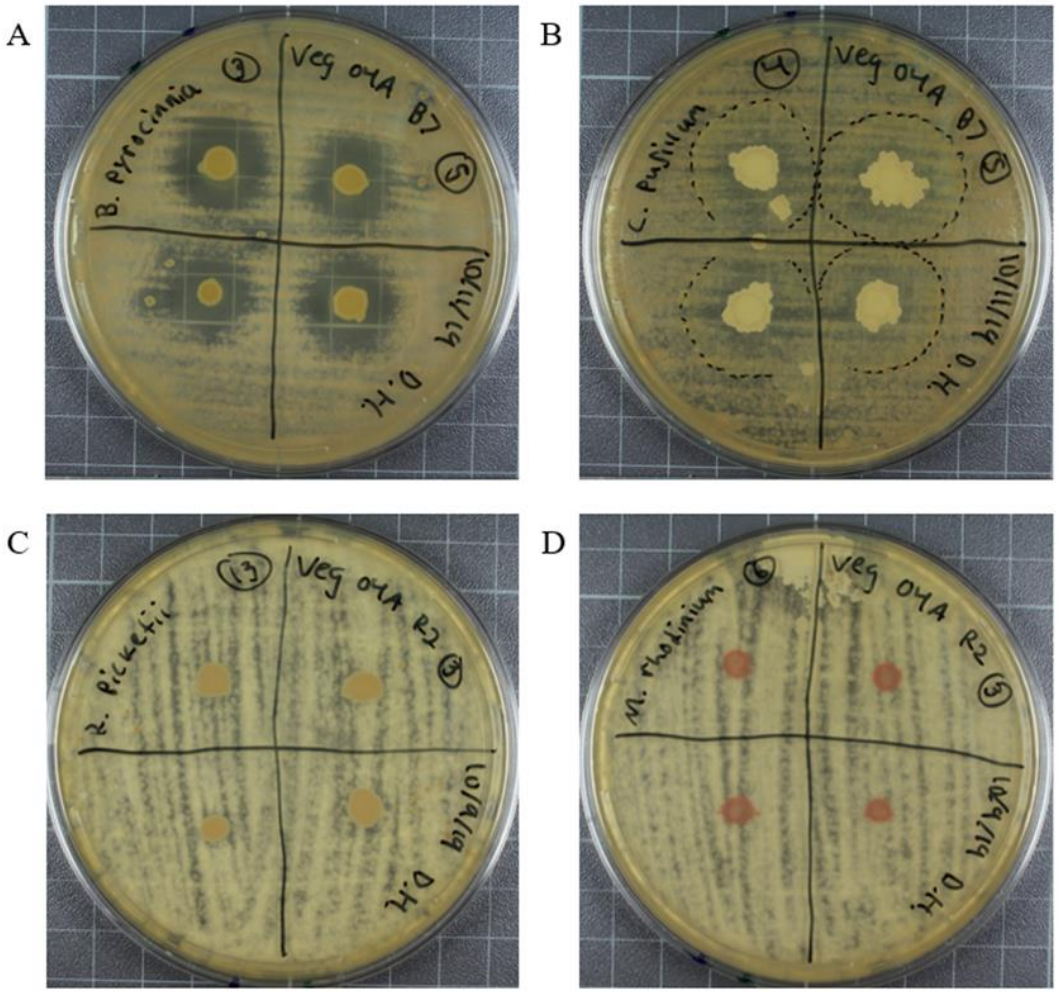

Figure 2.4. Antifungal assay plates showing examples of (A) Rating 4 growth of *Burkholderia pyrrocinia* on *Emericella parvathecia*, (B) Rating 3 growth of *Curtobacterium pusillum* on *Emericella parvathecia*, (C) Rating 2 growth of *Ralstonia picketii* on *Aspergillus sydowii* and (D) Rating 1 growth of *Methylobacterium rhodinum* on *Aspergillus sydowii*.

2.4 Discussion

The spaceflight environment has proven to be stressful to plants, yet long-term operations in deep space depend on successful plant growth for supplemental life support, food production, and psychological benefits. The isolation and detection of PGPB with spaceflight history provides an opportunity to improve plant growth through the development of a curated microbiome for the growth of plants aboard the ISS, on the moon, or another planet. Using several biochemical and phenotypic assessments of bacteria isolated from the Veggie crop production system, we have confirmed the potential for several PGPB to exist in conjunction with plants grown on the ISS. However it remains to be seen if these bacteria are indeed capable of encouraging plant growth within the spaceflight environment. Of the 21 bacterial isolates, 17 were shown to be capable of at least one plant beneficial function, though most performed 2 to 3 of these functions. Of those that appeared to generate indole, further studies will need to confirm the presence of IAA, especially those that did not exhibit other plant growth promoting functions, due to the assay used being general for all indoles.

These assessments provide a collection of culturable, space-viable PGPB for the selective application to space food production systems. The selection of microorganisms for our planned community could be made smaller and more manageable by the selection of "generalist" PGPB, *i.e.* those that perform two or

more of the plant-growth promoting functions evaluated herein. A potential minimal microbiome from this set of isolates would consist of just *P. agglomerans*, and *B. pyrocinnia*, both capable of phosphate solubilization, siderophore production, and ACC deamination. *P. agglomerans* also produces indoles while *B. pyrocinnia* provides antifungal capabilities.

Both species have been previously studied for plant growth promotion. Studies have shown that certain strains of *P. agglomerans* have nitrogen fixing capabilities, and produce not only IAA, but also the phytohormones abscisic acid, gibberellic acid, and cytokinins [110]. Inoculations of rice seedlings with the species increases overall growth rate and improved nutrient uptake preventing malnutrition stress [110,111]. Other studies show that *P. agglomerans* also has the ability to reduce heavy metal toxicity [112] and provide biocontrol against the plant pathogen *Ralstonia solanacearum* [113]. *B. pyrocinnia* is a member of the *Burkholderia cepacia* complex and has been investigated for its biocontrol potential against plant pathogen *Rhizoctonia solani* via production of the antibiotic pyrrolnitrin [114] and studies on its siderophores have shown the ability to improve seed germination, plant size and fruit size [115].

An inoculated microbiome can also be tailored for specific functions. ISRU approaches using regolith as a plant growth substrate on Mars will want to prioritize the acquisition of mineral nutrients, so the selection of microbes may be

expanded to include *C. flaccumfaciens* and *P. macerans*, both of which solubilize phosphate and produce siderophores. Hydroponic systems would not require the highly active nutrient scavenging bacteria, but inoculation with indole producing, ethylene reducing, and pathogen mediating bacteria can help increase biomass and yield in hydroponic systems [116,117]. Thus, including indole producing bacteria such as *P. fulva* and *P. macerans*, ACC deaminating bacteria such as *P. pabuli* and *R. picketii*, and antifungal bacteria such as *B. pseudomycoides* and *B. amyloliquefaciens* would improve the robustness and efficiency of the system.

Ultimately, the selection of needed PGPB functions will be determined by the location of Lunar and Martian settlement sites, as well as the method of plant growth. ISRU approaches will have to be tailored not just to the planetary body, but also the specific settlement site as locations will vary in mineralogy, which will also require the development of site-specific regolith simulants important for research in these systems [118,119]. The use of siderophore-producing and phosphate-solubilizing bacteria will be dependent on the amount of insoluble nutrients available in the on-site substrate, as well as what interactions applied fertilizers may have with the geologic material.

Compounds contained in on-site regolith may also create a need for plant growth promoting functions not investigated here, such as remediation of perchlorate compounds found on the Martian surface [120,121]. These compounds

have the potential to bioaccumulate in plant tissues [122] and be toxic to Martian settlers [123], but can also be a source of breathable oxygen when degraded by perchlorate-reducing bacteria [124].

Before implementing these bacteria into food production systems, each species will need to be validated for use. Further testing should include *in planta* studies to confirm that the strains investigated are plant growth promoting. Certain species investigated, such as *C. flaccumfaciens* have variants that are plant growth promoters [125] and others that cause disease such as bacterial wilt [126]. Species of interest for food production systems will also need to be screened for their potential as human pathogens, whether opportunistic or otherwise to ensure crew safety. However, it should be noted that the strains analyzed here are already present in the plant growth systems being tended to by astronauts on the ISS.

As research into BLSS continues, further investigation of PGPB will be crucial. The ISS has already shown that current sterilization and sanitization practices have been shown to be ineffective at preventing opportunistic microorganisms from infecting plants, and the stress effects caused by the space environment can be mitigated to improve plant growth and yield. As crops are selected for space food production systems, and sites are selected for settlement on the moon and Mars, this work will go towards providing the foundation for developing robust and sustainable agricultural systems.

Chapter 3
Effects of Simulated Microgravity on Bacterial Plant Growth Promoting Phenotypes

3.1 Introduction

3.1.1 Background

As space exploration advances in the coming decades, mission durations are planned to be months to years in duration. Mission resupplies will become increasingly prohibitive in terms of cost and logistics as missions move further from Earth, making mission success and crew survival dependent on integrated systems. To support this, the development and implementation of bioregenerative life support systems (BLSS) have been deemed ‘mission critical’. An important part of these systems will be robust and sustainable agricultural modules. Plants can be used in air revitalization, waste management, and most importantly food production.

However, many of these off-world environments pose significant challenges to successful plant growth such as low nutrient growth substrates, toxic components, minimal water, altered gravity, etc. Strategies to compensate and/or ameliorate these effects are crucial to advancing BLSS to viable technological readiness levels. Bacteria and other microorganisms often play vital roles in supporting the health of their hosts. Plants in their native environments are not unlike corals, i.e. highly interdependent on a diverse population of microbes that

inhabit specific tissues, in this case, plant tissues as well as the root surface and the proximal soil, i.e. the ‘rhizosphere’ [48]. Within these holobiont assemblages, microbes possess a variety of direct and indirect strategies for improving growth. Common plant growth promoting (PGP) phenotypes include improved nutrient uptake, pathogen prevention, growth hormone production, and stress mediation [56].

Plants grown in spaceflight (SF), such as aboard the International Space Station (ISS) experience a variety of abiotic and biotic stresses. For example, the behavior of water in microgravity can cause nutrient solutions to envelop plant tissues, creating hypoxic conditions in roots. Similarly, limited convective air currents can allow for the buildup of metabolic by-products that under these specific conditions are deleterious. For instance, the accumulation of oxygen derived from photosynthesis can increase photorespiration frequency thereby inhibiting carbon fixation. [15,16]. Meanwhile, the buildup of ethylene, the primary stress response hormone, can induce inappropriate immune responses, stunted growth, and other effects [17]. Between abiotic stress and opportunistic pathogens, crop loss is common in Veggie flight samples.

Protocols for sending anything to the ISS call for sanitizing and sterilizing all the materials involved to limit microbial contamination of the station. These protocols originate out of a concern for astronaut health to prevent the introduction

of human pathogens. Despite these efforts, routine monitoring of the microbiome of the ISS finds a spatially and temporally diverse population of microorganisms in this isolated environment. This is indeed the case for plants grown in the Veggie system where a diverse range of microbes have been identified [10,89]. The presence of these microbes can likely be attributed to two sources: interactions with the astronaut crew who carry their own microbiome on their skin as well as internally, and seed-borne endophytic bacteria that survived the sanitizing processes protected by the plant seed coat.

3.1.2 Microbes in Microgravity

Our previous work has found that a subset of these bacteria possesses PGP phenotypes common to plant-associated endophytes, including phosphate solubilization, siderophore production, indole production, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCd) activity [91]. Phosphate readily reacts with other compounds and forms insoluble salts, such as tricalcium phosphate, restricting its bioavailability, however many microbes are able to resolubilize phosphates via a variety of means such as weak acid production [54]. Siderophores are chelating agents which can bind to environmental metal micronutrients making them more bioavailable, and many siderophores play roles in biocontrol [53]. PGP bacteria can also influence plant physiology by manipulating phytohormones, such as the production of the common growth

hormone indole-3-acetic acid (IAA), or by interfering with ethylene production by breaking down ACC, the stress hormones metabolic precursor [68,69].

Having successfully identified bacteria with PGP phenotypes there is an immediate question which must be answered for their integration into BLSS and support crop production. How does microgravity impact PGP phenotypes? Microgravity investigations into prokaryotic phenotypes such as virulence and biofilm formation have shown that these factors often change both in SF as well as in simulated microgravity. *Salmonella typhimurium* has been observed to exhibit hypervirulence, while *Candida*, *Listeria*, *Enterococcus,* and MRSA exhibit hypovirulence in SF [38,39]. Unique biofilm architectures have also been observed in SF compared with ground controls, with the phenotype appearing coupled to the motility of the organism as a motility knockout did not form the same unique architecture [36]. Antibiotic resistance in microgravity depends on culture method/motility, with liquid cultures often showing increased resistance, while *E. coli* grown on agar plates were equally susceptible in SF and normal gravity [35]. Based on these investigations, as well as others, specific rules for microbial behavior under microgravity are unclear. As such, it is important to investigate each species individually, and with consideration toward media environment.

An immediate and crucially related question is: Are PGP bacteria capable of working and growing together in spaceflight to support plant growth? As to this

question, many of these bacteria were isolated from the same plants and are therefore likely to have interacted with each other. This could lead to synergistic effects, with bacteria possessing different phenotypes complimenting each other, or bacteria with shared phenotypes improving the microbiome's overall efficiency in that process. This suggests the possibility of creating the equivalent of a plant probiotic for ameliorating stress and improving yield in the space flight environment. Alternatively, they could be antagonistic toward one another, with one inhibiting the other, creating differential microbiomes between tissues or resulting in discrepancies in microbiome populations between individual plants. Also, given the stochastic nature of these populations, there is no guarantee the same strains will be present flight-flight, especially when different crops are used, as different species are likely to carry different endophytes. This makes it even more important to determine the compatibility of strains before use in a mixed consortium.

3.1.3 Testing Phenotypes in Consortia

To investigate these questions, we built a 3D clinostat to perform these experiments (Figure 3.1). 3D-clinorotation was selected over other means such as low fluid-shear (LFS) simulation as the latter reflects the growth of bulk liquid cultures in microgravity, rather than the surfaces or interior of roots, the interface of the plant-microbial associations with which we are concerned. [127]. These are

effectively semi-solid environments better replicated on solid agar media than liquid broth-based assessments. This is especially crucial as bacterial phenotypes tend to differ between solid and liquid media environments, especially in spaceflight [34,35,43]. To this end, PGP phenotype assays done in liquid culture will need to be adapted to agar plates which can be placed on the clinostat.

Figure 3.1. 3D clinostat assembled at Florida Institute of Technology.

Next, we considered which strains to investigate both individually as well as for co-culturing studies for this investigation. From our growing list of ISS-derived (ISSd) isolates possessing PGP phenotypes, 3 bacteria have been selected for these experiments based on published phenotypic data: *Pantoea agglomerans (Pa), Curtobacterium flaccumfaciens (Cf),* and *Burkholderia pyrrocinia (Bp).* These bacteria were selected for possessing at least three PGP phenotypes. *P.*

agglomerans possesses all 4 phenotypes; *C. flaccumfaciens* and *B. pyrrocinia* each possess 3 phenotypes (Table 3.1) [91]. Here, we aim to study how simulated microgravity (SM) and co-culture may affect these phenotypes. Determining how these microbes behave in co-culture is crucial to understanding the interactions occurring in SF. Plants have extremely diverse microbiota, hosting up to thousands of species [49]. As such, many of these strains were isolated from the same plants, and thus were capable of interacting synergistically or in opposition with each other and their host. Plants thrive on this diversity, as individual mutualists can play a variety of roles, and possessing mutualists with redundant capabilities improves the likelihood that the host sees the benefit from a desired phenotype.

Table 3.1. Plant growth promoting properties associated with the ISSd bacteria used in this study

Species	Gram Type (+/-)	Siderophore Production	PO_4 Solubilization	ACCd Activity	Indole Production	Refs.
Pantoea agglomerans	-	+	+	+	+	[91,128–135]
Curtobacterium flaccumfaciens	+	+	+	-	+	[91,136–139]
Burkholderia pyrrocinia	-	+	+	+	-	[91,140–142]

P. agglomerans is a Gram-negative bacteria found in a wide variety of environments and has extremely high strain variation [143]. Mostly associated with plants, the species consists of strains that are either beneficial or pathogenic and effect a wide range of plant hosts [144]. Beneficial strains have been observed to assist in potassium and phosphorous uptake, produce iron chelating siderophores, and contribute to nitrogen fixation [128–130]. Strains have also been found to produce ACCd, which interferes with ethylene production, as well as produce a variety of phytohormones including indole-3-acetic acid, abscisic acid, gibberellic acid, and cytokinins [130–132]. *P. agglomerans* has also been found to produce the antibacterial compounds phenazine and pantocin, as well as siderophores with antifungal properties [133–135]. Previous work on our ISS-derived (ISSd) strain used here has identified that it possesses ACCd activity, indole production, siderophore production, and solubilizes phosphate [91].

The most studied strains of *C. flaccumfaciens* are pathogens such as *C. flaccumfaciens* pv *flaccumfaciens* and *C. flaccumfaciens* pv *ortii*, which are commercially relevant strains as they are sources of bacterial wilt in many legumes [145]. While nearly half of *C. flaccumfaciens'* 50+ variants are plant pathogens, many of the remaining strains manifest PGP phenotypes in association with plants such as olive, cucumber, ryegrass, and wheat [139,146]. Among these strains phosphate solubilization appears to be a common trait, while there is strain variance in the production of indoles and siderophores [136–139]. The ISSd strain used here

has previously been identified as positive for phosphate solubilization, as well as indole and siderophore production, but ACCd negative [91].

The Gram negative soil microbe *B. pyrrocinia* has been well documented as a PGP biocontrol agent against fungal disease in a variety of plants including poplar, rice, and tobacco [142,147–150]. *B. pyrrocinia* achieves this by producing a variety of compounds, including volatile organic compounds such as dimethyl disulfide, antifungals such as occidiofungin, and siderophores such as ornibactin [142,147,149]. This species has also been recorded to solubilize phosphate, produce indole, and exhibits ACCd activity [140,141]. This ISSd strain was previously reported to produce siderophores, solubilize phosphate, and exhibit ACCd activity, however indole production was negligible. Also, this strain of *B. pyrrocinia* produced a strong inhibitory effect on *Aspergillus sydowii* and *Emericella parvathecia* strains isolated from Veggie, however these strains were not available for follow-up experiments here[91].

The goal of these investigations is to identify candidate bacteria for a plant-beneficial consortium that can be used to inoculate BLSS to improve overall yields. To this end, we must determine how the relevant phenotypes of these microbes are affected by microgravity, as well as co-culturing. Thus, on top of continued screening, we have adapted experiments to run on a microgravity simulator to compare phenotypes in different gravity environments, and to compare phenotypic

results of mixed cultures. The work with these three isolates establishes methods that will be used to screen the rest of the bulk phenotypes identified among the ISSd bacteria. These findings, along with those generated in future studies, will help to broaden the collective understanding of how microgravity affects bacterial behaviors.

3.2 Materials and Methods

3.2.1 Maintenance and preparation of bacterial stock cultures

All samples used here were requested from the OSDR list of available VEG-01 isolates, which were delivered as 2-5 day old cultures on tryptic soy agar (TSA) petri plates. Frozen samples were prepared by adding sterile glycerol to aliquots of turbid 18-48 hour cultures, dependent on growth rate of the bacteria, to a final glycerol concentration of 30% and stored at -80°C. Frozen aliquots are then thawed, as needed, to generate live stocks, then refrozen a maximum of 2 times before disposal and preparation of new freezer samples. Live bacteria stocks were maintained on TSA and recultured every 2-3 weeks to maintain viability. Cultures were grown at 30°C for 48 hours, then stored at room temperature, approximately 21°C.

3.2.2 Construction of 3D Clinostat

The 3D clinostat was designed and assembled at Florida Institute of Technology using a mix of purchased and printed components. Structural components including the two ‘arms’ of the device as well as the petri dish holders were modeled in CAD and 3D printed using polylactic acid (PLA) filament. Nema17 stepper motors driven by TMC2208 Stepper Drivers were installed on each of the two axes, under the control of an Arduino Nano Every controller. These motors were set to turn at 3 complete rotations per minute but can be adjusted by computer. Sample plates are arranged among 3 weight-balanced bays surrounding the inner axis of rotation, observable in Figure 3.1, where plates are held in place by slots along the axis and spring-loaded adjustable plates on the outer arm of the bay.

3.2.3 Preparation and plating of bacteria

For phenotypic screens, bacterial colonies were aseptically transferred from stock plates to assay plates or culture broth using a flame-sterilized wire loop. For SM co-culture, individual strains were cultured overnight, approximately 18 hours, in tryptic soy broth. Aliquots were centrifuged down and cell pellets were resuspended in isotonic saline 3 times to rinse out excess culture media. Optical density at 600 nm (OD_{600}) was measured for each suspension in triplicate, and the

average was used to calculate dilution to an OD_{600} of approx. 0.2. For single bacteria cultures this was then used in plating for assays. For cocultures, the optical density was treated as the effective concentration in order to make suspensions consisting of 0.2 OD_{600} for each bacteria. For all assays, plates were divided into seven sections, each dedicated to a specific combination of 1, 2, or all 3 bacteria (Figure 3.2). In the center of each section, 3 µL of bacteria suspension was pipetted and allowed to dry before inverting. For each experiment, half of all plates were placed onto the clinostat for SM treatment or placed, inverted, next to the machine as the normal gravity control (GC).

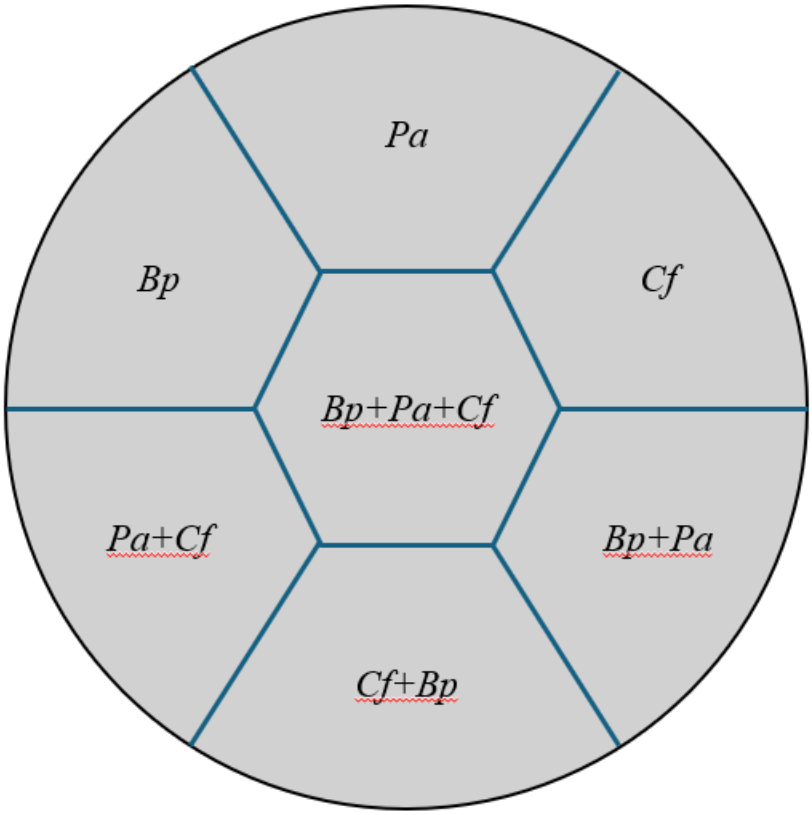

Figure 3.2. Illustration of plate layout for coculture experiments, separated into 6 outer sections with a 7th section in the center. Each plate includes a single species culture for comparison to combined cultures of two or three species. All plate assays follow this template for placement of cultures. Starting in the top half, leftmost section and moving clockwise, each section will contain *Bp, Pa, Cf, Bp+Pa, Cf+Bp, Pa+Cf,* and the center section will grow *Bp+Cf+Pa.*

3.2.4 Co-culture Growth Characteristics on TSA

As a control to compare effects that assay media may have on certain species, 12 replicate co-culture plates were prepared on TSA media and grown for 4 days at 21°C under SM and normal gravity. Images were taken with a black background after the growth period to compare morphology and measure the size of bacterial spots.

3.2.5 Siderophore production

Siderophore production assay media was prepared as previously described [91,104]. Chrome azurol S (CAS) dye solution was prepared using stock solutions of iron (III) chloride, chrome azurol S, and cetrimonium bromide, combined with a piperazine/HCl buffer. Assay media was prepared using 0.2x King B agar media, containing glycerol, peptone, K_2HPO_4, and $MgSO_4$ heptahydrate, supplemented with 10% by volume CAS dye solution (KBC). For screening assessment, plates were incubated for 1 week at 30°C. For SM co-culture assessment, 12 replicate plates were incubated for 3 days at 21°C with and without SM. Images were taken after the growth period to compare morphology and measure the size of bacterial spots and siderophore halo. Images were taken with a black background for size comparisons, and with a white background for halo analysis.

3.2.6 Phosphate solubilization

Phosphate solubilization assay media was prepared as previously described [91,104]. GY solid media was prepared from glucose and yeast extract, then supplemented while molten with solutions of K_2HPO_4 and $CaCl_2$ to form insoluble tricalcium phosphate, referred to as GY/TP media. For screening assessment, plates were incubated for 1 week at 30°C. For SM co-culture assessment, 12 replicate plates were incubated for 4 days at 21°C with and without SM. Images were taken with a black background after the growth period to compare morphology and measure the size of bacterial spots and solubilized halo.

3.2.7 ACC deaminase activity

ACCd activity assay media was prepared previously described [91,104]. Dworkin and Foster salts (DFS) solid media, glucose, gluconic acid, K_2HPO_4, Na_2HPO_4, $MgSO_4$ heptahydrate, citric acid, $FeSO_4$ heptahydrate, H_3BO_4, $MnSO_4$ monohydrate, $ZnSO_4$ heptahydrate, and $CuSO_4$ pentahydrate. Assay plates were supplemented by spreading an ACC stock solution to an effective concentration of 1 mM ACC, with unsupplemented plates used as negative controls. For screening assessment, plates were incubated for 1 week at 30°C. For SM co-culture assessment, 6 replicate plates were incubated for 4 days at 21°C with and without

SM. Images were taken with a black background after the growth period to compare morphology and measure the size of bacterial spots.

3.2.8 Indole Production

The screening protocol remained the same as previously described. King B broth was supplemented with tryptophan and bacteria were cultured for 48 hours at 30°C. Aliquots of the culture were centrifuged to remove bacteria and the supernatant treated with Salkowski's reagent to detect the presence of indole. Absorbance at 550 nm (A_{550}) was compared to a set of indole-3-acetic acid standards to determine indole concentration of bacterial supernatant [91,104]. For SM, the indole production assay had to be adapted from the previously used liquid culture format to an agar plate format to be placed into the microgravity simulator. To prepare King B with tryptophan (KBT) agar, King B agar media was prepared and supplemented with filter sterilized tryptophan solution while the agar was still molten, such that the concentrations are equivalent to previously described methods. 6 replicate plates were incubated for 3 days at 21°C with and without SM. Images were taken with a black background after the growth period to compare morphology and measure the size of bacterial spots. After imaging, the spots and surrounding agar (~1.5 cm diameter) was excised from the plate and placed in a tube with 1 mL 95% ethanol to extract indole. Ethanol was chosen as the extractant

based on its use as the solvent for the IAA stock solutions. After extracting overnight, the extractant was removed and used in the colorimetric assay to compare A_{550} with IAA standards, normalized to the area of the spot recorded in the spot size analysis.

3.2.9 Image Analysis

All image analysis was performed in ImageJ. Spot size was determined using the “analyze particles” function to find the area of each spot, then these values were used to calculate the reported diameters. For phosphate halo analysis, plates were highlighted with circle selection tool, then enhanced contrast; pixel saturation 3%. The circular selection tool was used to outline the halo, and the “measure” function was used to find the area of the halos, which was then used to calculate the reported diameters. For siderophore halo analysis, plates were highlighted with circle selection tool, then enhanced contrast; pixel saturation 3%. Then the “color threshold” adjustment was used to highlight colors outside hue range 127 to 147, which includes the blue color of unchanged media, thus highlighting the area of color change. The circular selection tool was used to outline the highlighted area, and the “measure” function was used to find the area, which was then used to calculate the reported diameters.

Graphs and analyses were generated in Graphpad Prism v10.2.3. Two-way ANOVA and Tukey's multiple comparisons test were run on all data sets to determine significant differences between coculture combinations and gravity treatments.

3.3 Results

3.3.1 Continued Screening of ISSd Isolates

Work presented here, performed at Florida Tech, analyzes isolates from VEG-01 missions requested through the OSDR, whereas our previously published screening results, performed on site at NASA Kennedy Space Center, used isolates from VEG-03 missions. Since our previous work was published, screening of ISSd isolates in our lab has continued to identify PGP phenotypes among the population. The work is continuous as new isolates are generated by Veggie microbial count studies and made available through the OSDR. Of the 13 species reported here, all have at least one of the four phenotypes (Table 3.2).

Table 3.2. Screening results of ISSd isolates for PGP phenotypes. '+' indicates positive for phenotype, '-' indicates negative for phenotype, 'N' indicates phenotype is not yet tested.

Species	Indole Production	PO_4 Solubility	Siderophore Production	ACC Deaminase
Acidovorax antharii	+	-	+	+
Acidovorax avenae	N	N	+	+
Bacillus arlettae	-	+	-	-
Bacillus fluminis	+	+	-	+
Brevindimonas vesicularis	+	+	-	-
Cupriavidus metallidurans	N	+	-	+
Enterobacter cancerogenus	N	+	+	+
Kocuria pulustris	-	N	-	+
Leifsonia shinshuensis	N	-	+	-
Methylobacterium lusitanum	N	N	+	+
Microbacterium laevaniformans	N	-	+	+
Micrococcus yunneneneis	+	-	+	+
Pantoea vagans	N	+	+	-

3.3.2 Growth on Tryptic Soy Agar

Comparing spot morphologies on general growth media can assist in determining if a particular strain is dominant in coculture, as well as for differences between gravity treatments, without the influence of assay media creating advantage to one bacteria. In this qualitative analysis, it was observed that morphologies did not noticeably differ between SM and GC. In this experiment, *Bp* was observed to have a matte yellow color, a wide, flat shape with a curved edge. *Pa* had an off-white color and a thin ring near the edge. *Cf* had a pale-yellow color

and was noticeably larger than *Bp*. When cocultured, *Bp+Pa* had a matte yellow center with a distinct ring in a closer to the edge compared to *Pa*. The coculture of *Cf+Bp* was identical to the growth of *Bp* on its own. *Pa+Cf* growth had the same color as *Cf*, with distinct ring off-white border, similar to but wider than the border of *Bp+Pa*. The combined coculture of all three had identical morphology to *Bp+Pa* (Figure 3.3). Statistical analysis of spot diameter showed that the growth of only *Pa* in single culture was altered by SM, having increased compared to GC, with all other comparisons between SM and GC being similar. Interestingly, all cocultures involving *Bp* were similar in diameter to the *Bp* single culture. Single and cocultures of *Pa* and *Cf* are roughly similar, with *Cf* in SM being larger than the rest (Figure 3.4).

The qualitative differences in morphology show that these bacteria can successfully cohabitate in some combinations, though *Bp* appears to be a potentially controlling member of the trio. The combination of *Bp* with *Cf* appears to remove morphology indicative of *Cf* such as the pale-yellow color or the smooth growth lacking a ridged edge. The other indicator of *Bp* controlling nature is the size; though mixed morphology was observed with *Pa*, all mixed cultures involving the *Bp* were statistically similar in size, while those without it were overall larger (Figure 3.3 & Figure 3.4).

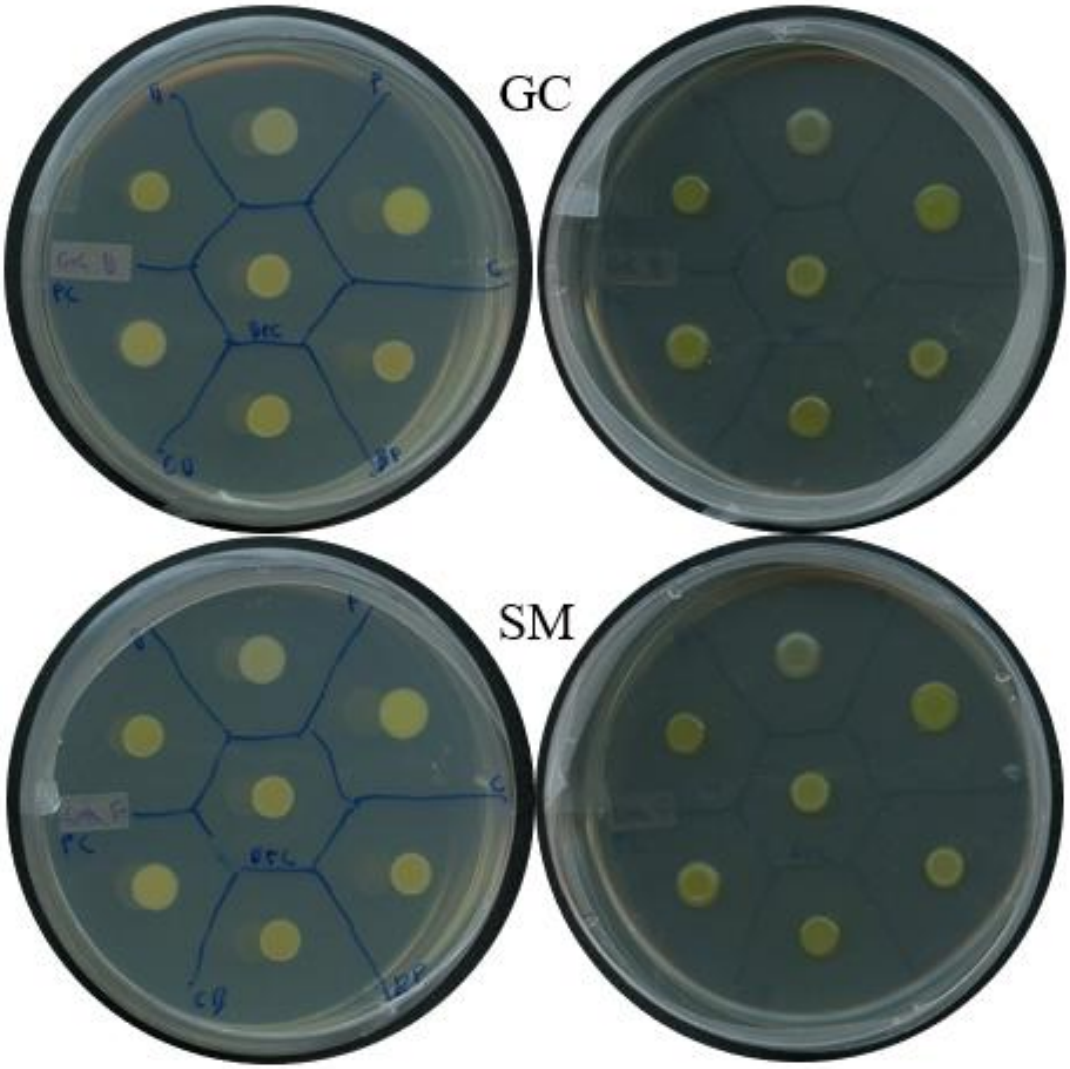

Figure 3.3: Representative images of bacterial growth on TSA, in GC (top) or SM (bottom) conditions, imaged from the bottom (left) and top (right) of the plate for qualitative analysis. For each plate, starting in the top half, leftmost section and moving clockwise, each section contains *Bp, Pa, Cf, Bp+Pa, Cf+Bp, Pa+Cf,* and the center section will grow *Bp+Cf+Pa.*

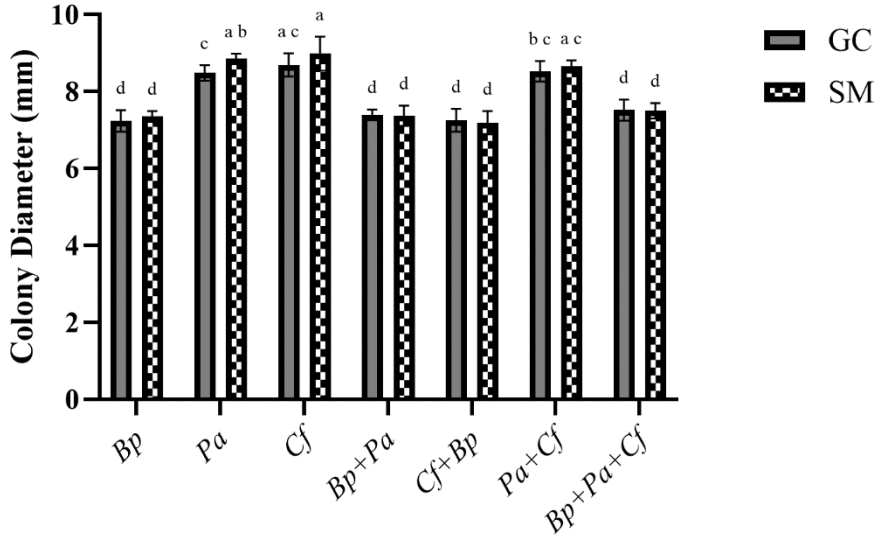

Figure 3.4. Spot diameter of bacterial growth on TSA with GC and SM treatments. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=12.

3.3.3 Siderophore Production

Most notably, *Cf* did not grow on KBC, unlike previously published findings, except on a single GC plate where two small spots formed in the area where *Cf* had been pipetted (Figure 3.5) [91]. *Bp* grew the same in both gravity treatments with it's typical yellow color, forming a shallow bowl with a raised edge. Meanwhile, *Pa* had a glossy off-white color with a distinct white center, a dome shape. The growth of *Bp+Pa* was matte off-white with a bumpy, cauliflower-esque texture, forming a wide dome with rough edges. The other coculture

combinations appeared identical to the culture not including *Cf*; *Cf+Bp* appeared identical to *Bp, Pa+Cf* appeared identical to *Pa*, and *Bp+Pa+Cf* appeared identical to *Bp+Pa* (Figure 3.5).

When comparing spot size between gravity treatments, most cultures were larger in SM than in GC, only *Bp+Pa* and *Bp+Pa+Cf* were statistically similar between treatments. When comparing culture combinations, cultures originally containing *Cf* were grouped with the cultures without *Cf*, in the same pattern as described with spot morphology. When comparing *Pa, Bp,* and their combination, the combined culture was most similar to *Pa*, while *Bp* had the smallest overall growth (Figure 3.6). Analysis of the halos, however, shows that only *Cf+Bp* halo was different between gravity treatments, though the *Bp* halos do have a moderate, though non-significant increase in SM (Figure 3.7).

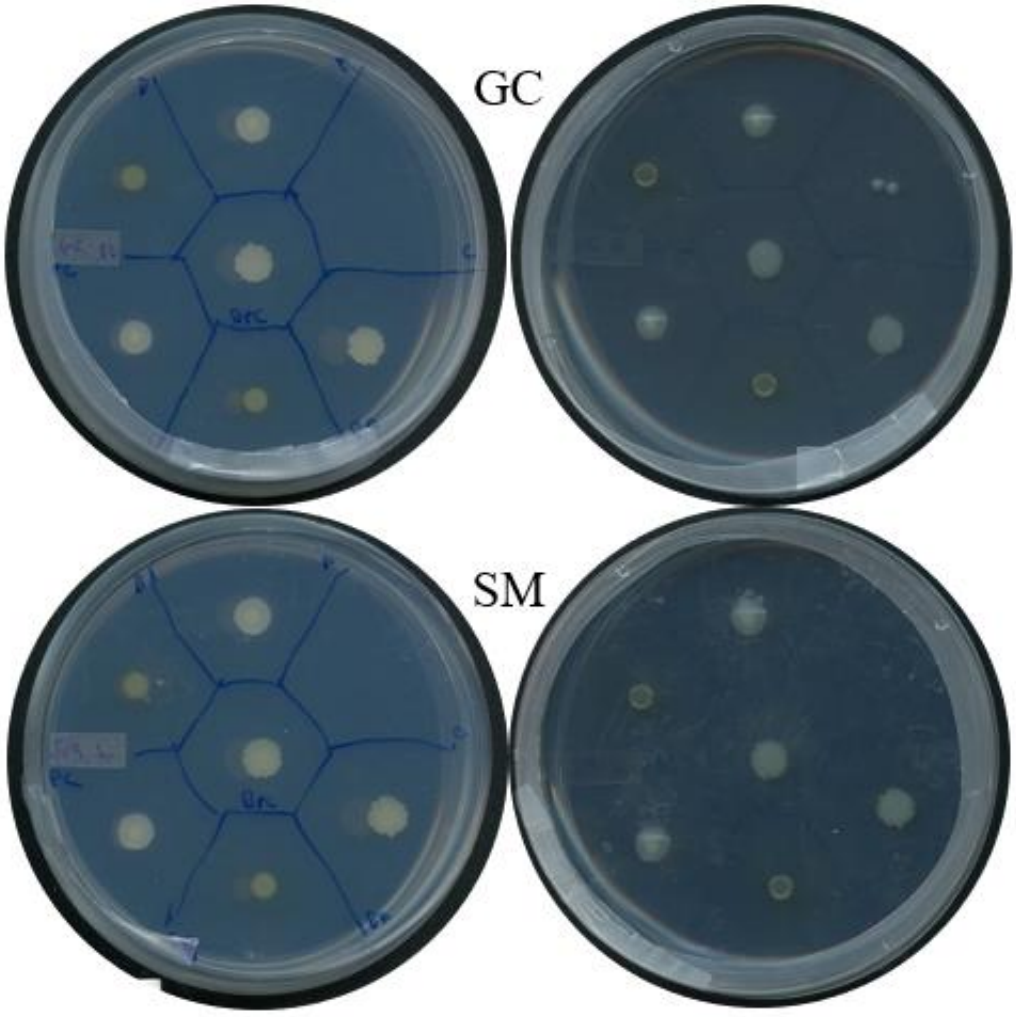

Figure 3.5. Representative images of bacterial growth on KBC in GC (top) or SM (bottom) conditions, imaged from the bottom (left) and top (right) of the plate for qualitative analysis. For each plate, starting in the top half, leftmost section and moving clockwise, each section contains *Bp, Pa, Cf, Bp+Pa, Cf+Bp, Pa+Cf,* and the center section will grow *Bp+Cf+Pa.*

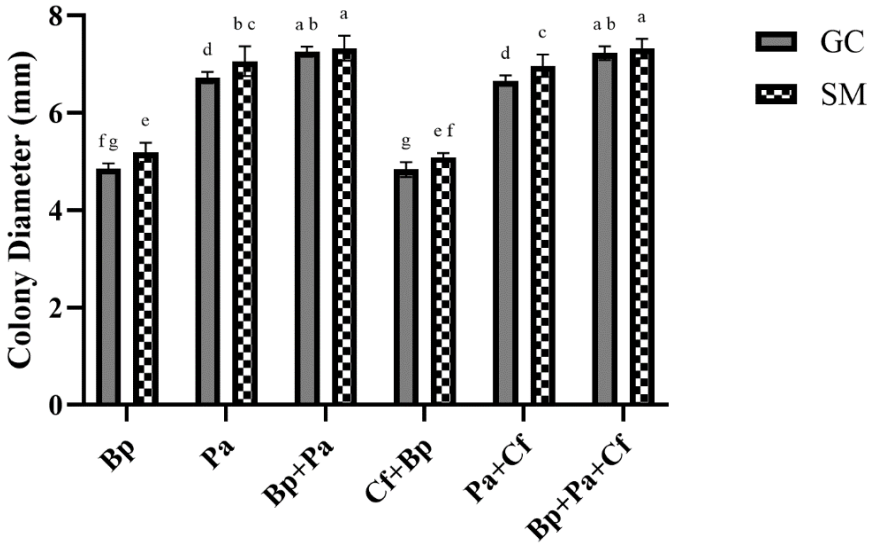

Figure 3.6. Spot diameter of bacterial growth on KBC, with GC and SM treatments. *Cf* not shown due to lack of growth. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=12.

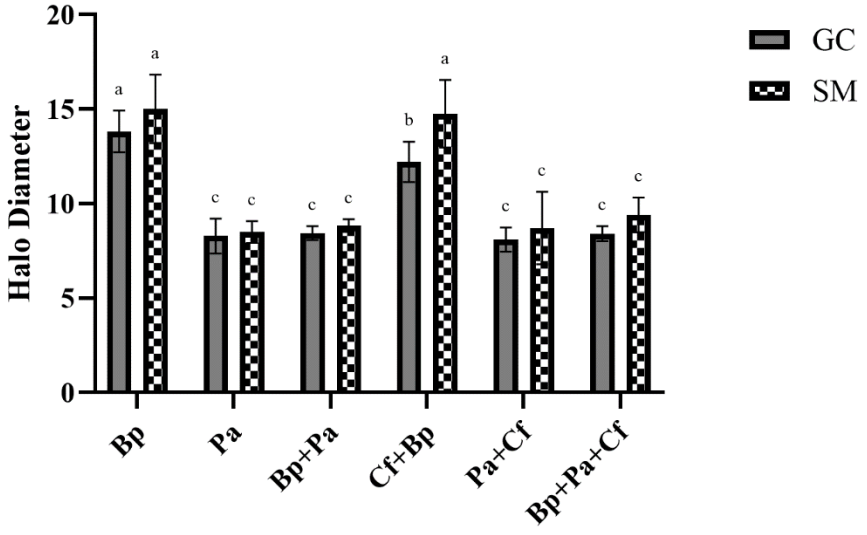

Figure 3.7. Siderophore halo diameter resulting from bacterial growth on KBC, with GC and SM treatments. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=12.

3.3.4 Phosphate Solubilization

All cocultures involving *Pa* appear identical to the single culture of *Pa*, a glossy, white spot with a textured edge in both gravity treatments, but a raised flat center on GC plates or an overall dome shape in SM. Meanwhile, the coculture of *Cf*+*Bp* is identical to *Bp* single culture; matte, slightly beige off-white spot, with a flat center and raised edges, identical in both GC and SM. Finally, growth consistent with *Cf* was only observed in single culture, a consistent glossy, slightly yellow off-white color in both GC and SM, but a slight dome in GC while flat with raised edges in SM (Figure 3.8).

Analysis of spot size reveals an increase in diameter of SM spots compared to corresponding GC growth in each culture combination except *Bp* and *Cf*+*Bp*. Comparing culture combinations, cocultures involving *Pa* were in the same statistical grouping as the single culture of *Pa* in the matching gravity treatment. The combination of *Cf*+*Bp* was statisically the same as *Bp*, and *Cf* in SM, all of these being larger than *Cf* in GC (Figure 3.9). Clear halos resulting from phosphate solubilization in the media were only observable in the four cultures involving *Pa*. Of these, only the triple coculture showed a statistically significant increase in diameter due to SM treatment; though all SM halos are in the same statistical grouping while the halos of *Pa*, *Bp*+*Pa*, and *Pa*+*Cf* in GC share statistical groupings with both SM and GC of *Bp*+*Pa*+*Cf* (Figure 3.10).

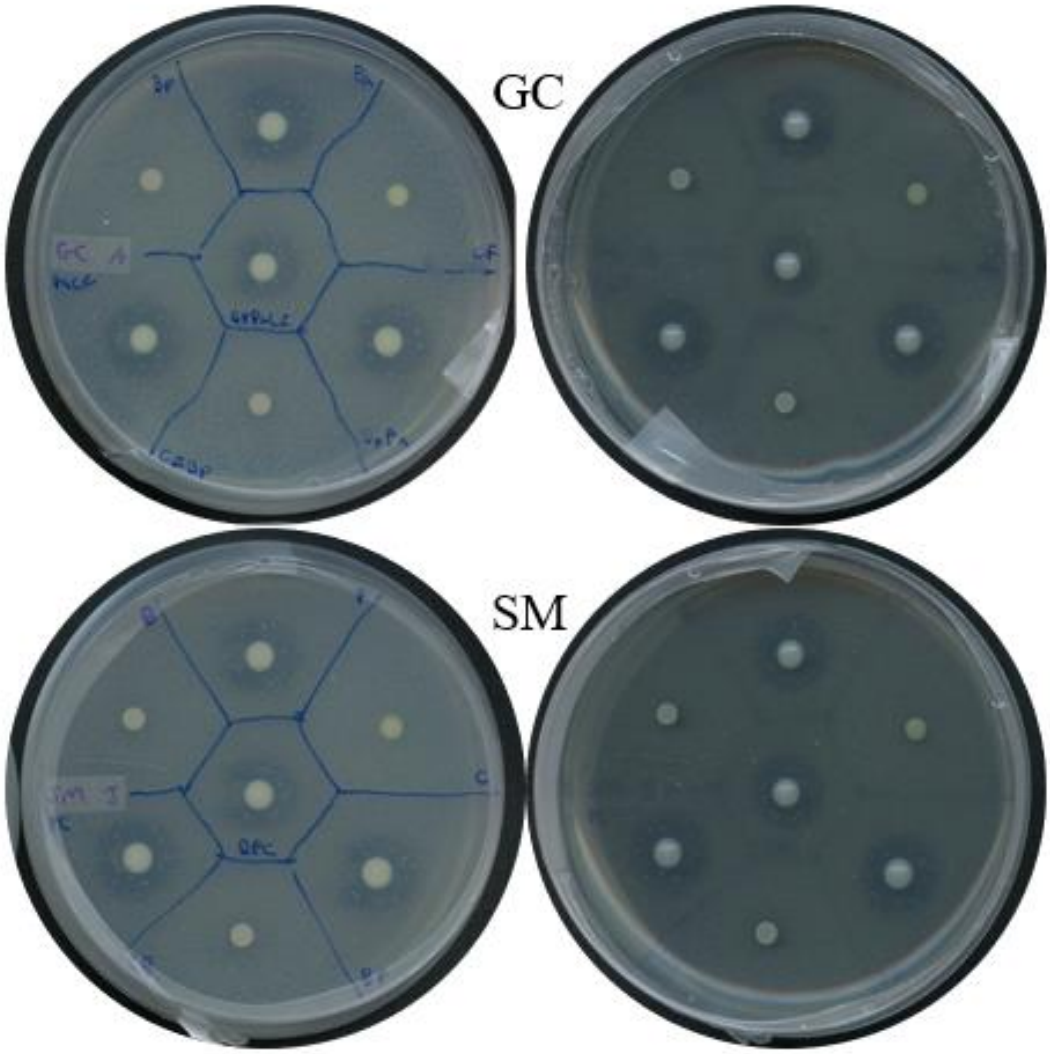

Figure 3.8. Representative images of bacterial growth on GY/TP media, in GC (top) or SM (bottom) conditions, imaged from the bottom (left) and top (right) of the plate for qualitative analysis. For each plate, starting in the top half, leftmost section and moving clockwise, each section contains *Bp, Pa, Cf, Bp+Pa, Cf+Bp, Pa+Cf,* and the center section will grow *Bp+Cf+Pa.*

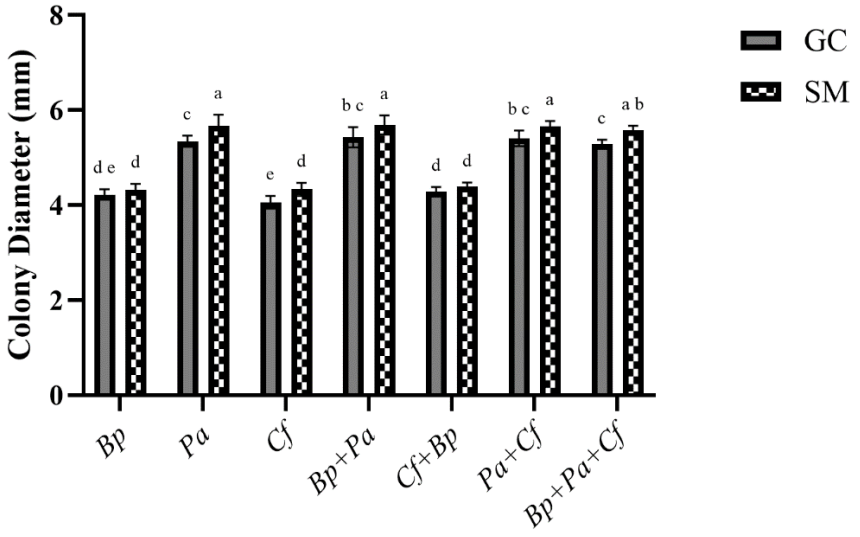

Figure 3.9. Spot diameter of bacterial growth on GY/TP with GC and SM treatments. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=12.

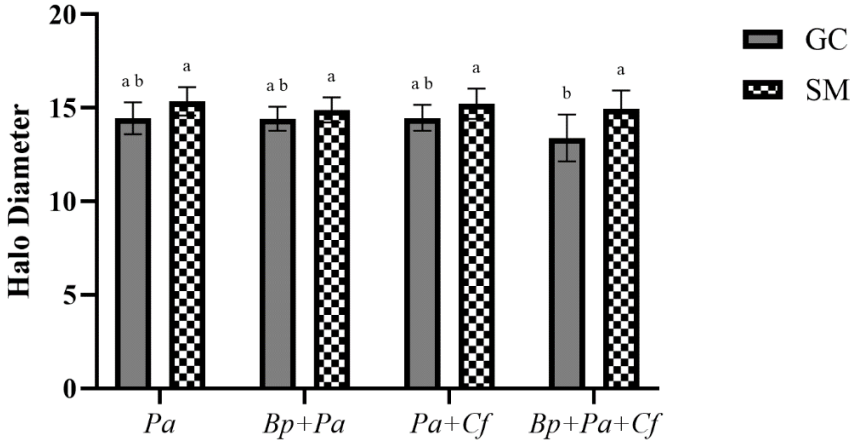

Figure 3.10. Cleared phosphate halo diameter resulting from bacterial growth on GY/TP with GC and SM treatments. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=12.

3.3.5 ACC Deaminase Activity

On plates with no ACC, only the spots of *Pa* and *Pa+Bp* had any significant growth, both having a matte white color, flat with a slightly raised edge, though *Pa+Bp* was slightly less opaque. The other spots had a translucent film, likely the result of minute growth using trace nitrogen leftover after the repeated resuspension in saline. However, spots containing *Pa+Cf,* while not measurable, do appear to have minute amounts of growth at the edge of the spot, which is consistent across all plates. These results are identical in both SM and GC (Figure 3.11). Supplementation with ACC also allowed for measurable growth in all cultures except *Cf,* which was previously reported to be ACCd negative. In both gravity treatments, the morphological characteristics of *Pa* remain the same with ACC as without, and the growth of *Bp+Pa*, *Pa+Cf,* and *Bp+Pa+Cf* with ACC are similar to *Pa*, though consistently less opaque in the center. Growth of *Bp* is consistent between gravity treatments, having a white color with a less opaque center and distinct edges. The color of *Cf+Bp* is consistent between gravity treatments, being effectively a dulled version of *Bp*'s color (Figure 3.11).

Spot size analysis shows that gravity treatment does not alter the diameter of any culture combination. However, it does show more subtle differences when comparing mixed cultures to single cultures. The growth of *Bp+Pa* in GC is statistically similar to *Pa*, but in SM is similar in size to *Bp*. Regarding cocultures

involving *Cf*, *Cf+Bp* is similar to *Bp*, however *Pa+Cf* is also similar to these, which is to say it is reduced compared to *Pa* (Figure 3.12). This, combined with the translucent center of *Pa+Cf*, suggests the ACCd negative *Cf* may be inhibiting *Pa* which may be sharing the nitrogen it gathers from ACCd activity and nitrogen fixation (Figure 3.11 & Figure 3.12).

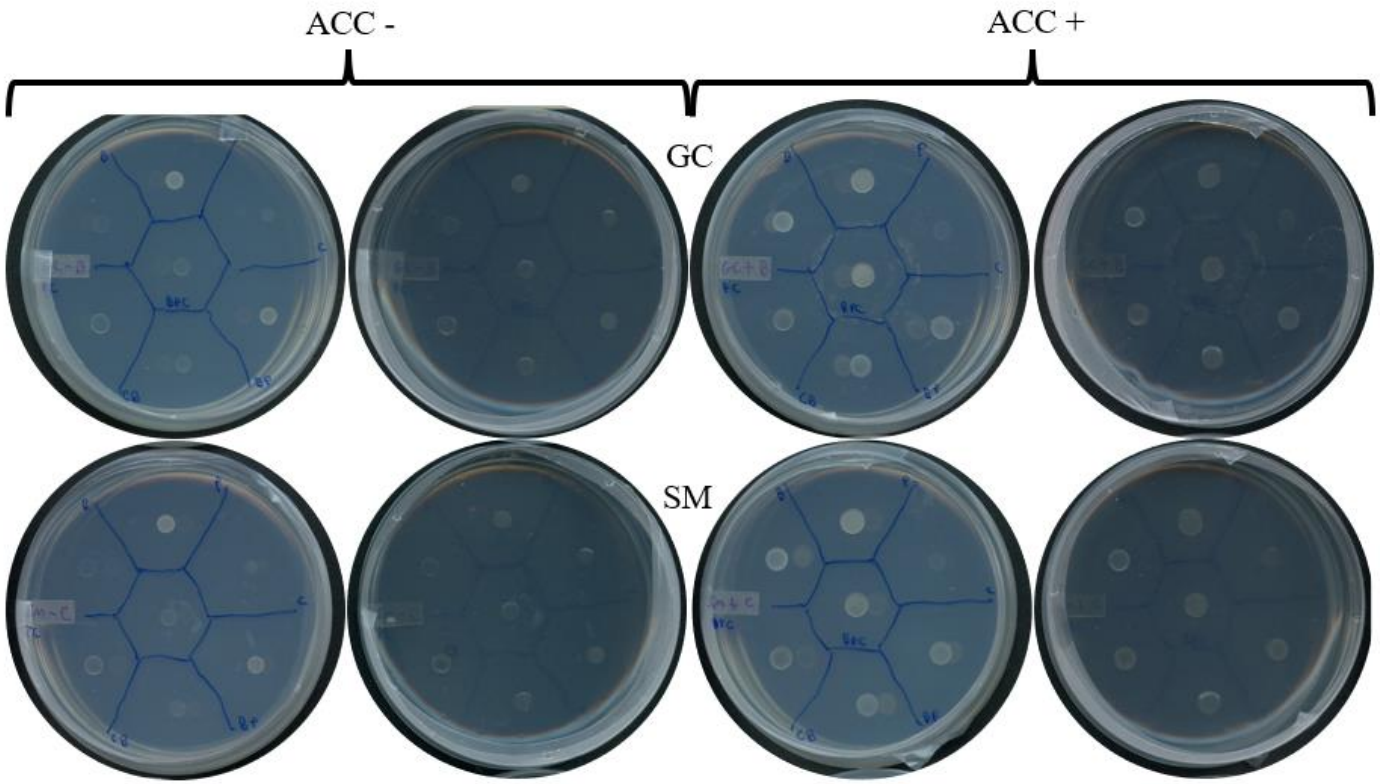

Figure 3.11. Representative images of bacterial growth on DFS media, in GC (top) or SM (bottom) conditions, without (left four) or with (right four) ACC. imaged from the bottom (left of each pair) and top (right of each pair) of the plate for qualitative analysis. For each plate, starting in the top half, leftmost section and moving clockwise, each section contains *Bp, Pa, Cf, Bp+Pa, Cf+Bp, Pa+Cf,* and the center section will grow *Bp+Cf+Pa.*

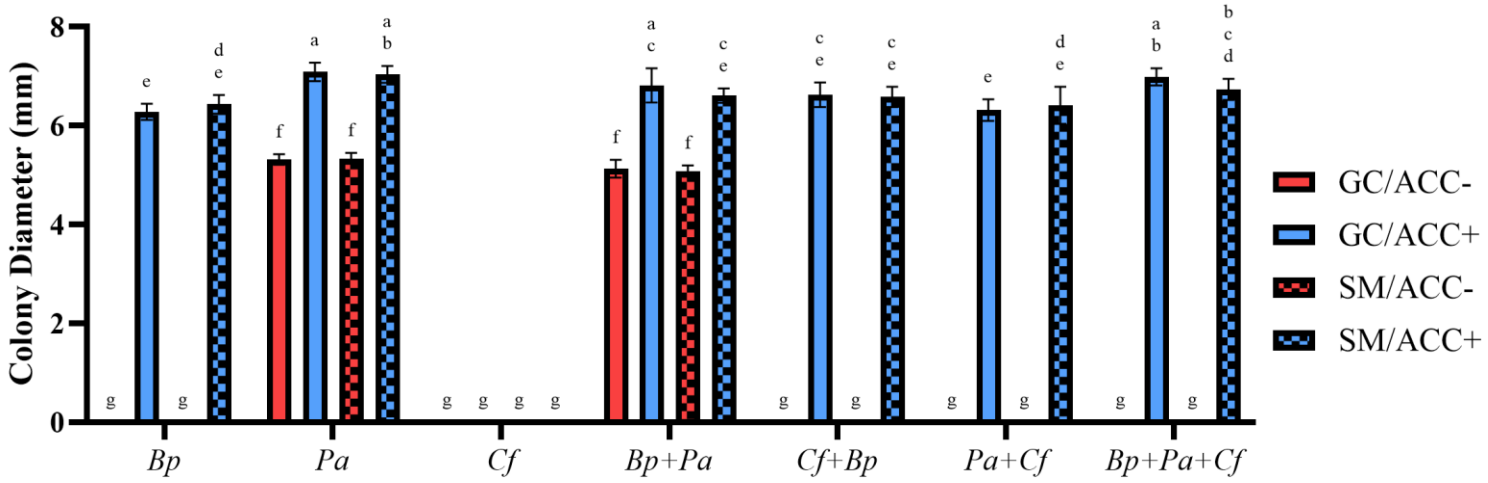

Figure 3.12. Spot diameter of bacterial growth on DFS media, with and without ACC supplementation, and with GC and SM treatments. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=6. Statistical group "g" did not exhibit measurable growth.

3.3.6 Indole Production

Qualitative analysis of the spot morphology revealed no differences between gravity treatments. *Bp* had an off-white with a slightly pronounced edge, *Pa* was glassy and white with a dome shape, and *Cf* was a glassy and pale yellow with a slight dome shape. Interestingly, *Bp+Pa* and *Bp+Pa+Cf* had very similar texture and shape, with rough texturing and wavy edges, the differences being the former was off-white while the latter had a yellow hue. *Pa+Cf* was identical to *Pa*, and *Cf+Bp* had morphology identical to *Bp* except it's color matched closer to *Cf* (Figure 3.13).

Spot size analysis revealed only *Pa+Cf* was altered by gravity treatment, being increased in SM. Other cultures show slight increases if any change, though none that are statically significant. Statistically grouped with *Pa+Cf* in SM were both *Pa* cultures, though *Pa* in GC was also grouped with both *Bp+Pa* gravity treatments and *Bp+Pa+Cf* in SM. Cultures of *Bp*, *Cf*, and *Bp+Cf* were all of similar size (Figure 3.14).

This method of extracting indole from the agar yields a smaller amount of indole than the broth culture method, but that is likely due to the lower relative growth on agar versus broth culture. This method still reveals differential indole production, as *Pa* and *Pa+Cf* yield statistically higher concentrations of indole compared to other cultures. While *Cf* indole appears higher than *Bp* and all

cocultures contains *Bp*, it is not statistically relevant (Figure 3.15). Analysis of indole concentration per mm^2 revealed only *Pa* was statistically altered between gravity treatments, being reduced in SM. Other cultures show slight differences, often reduced in SM, except for *Pa+Cf* which appears to be slightly increased. Also notable about *Pa+Cf* is that both gravity treatments were grouped with *Pa* in GC, which is significantly more than *Cf* alone. *Bp* had the smallest amount of indole per mm^2, which is consistent with previous findings being extremely low, however, it is grouped with *Bp+Pa* which implicates an effect on the indole production of *Pa* in the coculture (Figure 3.16).

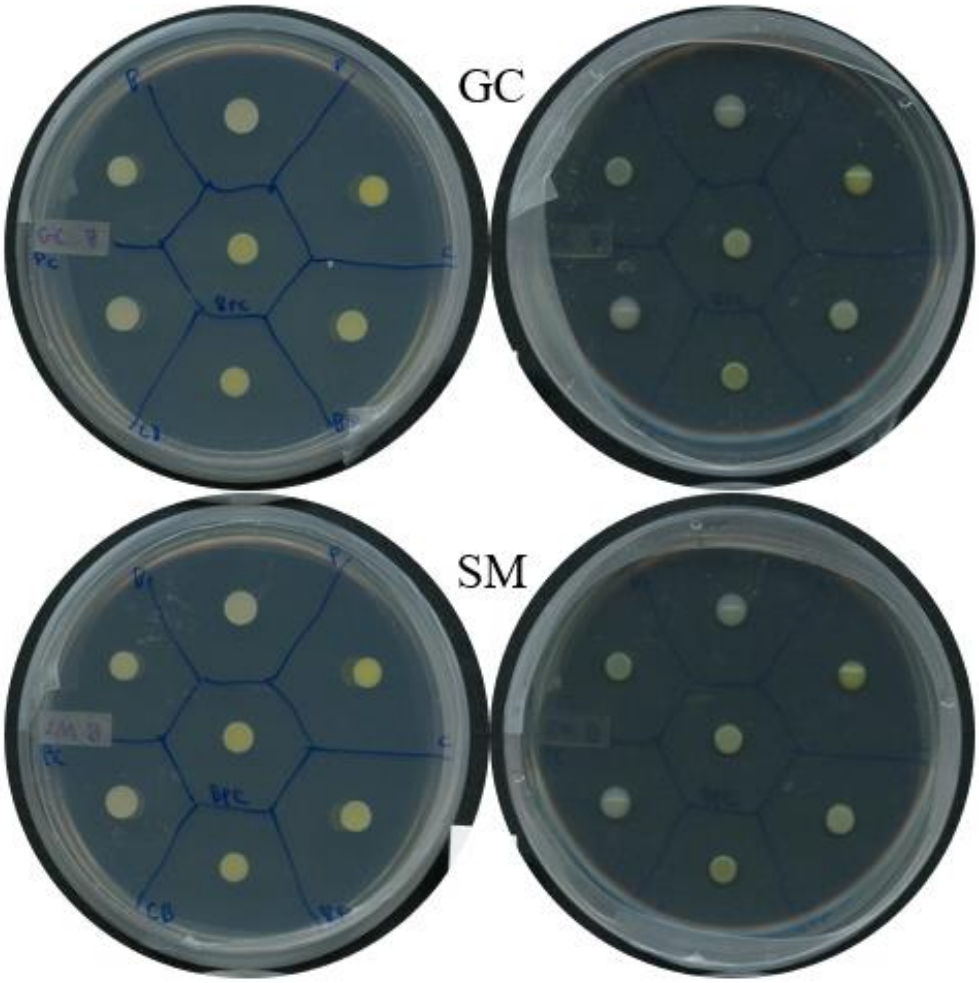

Figure 3.13. Representative images of bacterial growth on KBT media, in GC (top) or SM (bottom) conditions, imaged from the bottom (left) and top (right) of the plate for qualitative analysis. For each plate, starting in the top half, leftmost section and moving clockwise, each section contains *Bp, Pa, Cf, Bp+Pa, Cf+Bp, Pa+Cf,* and the center section will grow *Bp+Cf+Pa.*

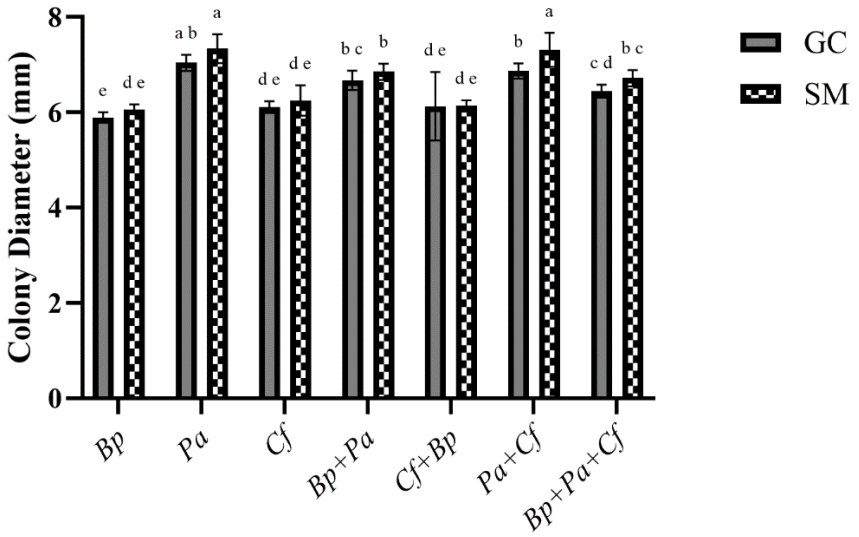

Figure 3.14. Spot diameter of bacterial growth on KBT with GC and SM treatments. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=6.

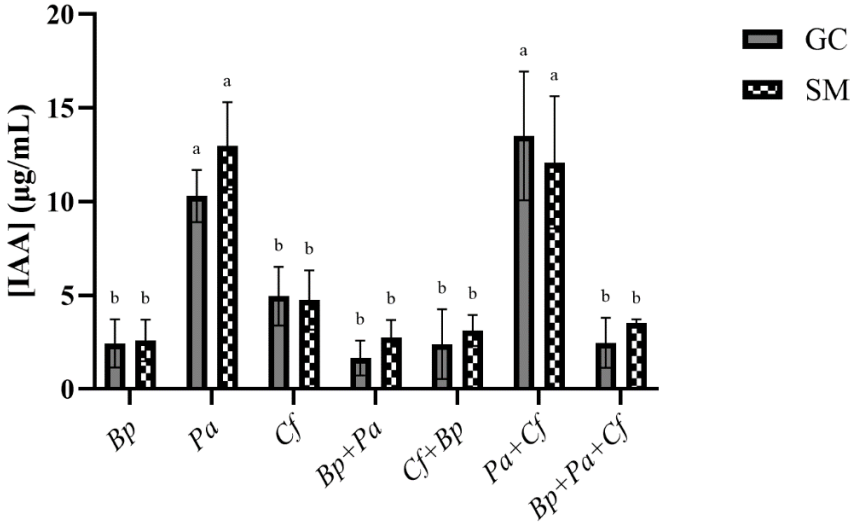

Figure 3.15. Concentration of indole extracted from agar surrounding spots grown on KBT with GC and SM, calculated from a standard curve ($R^2 > 0.97$) of IAA ranging from 0.244 to 125 µg/mL. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=6.

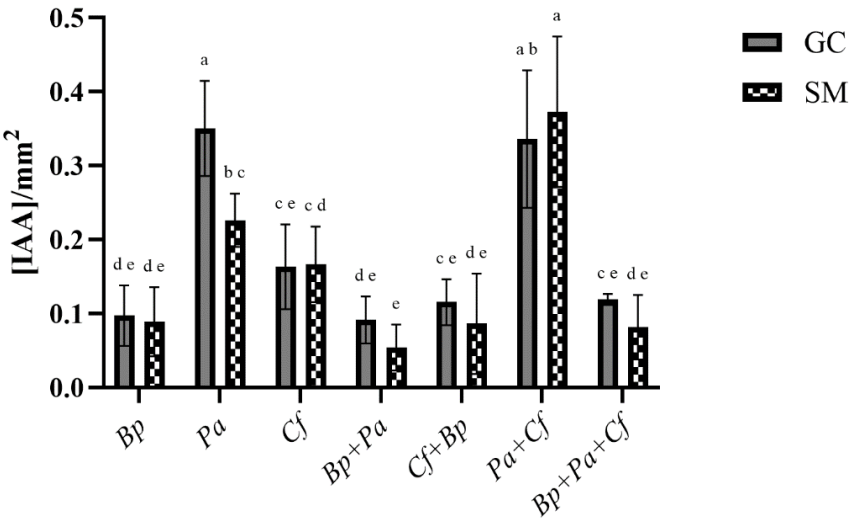

Figure 3.16. Concentration of indole extracted from agar surrounding spots normalized to spot area. Letters indicate statistically similar groupings as determined by two-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=6.

3.4 Discussion

These investigations aim to reveal how SM and coculture affects PGP-related phenotypes of these ISSd isolates. Through this we seek to understand what effects these bacteria may have had on the plants grown on ISS, and why these plants supported these bacteria as members of their microbiome. Indeed, it was observed that in multiple cases SM had an impact on the bacterial growth and expression of the phenotypes, and coculture resulted in unique morphologies and unique expression of phenotypes

Also notable was the behavior of *Pa* in the ACC deaminase experiment. Though it was not reported in our previous work, the growth of *Pa* on nitrogen and

ACC free media indicates that this strain likely fixes atmospheric nitrogen, which would normally invalidate a positive ACCd activity result. However, analysis of spot size reveals that supplementation with ACC significantly increases the size of the spot, supporting the ACCd activity of *Pa* (Figure 3.12). Previous images have since been revaluated and confirm that this phenotype was indeed present in prior studies but was not reported as the previous study was not concerned with nitrogen fixation.

It was previously reported that *Cf* could produce siderophores, however in this study no growth was observed on the media. This was confirmed over multiple iterations of the experiment, though plates allowed to incubate for 7 days at 30°C showed small growth (data not shown). By this point, however, halos of other spots were too large to measure. A possible reason for this is strain variance, which as previously discussed, *Cf* has a high rate of variance. The strains used in previous studies at Kennedy Space Center were mostly derived from VEG-03 missions. When these studies moved to Florida Tech, only samples from VEG-01 missions were available for request through the OSDR. At the time, it was assumed that the species isolated from different missions would be identical, however this appears to be untrue, emphasizing the need for thorough characterization of isolates, including new isolates of previously identified species. The presence of unique strains among these ISSd isolates not only indicates multiple instances of these same species

circumventing sterilization efforts, but also that these isolates may have different PGP characteristics or may not even have PGP activity.

3.4.1 Coculture Interactions.

The ability for the microbes to cooperate appears dependent on nutrient conditions. The qualitative analysis of spot morphology is the primary determiner of successful cohabitation between species. Unique morphologies when compared to the single cultures indicate the presence of multiple species in mature cocultures. This can be as subtle as the change in hue in what is otherwise identical spots when *Cf* is added to *Pa* on TSA (Figure 3.3), to the drastically altered textures such as when *Pa* and *Bp* are combined on KBT (Figure 3.13). These types of differences appear to be common on general growth medias such as TSA and King B, however the addition of selective agents such as iron-limiting CAS dye or insoluble phosphate appear to give rise to competitive relationships.

For example, the qualitative analysis on GY/TP media shows a hierarchical competive relationship between the strains. The identical morphologies between mixed and single cultures indicates that under soluble phosphate limiting conditions, *Pa* becomes the dominant presence between these three. In its absence, *Bp* will dominate over *Cf* (Figure 3.9). However, on KBC media *Pa* and *Bp* are cooperative; while the halo size of the coculture is comparable to *Pa*, the spot size

is larger than either constituent species, indicating an improved efficiency in the uptake of the growth-limiting nutrient (Figure 3.6 & Figure 3.7).

These observations indicate that these isolates should be able to cohabitate in a host plant that provides the bacteria with basic carbon sources. However, should an event occur in which the system becomes nutrient limited or otherwise stressed, such as accidentally skipped waterings, or nutrients reacting with solid substrates, certain bacteria may not survive without assistance from the host of fellow symbionts. This emphasizes the need for a diverse consortium whose member interactions have been thoroughly investigated and that redundant bacteria with desirable PGP phenotypes are included in case of species loss.

3.4.2 How does microgravity impact PGP phenotypes?

In terms of morphology, gravity treatments did not notably alter growth. Quantitative analysis, however, is more telling, as altered growth is indicative of nutrient uptake efficiency, especially in selective, nutrient-limited medias such as KBC, GY/TP, and DFS where altered growth is indicative of altered phenotypic expression. For example on GY/TP media, the halo of *Bp+Pa+Cf*, plus every spot except *Cf+Bp* grew larger in SM, indicating improved phosphate solubilization in all these cases. The increased halo size of *Bp+Pa+Cf* suggests that despite the morphology being identical to *Pa*, traces of the other two may be cohabitating in

the spot, and the combined upregulation of the phenotype caused the increased halo size not observed in other spots. This observation of increased spot size due to SM holds true in other medias, especially of siderophore production on KBC where diameter is increased, though no differences in size were observed on DFS indicating no alteration to the phenotype. Summarized description on the upregulation and downregulation of these phenotypes are described in Table 3.3.

On non-selective medias, change in growth is indicative of general growth rather than any relation to a particular PGP phenotype. On KBT, *Pa+Cf* is the only culture to alter growth, increasing in SM. When comparing abundance of indole, *Pa* is the only one altered, and is the only case of a phenotype being down-regulated in this study, though the indole levels are not so low as to be completely ineffective in PGP scenarios (Table 3.3).

Table 3.3. Summary of changes to growth and phenotypes of cultures in SM as compared to GC. D indicates diameter, P indicates phenotype, + indicates upregulation, - indicates down regulation, NC indicates no change.

	Bp	*Pa*	*Cf*	*Bp+Pa*	*Cf+Bp*	*Pa+Cf*	*Bp+Pa+Cf*
TSA D	NC	+	NC	NC	NC	NC	NC
KBC D	+	+	NC	NC	+	+	NC
KBC P	NC	NC	NC	NC	+	NC	NC
GY/TP D	NC	+	+	+	NC	+	+
GY/TP P	NC	NC	NC	NC	NC	NC	+
DFS D	NC	NC	NC	NC	NC	NC	NC
KBT D	NC	NC	NC	NC	NC	+	NC
KBT P	NC	-	NC	NC	NC	NC	NC

These observations confirm that phenotypes can be altered with respect to gravity conditions, though only in select cases. *Pa* appears to be the most effected, its growth increased in SM on 3/5 medias, and its indole production downregulated. When including its cocultures, *Pa* is involved in 10/14 SM alterations. Meanwhile *Cf* is involved in 8/14 alterations and *Bp* appears the most resistant to change under SM at 6/14 alterations. Most importantly, all phenotypes are maintained at PGP-relevant levels, if not upregulated.

When compared to other SM studies primarily performed in liquid culture, the fact that growth is largely unchanged, or has increased in some instances, is notable. Most SM performed in LFS vessels has shown decreased growth, which has not been observed in solid media. It was also noted here that siderophore production was either unchanged or increased, yet a study by Golaz et al. found a reduction in siderophore production by *Paraburkholderia phymatum* grown in a LFS vessel on a random position machine [85].

These observations exemplify why the experimental setup is important. We seek to understand these interactions as relevant to a plant host. The microbes would be adhered to the surface of, or embedded in the tissues of their host, which is better replicated by solid media rather than LFS. Though, LFS could replicate bacteria suspended in water or nutrient media surrounding plant tissues. Traditionally, microgravity experimentation on microbes rarely uses solid media,

but the culture methods can create artifacts in these datasets that may not be biologically relevant, thus it is important to consider the *in vivo* environment when designing *in vitro* experiments.

Chapter 4
Plant Growth Effects of ISS Bacterial Isolates

4.1 Background

NASA currently aims to establish crewed stations in orbit of and on the Moon by the 2030s, with manned missions to Mars planned for shortly after that [93]. To facilitate this, researchers around the world conduct work toward the development of bioregenerative life support systems (BLSS) [46,151,152]. A crucial factor in BLSS will be plant growth modules, which will serve functions in air revitalization, waste management, and most importantly food production. As missions move further from Earth, resupply missions will become economically and logistically prohibitive. As such, it is imperative that long term stations and deep space transport vehicles are equipped with robust, sustainable food production capabilities.

In space, plants face numerous abiotic and biotic stressors. Space radiation can cause mutations, while microgravity can cause expressional changes, and the behavior of fluid dynamics in microgravity can cause a variety of issues. Water in spaceflight (SF) clings to hydrophilic surfaces, creating hypoxic conditions for roots when nutrient fluids are added to hydroponic systems. The lack of convective currents in microgravity also causes issues when volatile compounds are allowed to build up around leaves, such as ethylene, oxygen which can cause photorespiration, or water vapor which can condense on the leaves creating hypoxic conditions in the

aerial portions of the plant. The buildup of ethylene is especially problematic. As the hormone primarily responsible for stress response, excess ethylene inhibits growth factors, and can cause inappropriate immune responses [17]. These issues can lead to reduced growth of individual plants, as well as whole crop loss to opportunistic phytopathogens [23]. Controls such as ethylene scrubbers and fans have been implemented to combat ethylene buildup, and clay root substrates have been implemented to alleviate water-logging, however the mechanical systems can undergo temporary failures leading to stress events [13,106].

Microbes can provide numerous benefits to host organisms, be they animal or plant. In their native environments, plants rely on a diverse population of microbes in the rhizosphere and phyllosphere, *i.e.* along the roots and shoots [48,153]. These microbes play key roles in plant health and development, acting as biocontrol against would-be pathogens, assisting in nutrient uptake, and assisting in phytohormone regulation [56]. Inoculation of BLSS agricultural units with plant growth promoting (PGP) properties may be able to help ameliorate stress events and improve overall yields.

Current NASA protocols require all materials sent to the International Space Station (ISS) to be as sterile as possible. For the plant systems, this means heat or ethylene oxide sterilizing treatments for most of the materials, and surface sanitization of the seeds [106]. Despite these efforts, there remains a diverse

microbiome within the plant growth systems aboard the ISS [10,89]. These microbes are likely "stowaways" from two possible sources: the human-associated microbes of the astronaut crew tending the plants, or seed-borne endophytes that were protected from sanitization inside the host seed.

It is from this population that we first proposed to derive candidates for a consortium of plant growth promoting bacteria with spaceflight history which could be used to inoculate BLSS systems. Our previous investigations have revealed a significant portion of these ISS-derived (ISSd) possess PGP related phenotypes [Chapter 2] [91]. Most recently, we have found that these bacteria do experience moderate changes in these phenotypes in simulated microgravity. However, it is critical to note that no specific phenotype was consistently regulated between species, suggesting that these processes are not themselves directly regulated by gravity. Yet gravity does play some role in different species which can ultimately influence these processes that remain undetermined.

However, before moving to microgravity experiments, we sought to confirm that the PGP strains we have identified actually play this role. As the ISSd bacteria were isolated from healthy plants, we hypothesize that these isolates are beneficial and will improve plant growth. However, many plant pathogens share phenotypes with PGP bacteria, and it is possible that some of these isolates may be opportunistic pathogens that simply never had the opportunity to invade a host or

initiate virulent phenotypes. The experiments presented here seek to confirm the identity of these bacteria as plant growth promoters as the next step in their potential use in a controlled microbiome.

4.1.1 Species Selection

This population of microbes has been isolated from healthy plants grown in the Veggie system on the ISS over numerous missions since 2014, and have not been recorded to be infectious to the crew working with the plants. These samples are available for request through NASAs Open Science Data Repository (OSDR) for scientists to work with. Supplementary data of our previous work, published or presented, is available through the OSDR researchers to reference in their own investigations of these bacteria. Previously, our lab has identified a large, and still growing number of isolates that have PGP phenotypes [14, chapter 3]. These phenotypes include phosphate solubilization, siderophore production, indole production, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCd) activity. We have investigated three of these isolates, *Pantoea agglomerans* (*Pa*), *Curtobacterium flaccumfaciens* (*Cf*), and *Burkholderia pyrrocinia* (*Bp*) for effects of simulated microgravity on the phenotypes they express in single and mixed culture. These previous investigations work have shown that the PGP phenotypes of these isolates can be altered by simulated microgravity, and that the combination

of bacteria can cause unique expression of phenotypes compared to those of single cultures. For example, the presence of the ACCd negative *Cf* when cultured with *Pa* results in smaller growth than *Pa* on its own in the ACCd activity assay [chapter 3].

P. agglomerans is a Gram negative bacteria with high strain variation and is found in a variety of environments [143]. Symbionts are found among a wide range of hosts such as rice, beet, gypsophila, and wheat, with different strains classified as mutualist or pathogenic [128,154,155]. Common PGP phenotypes in this species includes potassium and phosphorous solubilization, siderophore production, nitrogen fixation, ACC deaminase activity, and production of a variety of phytohormones [128–132]. Strains have also been identified that produce commercially relevant antibiotics such as phenazine and pantocin [133–135]. Other strains have shown PGP effects such as improved grain yield and reduced salt stress in rice, and increased dry weight of sugarcane and tomato [128,129,156,157]. Pathovars of *P. agglomerans* are commonly found as the cause of bacterial gall formation, though the virulence factors appear to be located on a transmittable plasmid, indicating propensity for horizontal gene transfer [154]. Previous work on our ISSd strain used here has identified that it possesses ACC deaminase activity, indole production, siderophore production, and solubilizes phosphate [91].

Gram-positive *C. flaccumfaciens* has more than 50 unique variants, around half of which are responsible for diseases such as bacterial wilt [145,146]. Many pathovars are commonly found in legumes, while PGP strains have been identified in olive, cucumber, and various grains [136–139,145]. Examples of PGP strains have been shown to improve growth and reduce disease in cucumber, and improve growth and salt stress resistance in barley [137,158]. There is high variance in the presence of PGP phenotypes, though most strains appear capable of phosphate solubilization [136–139]. The ISSd strain used here has previously been identified to be phosphate solubilization, indole production, and siderophore production positive, and ACC deaminase negative [91].

B. pyrrocinia is a Gram negative soil microbe that has been isolated from a variety of plants and used as a biocontrol agent against fungi due to its production of multiple anti-fungal agents including dimethyl disulfide, occidiofungin, and ornibactin [142,147–150]. The species has been observed to reduce the incidence of rice sheath blight, reduce disease incidence in poplar, and improve salt tolerance in peanut [140,148,149]. This species has also been recorded to solubilize phosphate, produce indole, and exhibit ACC deaminase activity [140,141]. This ISSd strain was previously reported to produce siderophores, solubilize phosphate, and exhibit ACC deaminase activity, however indole production was negligible. Also, this strain of *B. pyrrocinia* produced a strong inhibitory effect on *Aspergillus sydowii*

and *Emericella parvathecia* strains isolated from Veggie, however these strains were not available for follow-up experiments here[91].

Understanding if and how these potential plant growth promoting phenotypes translate into effects on host plants is critical to incorporating this strategy into space agriculture and BLSS. Many PGP phenotypes are actually shared between beneficial and pathogenic symbionts, and as discussed, many plant-associated bacteria have both pathogenic and beneficial variants. Interference in ethylene response is necessary in circumventing the host immune response in colonization of host tissues, and the primary method is through ACC deaminase activity [69,73]. Many pathogens also produce growth hormones such as auxin and cytokinin to induce formation of tumor-like gall, however these same processes are used by nitrogen fixing mutualists in nodule formation [159]. While these bacteria were isolated from healthy plants, it is possible that some of them were opportunistic pathogens who never became virulent, or that other bacteria were acting as biocontrol preventing virulence.

Here, we investigate the effects of these three samples on plant growth. Using the model organism *Arabidopsis thaliana,* we investigate how single microbe inoculation affects the plants. We also investigate how co-inoculation with these strains affects the growth and development of these plants. We will also investigate how these microbes may affect the growth of red romaine lettuce

(*Lactuca sativa* cv ‘outredgous’), which is commonly grown in Veggie, and the host source of many of these isolates. The characterization of these ISSd isolates in the presence of prospective host plants is critical to ascertain their efficacy as inoculants in a PGP consortium for BLSS. More importantly, these results may also reveal traits that could explain their originating host’s vigor in SF, as reduction in stress response could assist plants in mounting successful immune responses to opportunistic pathogens.

4.2 Materials and Methods

4.2.1 Seed Preparation

Seeds were sanitized by treatment with 2% bleach for 3 minutes, triple rinsing with sterile water, then treatment with 70% ethanol for 3 minutes, followed by triple rinsing with sterile water. Seeds are then transferred into microcentrifuge tubes to have inoculum added. To prepare inoculum, individual strains were cultured overnight, approximately 18 hours, in tryptic soy broth at 30°C. Aliquots were centrifuged down and cell pellets were resuspended in isotonic saline 3 times to rinse out excess culture media. Optical density at 600 nm (OD_{600}) was measured for each suspension in triplicate, and the average was used to calculate dilution to an OD_{600} of approx. 0.2. For single bacteria inoculation, this was then added to seeds. For co-inoculation, the optical density was treated as the effective concentration in order to make suspensions consisting of 0.2 OD_{600} for each

bacteria. Seeds were set to incubate with the inoculum for 1 hour, before being strained and plated on nutrient media.

4.2.2 Seed germination analysis

All plants were grown at approximately 21°C, room temperature. Seeds were plated in approximate 8x8 grids on two plates per treatment for *A. thaliana*, or 5x5 grids on three plates per treatment for *L. sativa*, on 0.5x Hoagland's No. 2 agar media (Caisson Labs product number HOP01, PTP01) and placed under white LED light (Fecida GL-CR600, full specutrom indluding UV, IR) with a 12:12 light: dark cycle to germinate. Plates were sealed with parafilm to prevent contamination. *A. thaliana* plates were imaged at 5 days after planting (DAP) for total germination before transfer to root analysis plates. *L. sativa* plates were imaged every day for 5 days for germination rate analysis.

4.2.3 *A. thaliana* root analysis

At 6 DAP seedlings on germination plates whose cotyledons had extended away from the media surface and opened were selected for aseptic transfer to fresh 0.5x Hoagland's No. 2 agar media. This selective measure ensures that seedlings are in comparable developmental stages to limit variation in germination timing. Three seedlings were transferred to each plate, 12 replicate plates per treatment for a total of 36 seedlings per treatment, and placed on stands to grow for the remainder of the experiment. Plants were moved to grow racks with white fluorescent light (Phillips F40T12/Daylight/5000K) with a 16:8 hour light: dark

cycle. Plants were first imaged for root length analysis 24 hours after transfer at 7 DAP, then again at 14 DAP.

4.2.3 Inoculum dose analysis

To prepare inocula of varying dosages, colony forming unit (CFU) assays were performed to determine the correlation between OD_{600} and cells/mL for each species. These tests determined that for these three species, cultures in TSA with an OD_{600} of 0.2 have approximately 10^8 cells/mL. With this information *L. sativa* seeds were treated with single species doses of 10^8, 10^7, or 10^6 cell/mL of each bacteria. To prepare inoculants, overnight bacteria cultures were resuspended three times in isotonic saline, fixed to an OD_{600} of approximately 0.2, then serial diluted in sequence to generate the 10^7 and 10^6 dilutions. Seeds were soaked in inoculant for 1 hour before being transferred to nutrient-less water agar plates, 30 seeds per plate, 3 plates per treatment, or to three magenta boxes per treatment which had been filled with 0.5x Hoagland's No. 2 agar media. At 6 DAP 10 seedlings from germination plates would be harvested for mass analysis. Plants in magenta boxes were grown for 4 weeks before harvesting from the base of the leaves for edible biomass analysis. Plants were grown under previously described white fluorescent light with a 16:8 hour light: dark cycle.

4.2.4 Plant imaging and analysis

Images were analyzed in ImageJ, using the segmented line tool to trace and measure the primary root length for root analysis. Germination images were used to

notate hypocotyl emergence based on visual evaluation. Graphs and analyses were generated in Graphpad Prism v10.2.3. One-way ANOVA and Tukey's multiple comparisons test were run on all data relevant sets to determine significant differences between inocula.

4.3 Results

4.3.1 Effects of co-inoculation on *A. thaliana*

All treatments had greater than 86% germination, though only the uninoculated treatment was under 90% germination (Figure 4.1). This indicates a potential improvement to germination efficiency, especially by *Bp* (98%), as well as *Cf*+*Bp* and *Pa*+*Cf* (both 96%). At 7 DAP, plants appear healthy, though root analysis shows clear effects on early development (Figure 4.2 and Figure 4.3). Plants inoculated with *Pa* and *Cf* individually have the greatest root length compared to all other treatments. Though, the *Pa*+*Cf* treatment is statistically grouped with both its single inoculant and the uninoculated treatments. Conversely, *Bp* appears to have negatively impacted growth in both single and co-inoculation, as all treatments involving *Bp* except *Bp*+*Pa* are statistically reduced compared to the uninoculated treatment.

The negative impact of *Bp* is further confirmed at 14 DAP, by which time an inhibitory biofilm has formed around the roots of all treatments involving the strain (Figure 4.4 and Figure 4.5). This biolfilm obscures the roots, limiting the

number of measurable roots as low as N= 7 in some treatments (Figure 4.4). This biofilm was coupled with stunted growth in the afflicted plants, with barely any growth between 7 and 14 DAP. The unafflicted groups, however, appear healthy and have grown significantly. By 14 DAP, the uninoculated treatment has caught up to *Pa*, while *Cf* has outgrown all other treatments.

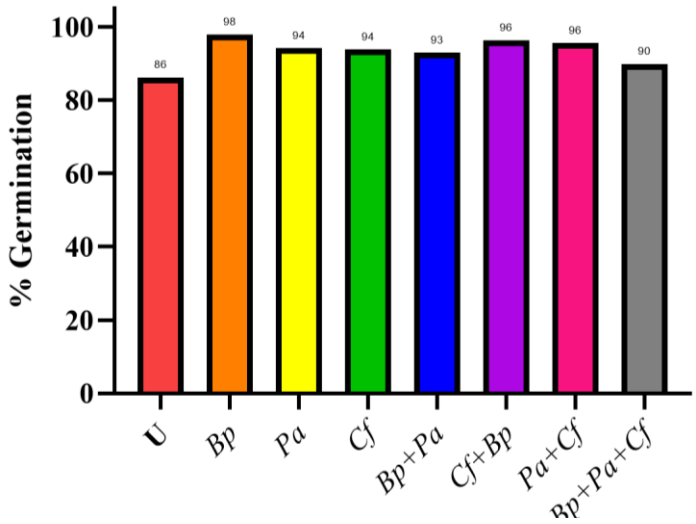

Figure 4.1. Percentage of germinated *A. thaliana* seeds at 6 DAP. Bars represent total germination of treated seeds split across two plates. Differences between replicate plates were not statistically significant. Graph generated in Graphpad Prism v10.2.3. N≈ 135 seeds.

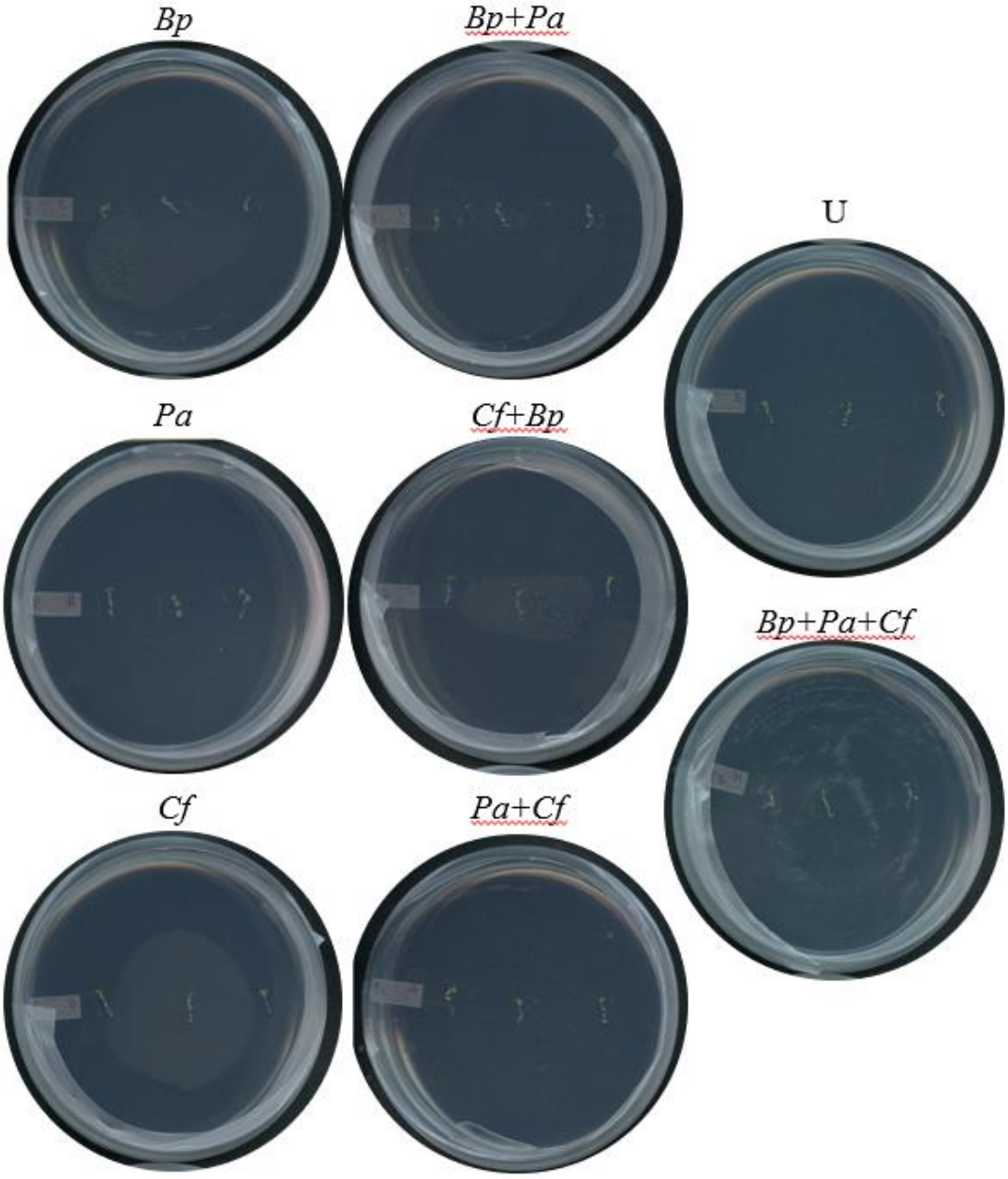

Figure 4.2. Representative images of A. thaliana seedlings at 7 DAP. ***No notable biofilm development present.***

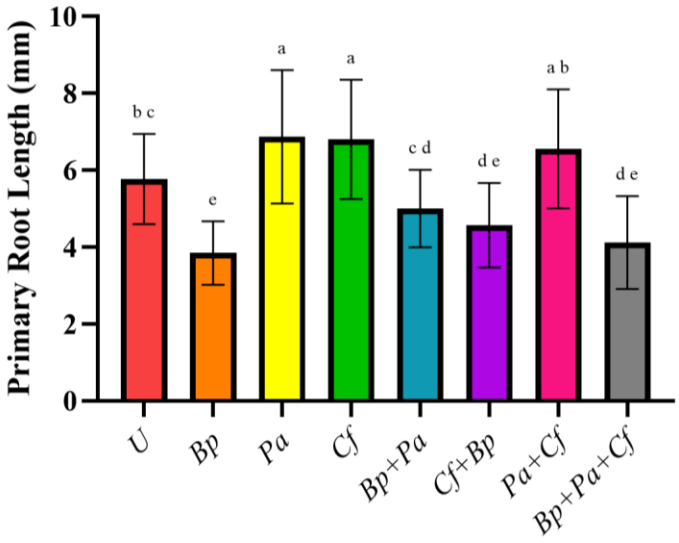

Figure 4.3. Primary root length of treated *A. thaliana* seedlings at 7 DAP. Letters indicate statistically similar groupings as determined by one-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N=36 seedlings.

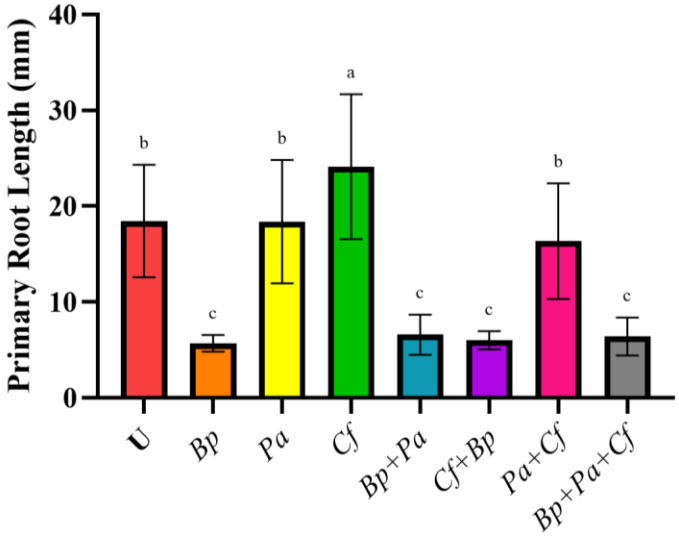

Figure 4.4. Primary root length of treated *A. thaliana* seedlings at 14 DAP. Letters indicate statistically similar groupings as determined by one-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N= 36 seedlings for U, Pa, Cf, Pa+Cf; N= 21 for Bp+Pa; N= 8 for Cf+Bp; N=7.

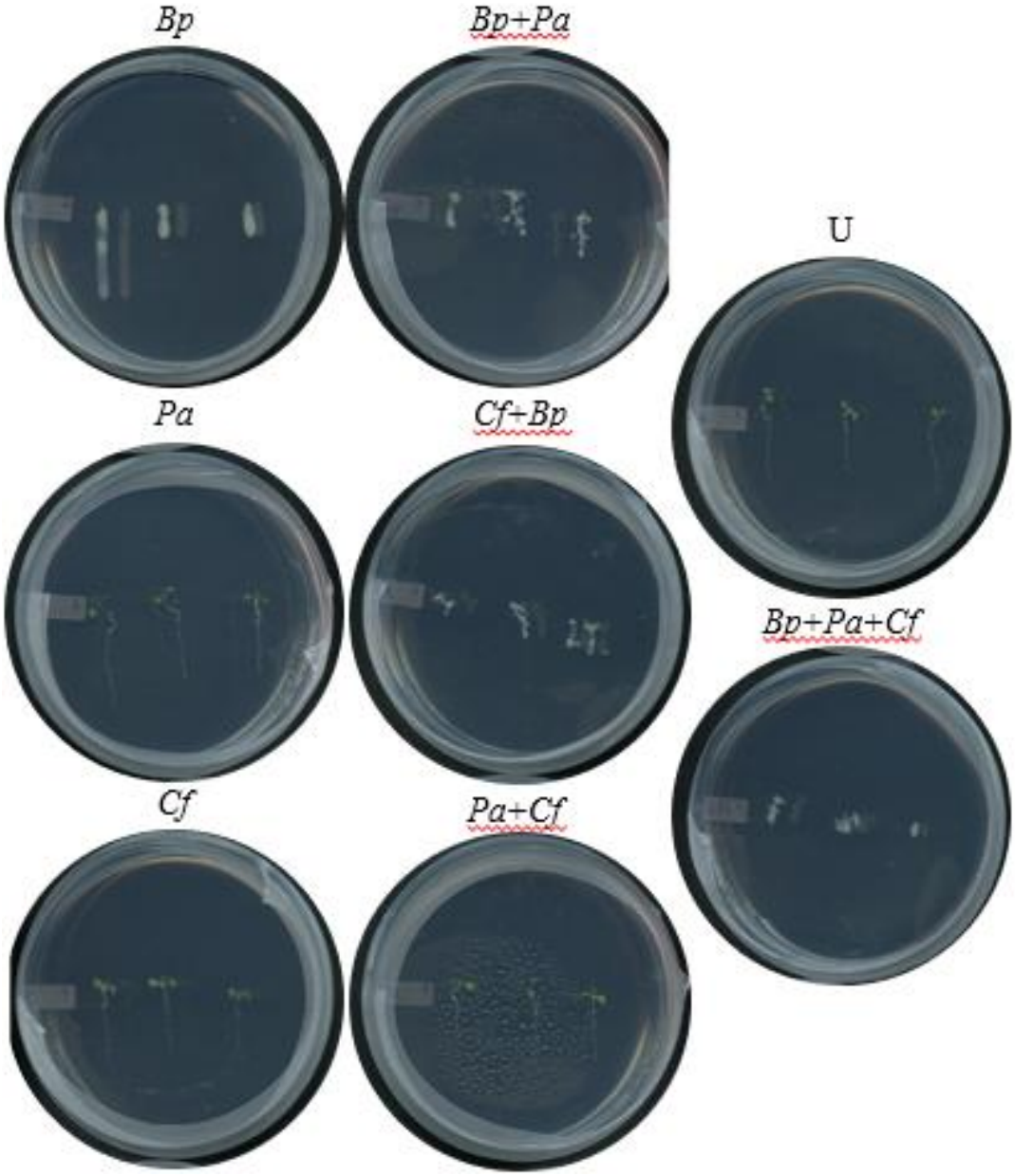

Figure 4.5. Representative images of A. thaliana seedlings at 14 DAP. Notable biofilm development at 14 DAP on *Bp, Bp+Pa, Cf+Bp,* and *Bp+Pa+Cf.*

4.3.2 Effects of co-inoculation on *L. sativa* germination

Effects on the germination of *L. sativa* can be observed as early as 2 DAP, where *Pa+Cf* has germinated earlier than other treatments, especially the uninoculated control (

Figure **4.6** and Figure 4.7). However, by 4 DAP many of the other treatments have caught up to Pa+Cf, however by 5 DAP, Bp appears to negatively impact germination, showing that negative effects are not specific to A. thaliana (

Figure 4.6). These impacts are even found in *Cf+Bp*, as it is statistically grouped with both *Cf* and *Bp*, though *Bp* is statistically reduced compared to *Cf*, indicating *Cf+Bp* is moderately reduced compared to *Cf*, and significantly reduced compared to uninoculated treatments (Figure 4.8). However, these effects may be due to the concentration of bacteria used.

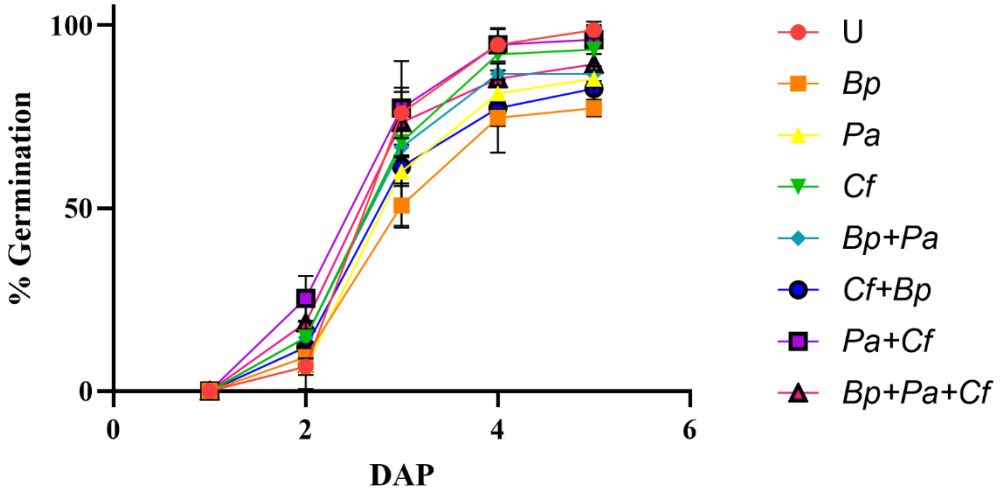

Figure 4.6. The germination rate of *L. sativa* over 5 days as determined by hypocotyl emergence. N= 3 replicate plates of 25 seeds each. Figure generated using Graphpad Prism v10.2.3.

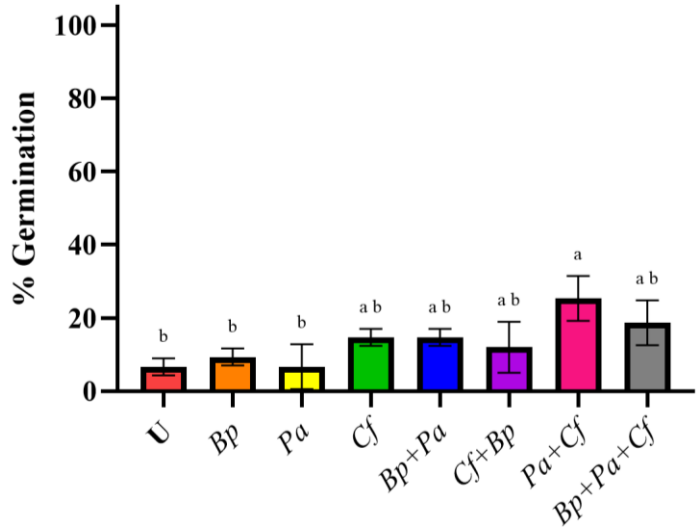

Figure 4.7. The germination rate of *L. sativa* at 2 DAP as determined by hypocotyl emergence. Letters indicate statistically similar groupings as determined by one-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N= 3 replicate plates of 25 seeds each.

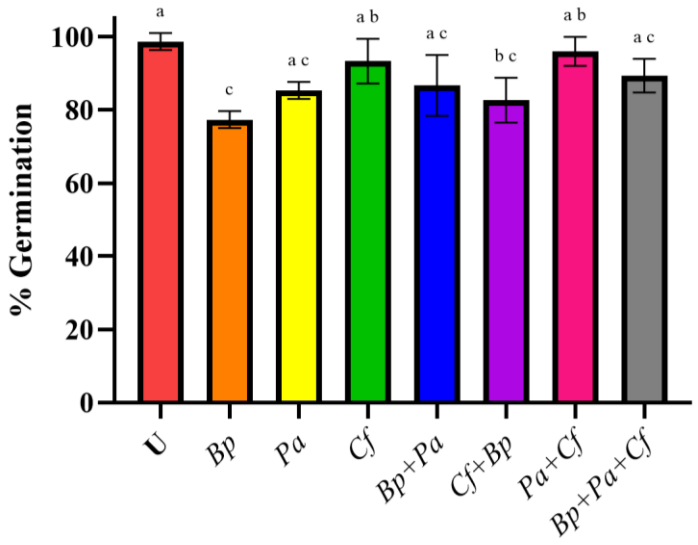

Figure 4.8. The germination rate of *L. sativa* at 5 DAP as determined by hypocotyl emergence. Letters indicate statistically similar groupings as determined by one-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N= 3 replicate plates of 25 seeds each.

4.3.3 Effects of inoculum dose on *L. sativa*

In terms of germination rate, the presence of bacteria appears to delay germination in the first 2-3 DAP regardless of species or concentration. By 6 DAP, overall germination in *Pa* and *Cf* statistically indistinguishable from controls, whereas germination in *Bp* treatments still was delayed in both 10^7 and 10^6 cells/mL treatments (Figure 4.9).

Seedling mass appears to be impacted in all bacterial treatments, though impact appears dependent on dosage of the inoculum (Figure 4.10). Seedling mass in *Bp* treated seedlings were all negatively impacted, with all dosages significantly reduced compared to the control plants, though the 10^6 cells/mL dose was impacted

less than the higher doses. Conversely, seedlings from *Pa* and *Cf* treatments appear unaffected or larger than the control seedlings. Seeds treated with *Pa* are moderately larger with 10^8 and 10^6 cells/mL doses and significantly increased with a 10^7 cells/mL dose. In *Cf* treatments, the notable beneficial effects appear inversely proportional to dosage, with 10^8 cells/mL having no change, 10^7 cells/mL having a moderate increase, and 10^6 cells/mL having a statistically significant increase.

Though statistical analysis of 4-week-old edible leaf mass only revealed significant differences in *Cf* treatments, it should be noted that a 10^7 cells/mL dose of *Bp* and a 10^8 cells/mL dose of *Pa* did increase the average biomass (Figure 4.11). This analysis shows that a 10^6 cells/mL dose of *Cf* has the most benefit to mature plants, though a 10^7 cells/mL dose of *Cf* has the most detriment to mature plants.

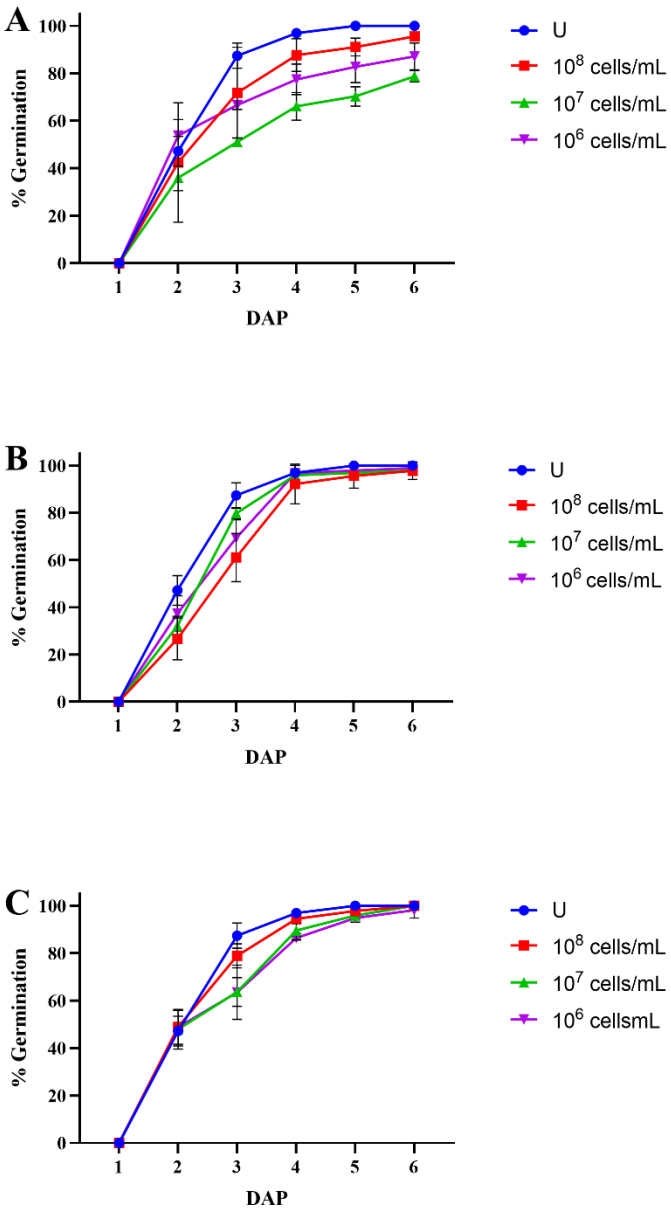

Figure 4.9. Germination rates of *L. sativa* inoculated with varying concentrations (Cells/mL) of (A) *B. pyrrocinia*, (B) *P. agglomerans*, and (C) *C. flaccumfaciens*, compared with an uninoculated control *(U)*. N= 3 plates of 30 seeds each. Figure generated using Graphpad Prism v10.2.3.

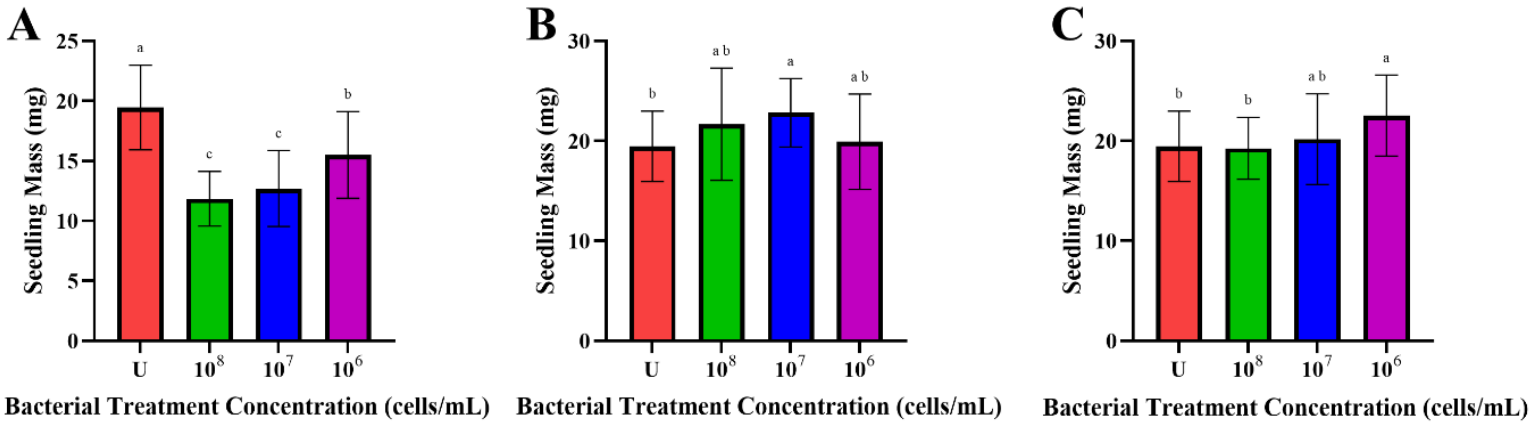

Figure 4.10. Seedling mass at 6 DAP of *L. sativa* inoculated with varying concentrations *(*cells/mL*)* of (A) *B. pyrrocinia*, (B) *P. agglomerans*, and (C) *C. flaccumfaciens*, compared with an uninoculated control (U). Letters indicate statistically similar groupings as determined by one-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N= 30.

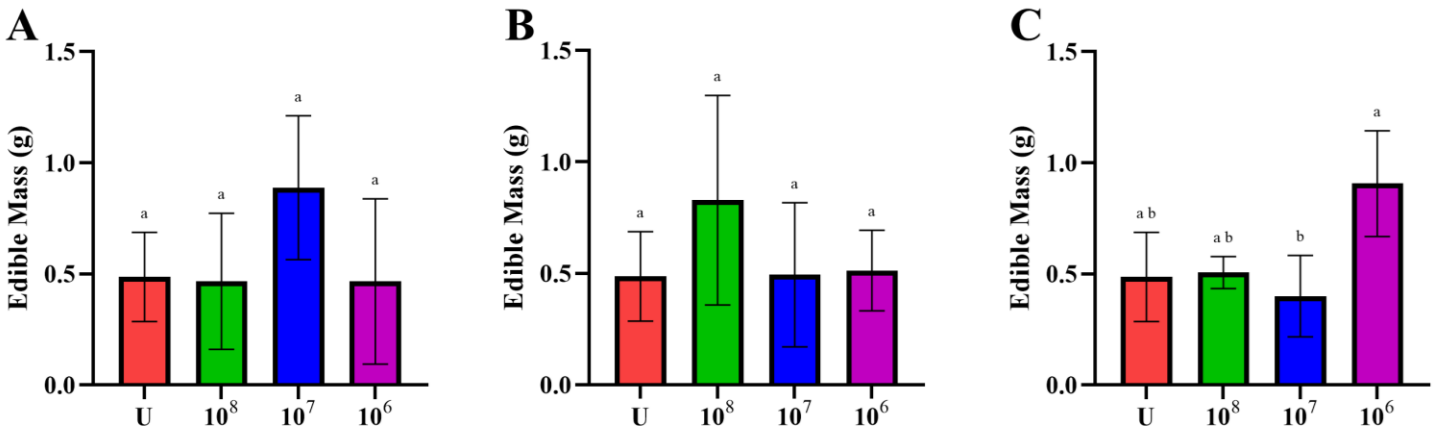

Figure 4.11. Edible leaf mass of *L. sativa* at 4 weeks of growth, inoculated with varying concentrations *(*cells/mL*)* of (A) *B. pyrrocinia*, (B) *P. agglomerans*, and (C) *C. flaccumfaciens*, compared with an uninoculated control (U). Each plant yieled 5-8 leaves with no statistical difference in leaf number between treatments. Letters indicate statistically similar groupings as determined by one-way ANOVA and Tukey's tests performed in Graphpad Prism v10.2.3. N= 3.

4.4 Discussion

These investigations sought to determine how these ISSd bacterial isolates interact with each other in the context of a host plant. Though it was first thought that these isolates would all be beneficial, we have determined that *Bp* has the potential to be problematic, if not an opportunistic pathogen. The consistent biofilms formed in *A. thaliana* co-inoculation show that it is readily hostile to this host, however these biofilms were not noticed in any experiments with *L. sativa* indicating that this may be a host specific response (Figure 4.2). It is also notable that the biofilms were not as consistently dense in co-inoculation with *Cf*, as this group had an N of 21 measurable roots, versus the N of 7-8 for other treatments involving *Bp*, though this was not sufficient to improve the stunted growth observed in all *A. thaliana* treatments involving *Bp* (Figure 4.4). Despite the lack of visible biofilms in *L. sativa*, negative impacts were observed in all early-development experiments. This strain delayed germination significantly on its own and in conjunction with *Cf*, and moderately in other co-inoculations it was involved in (

Figure **4.6** and Figure 4.8).

Biofilms are common among plant associated microbes and are often involved in the colonization process by both pathogenic and beneficial microbes [160]. Biofilm formation in many bacteria, including the *Burkholderia* genus, is

quorum sensing (QS) regulated [161]. QS is the ability of bacteria and other microbes to detect the relative population size around them using short-lived chemical signals. Many quorum sensing behaviors function as a concentration-dependent switch, altering behavior when a certain threshold is met, and these chemical signals can be influenced by surrounding species and host organisms [162].

Pa also possesses a relevant QS phenotype; the species forms tightly packed aggregates called symplasmata which has been shown to be involved in its colonization and subsequent support of plant growth [111]. While it is possible this strain of *Bp* happens to be an opportunistic pathogen, an alternative hypothesis to be considered is that dosage of the inoculum is too large, causing biofilm formation on underdeveloped plants resulting in detrimental effects. These factors are the reasons we investigated inoculum dosage, to determine if this would have a significant effect on the growth and development of these plants. Indeed, inoculum dosage does seem to alter the *Bp*'s effects, as the seedling mass increases inversely proportional to the dose, with 10^6 being statistically larger than either larger dose, though these are all still significantly smaller than the uninoculated (Figure 4.10). However, these impacts do not appear in the 4-week-old plants, with edible biomasses all being comparable, showing that the negative impacts in juvenile plants do not necessarily translate to impacts in mature plants (Figure 4.11).

These experiments have verified that this ISSd strain of *Cf* is a plant growth promoter. In the co-inoculation studies, it performed approximately equal to, if not better than the uninoculated controls, and performed the best out of the three species overall. It significantly increased root growth in *A. thaliana* by 7 DAP compared to the uninoculated, and had outpaced all other treatments by 14 DAP (Figure 4.3 and Figure 4.4). In *L. sativa* at 2 DAP it had an immediately noticeable impact on germination, and in conjunction with *Pa* significantly increased the germination early on implicating a synergistic effect (

Figure 4.6 and Figure 4.7). However, it is possible that these benefits were subdued by over-inoculation, as the dosage experiments revealed that an inoculum of 10^6 cells/mL was most effective at improving plant growth in juvenile and mature stages (Figure 4.10 and Figure 4.11).

Analysis of *Pa* shows that it is most beneficial to young plants. In *A. thaliana* it had larger roots than the uninoculated plants at 7 DAP, having similar lengths to the *Cf* treated plants (Figure 4.3). By 14 DAP, however, it had not kept up with *Cf* and the uninoculated plants had caught up in size (Figure 4.4). In co-inoculation with *Cf*, *Pa* appears to negate the effects *Cf* would have on root length, with the combination maintaining similarity with *Pa* between 7 and 14 DAP (Figure 4.3 and Figure 4.4) In the germination of *L. sativa* at 2 DAP, it did not improve germination on it's own, but all of the co-inoculations involving *Pa*

germinated moderately to significantly faster than the uninoculated treatment (Figure 4.7). Effects of *Pa* do not appear to be dose dependent, as the only notable statistical differences were in the seedling mass where a 10^7 cells/mL dose did increase mass compared to the uninoculated, but the other doses where statistically similar to both the uninoculated and 10^7 cells/mL treatment (Figure 4.10). These improvements did not, however, translate to improved growth in 4-week-old plants (Figure 4.11).

In summary, these investigations support our hypothesis that PGP bacteria exist within this ISSd population, however, this is not true for every strain identified with PGP phenotypes. The data presented here supports the conclusion that *Pa* and *Cf* are PGP, however, *Bp* may be an opportunistic pathogen, and its use as an inoculant may prove a hinderance, especially if it is more pathogenic to hosts other than *L. sativa* as suggested here.

These findings have yielded promising results for the prospect of using these ISSd isolates in a probiotic consortium for BLSS. However, this work also reveals the need to thoroughly characterize the interactions. A BLSS designed for mass food production will utilize a variety of species to ensure adequate nutrition and limit palate fatigue. Each plant used in a biodiverse system will have to be included in inoculation experiments to determine host-specific interactions and prevent detrimental effects. However, with careful consideration in this ecological

engineering endeavor, these microbes have the potential to improve the health and yield of BLSS.

Chapter 5
Discussion

Earth's gravity has remained a relative constant throughout the evolution of life on our planet. Over that time, many living systems have evolved to utilize this phenomenon to coordinate various biological processes, gravitropism in plants being one of the most well-established examples. Understanding the extent to which gravity impacts various processes is often best realized by the removal of this phenomenon either by simulation or in actual microgravity. A variety of microorganisms have been studied to date to provide a better understanding of the effects of gravity on the evolution, growth, and metabolism of these smallest forms of life. Such studies are also important for future long duration space exploration missions where microgravity is likely to be a persistent concern. Most of the efforts to understand bacterial growth in these conditions have focused on liquid culture-like studies but this fails to provide a realistic view for the conditions in which many microorganisms of interest persist, in thin films at interfaces common with prospective host organisms.

Through the work presented in this dissertation, I have strived to improve our collective understanding of how microbes behave in microgravity in the context of a holobiont community. As sustainable plant growth will be integral to bioregenerative life support systems (BLSS) in expanding space exploration

efforts, I have selected plants routinely grown on the International Space Station (ISS) to be the focal point of these studies. Microbes isolated from space-flight (SF) grown plants are available for sample request through the Open Science Data Repository (OSDR), and through experimentation with these samples I attempted to answer four major questions:

- Question 1: Do bacteria with plant growth promoting phenotypes persist in the microbiome of plants grown on the International Space Station? (CHAPTER 2)
- Question 2: Do plant growth promoting phenotypes occur under microgravity conditions? (CHAPTER 3)
- Question 3: Do bacteria with plant growth promoting phenotypes interact under microgravity? (CHAPTER 3)
- Question 4: Are bacteria with plant growth promoting phenotypes able to improve plant growth (CHAPTER 4)

To answer these questions, I have developed a testing 'pipeline' to guide the workflow of analyzing these ISS derived (ISSd) isolates (Figure 1.5). The first step of this pipeline is to screen these isolates for plant growth promoting (PGP) phenotypes, the first iteration of which is presented here as Chapter 2, characterizing siderophore production, indole production, phosphate solubilization

and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCd) activity. These are well-established plant growth promoting phenotypes in terrestrial plant-microbial interactions.

However, the SF environment can have significant impacts on bacterial growth and other phenotypes which drove us to consider if these phenotypes were preserved under simulated microgravity (SM) the primary subject of Chapter 3. As these phenotypes could be modified by the presence or absence of other microbes, we considered both pure as well as mixed cultures of multiple species derived from the International Space Station. These mixtures were considered both to mimic the more heterogeneous environment at the root surface but also to test the potential to use multiple species of PGP bacteria to improve plant growth synergistically, the subject of Chapter 4.

While microgravity can affect the bacterial expression of these PGP phenotypes, it is also notable that the nutrient content of the medium has major effects on the success of a bacteria to thrive in a biodiverse environment. This is highly relevant to consider, as microgravity has been shown to cause alterations in the metabolic profile of plants which the microbes feed off of. These metabolic changes could result in the observed shifts in the ratios of distinct taxa in SF, and could cause effects on microbial behaviors larger than microgravity alone would.

5.1 Pipeline Step 1: Characterization of ISSd Isolates

With the current protocols to sterilize and sanitize anything sent into space, the stochastic population of microbes in Veggie likely derives from two sources; the astronaut crew who possess their own natural skin and oral microbiomes that could spread to the plants during watering and tending procedures, and seedborne endophytes which are protected from sanitization by the seed coat. The former are likely commensal with the plants, taking up uninhabited spaces left by sterilization but otherwise neither helping nor hurting the host. Such transfers are more likely to occur in extremely isolated conditions such as the International Space Station. For example; *Acinetobacter genomospecies* 3 was originally isolated in VEG-01 flight samples, but not found in ground samples [10]. This particular species is typically isolated from anthropogenic sources and can be a cause of nosocomial skin infections [163]. The fact that no infections have been reported amongst astronauts indicates it is likely this strain is acting as a commensalist member of the astronaut skin microbiome and was likely transferred to the plants.

Endophytic microorgansms, contained with the surface sterilized seeds, are a more likely source of PGPB. Indeed, among these isolates, a significant number of bacteria possessing PGP properties have been identified through these screens. Many of these species are established in literature to be plant-associated, though no species have been identified with PGP traits novel among these ISSd strains (Table 2.1, Table 3.1, Table 3.2). It is therefore reasonable to assume that exp0osure to the

microgravity environment did not adversely impact the PGP activity of these post flight isolates. The alternative hypothesis that space flight resulted in a gain of function seems unlikely. However, exploring the frequency of these phenotypes among these populations over multiple generations in spaceflight would help discern between these two models.

It is crucial to note that while the same species may be isolated between expedition to expedition, subspecies variations can impact the number of PGP microorganisms available to each individual host. For example. the sample of *Curobacterium flaccumfaciens* (*Cf*) used in the experiments described in Chapter 2 was evaluated at Kennedy Space center and had been isolated from a VEG-03 expeiment, while the sample used in Chapter 3 was isolated from a VEG-01 experiment and was requested through the OSDR for experimentation at Florida Tech. The former strain produced large siderophore halos in the siderophore production assay, comparable to the other two species used in chapter 3 (Table 2.1). However, the latter strain did not grow on the siderophore assay media (Figure 3.5). This indicates repeat instances of the same species circumventing the pre-flight sanitizing processes, supporting the position that seedborne endophytes are a prime source of these isolates. It also illustrates the need to distinguish between subspecies variation and any possible gain or loss of function in PGP phenotypes.

5.2 Pipeline Step 2: Phenotypes Under Simulated Microgravity

Analysis of growth and phenotype expression showed that alterations do occur under SM, though not consistently. Of the 3 tested here, all experienced changes due to SM, though no bacteria had all phenotypes be altered, nor was any single phenotype universally altered (Table 3.3). This is consistent with other microgravity studies finding different species to have unique reactions to SF and SM [38,39,43,44]. As such, when focusing on a single phenotype, it is likely to find upregulation, downregulation, or no change among differing species of a population, which is consistent with my findings here. The cross-species differences in each phenotype indicate that each phenotype may not be directly affected by microgravity, but there is likely some underlying molecular process(es) being affected which manifests by affecting different expressional processes. Because each species experiences unique reactions to microgravity, it is important to assess each strain to gain a full understanding of its behaviors and potential interactions.

Individually, *Burkholderia pyrrocinia* (*Bp*) and *Cf* only altered growth or expression in 1 assay each. *Bp* grew larger in microgravity in the siderophore production assay indicating improved nutrient uptake in the iron limited media in

SM (Figure 3.6). Similar trends were observed in *Cf* in the phosphate solubilization assay, where it also grew larger in SM indicated improved nutrient uptake (Figure 3.9). *Pantoea agglomerans* (*Pa*) was the most susceptible to changes in SM, having a larger spot size on tryptic soy agar, in the siderophore production and phosphate solubilization assays, and producing lower amounts of indole relative to spot size.

Coculture analysis revealed unique growth patterns and some unique interactions of phenotypes. For example, in the siderophore assay the cocultures of *Bp+Pa* and *Bp+Pa+Cf* shared a unique spot morphology and grew larger than any individual species culture when comparing similar gravity treatments (Figure 3.6). In the ACC deaminase activity assay, the coculture of *Pa+Cf* was reduced compared to *Pa*, indicating that the presence of *Cf*, which is ACC deaminase negative, may be causing reduced efficiency in *Pa*'s activity. (Figure 3.12). In the indole production assay, growth of the *Bp+Pa* coculture on King B with tryptophan media was both reduced compared to *Pa* but increased compared to *Bp* (Figure 3.14). While not indicative of indole production, this does indicate that interactions can be dictated by the nutrient content of their environment given that coculture growth of multiple combinations with *Bp* on TSA, another general media, were similar to *Bp* rather than its cohabitant(s) (Figure 3.4). It is also notable that the coculture of *Pa+Cf* does not reduce indole production in SM like *Pa*, instead it remains similar to GC, if not slightly increased.

These results indicate that when alterations to these phenotypes do occur, they are to increase activity. In some cases however, this is in opposition to other findings. While I found that SM does affect siderophore production, it is to increase siderophore production, a study by Golaz et al. found a reduction in siderophore production by *Paraburkholderia phymatum*, however, this experiment was performed in liquid culture flasks on a random position machine [85]. This low fluid-shear environment has the effect of limiting diffusion of nutrients and metabolites through the media, resulting in low nutrient availability and artificial buildup of secondary metabolites (Figure 1.1) This type of SM is more relevant to a free-floating cell detached from any surface such as in the water or nutrient solution surrounding the plant tissues. The environment I am investigating here is that of microbes adhered to the surface of, or embedded in the tissues of plants in microgravity, which is not well simulated by liquid culture, hence the use of solid media in these studies. These types of observations exemplify why it is important to consider the natural environment when designing *in vitro* experiments.

5.3 Pipeline Step 3: Plant Growth Effects

Verifying that these isolates promote plant growth *in vivo* is potentially the most important step in this process. Many plant pathogens share *in vitro* phenotypes with PGP bacteria, the difference is in how these microbes utilize the

capabilities in a host plant. The findings here definitively confirm the presence of a plant growth promoting bacteria among the ISSd population: *Curtobacterium flaccumfaciens*. Inoculation with *Cf* yielded positive effects on growth of both *Arabidopsis thaliana* and *Lactuca sativa*, increasing root length in *A. thaliana*, seedling mass in *L. sativa*, and edible biomass in mature *L. sativa* (Figure 4.3, Figure 4.4, Figure 4.10, Figure 4.11). However, when co-inoculated with *Pa* or *Bp*, these same effects were not observed on growth, though *Pa+Cf* did enhance early germination rates of *L. sativa* (Figure 4.7). While not as extensive, *Pa* also had notable effects, particularly on early-stage growth of both plants, indicating that it can play an important role in the early development of a host plant.

Conversely, *Burkholderia pyrrocinia* has been identified as a potentially phytopathogenic bacteria. Inoculation with *Bp* consistently lowered germination rates, reduced seedling sizes, and in *A. thaliana* formed inhibitory biofilms, all regardless of the presence of *Pa* or *Cf* (Figure 4.5, Figure 4.8, Figure 4.9, Figure 4.10). Though, co-inoculation with *Cf* did appear to thin the biofilm enough more easily measure roots. However, these negative impacts were not observed in mature *L. sativa* plants, and reduced inoculum dose reduced severity of the negative impacts. Reduced inoculum dose even further improved the beneficial effects observed in *Cf* (Figure 4.10, Figure 4.11).

5.4 Conclusions

These investigations reveal the complicated dynamics of this uniquely sourced population of microbes, and in doing so show that understanding how microbes behave in microgravity will be important in understanding how life adapts to SF. The implications of these observations should be considered as space crop production systems become more complex, and missions become more reliant on them. The wrong microbe in the wrong place could mean mission failure for a food production system, but by understanding how these microbes behave with their hosts, these systems can be made more robust.

The repeated isolation of many of these species over multiple SF experiments indicates that these microbes will continue to be found in these systems even as plant growth systems are implemented on the Moon and Mars. This will be especially true if the same plant species are grown from the same seed source in future systems. However, changing seed type or source is unlikely to reduce the microbial load, only alter the species that are present. Instead of continuing to combat the very presence of these microbes, future BLSS can leverage these microbes by intentionally inoculating systems with the beneficial microbes identified through these investigations.

In spaceflight, most microbial experiments use liquid broth culture media over solid agar [43]. This is due to established methods for testing functions of

interest, as well as the ease of cell fixation for sample return. However, the fluid media environment does not adequately reflect the full range of the environments in which most of these processes will ideally occur, such as inorganic surfaces including ISS hardware, and solid matrices of host cells. This is not surprising as it reflects the inherent bias in much of the microbiology research conducted on Earth, i.e. in nutrient-rich liquid cultures, an environment largely unique to the laboratory.

My experiments highlight the importance of choosing appropriate methodologies for understanding host-microbial associations, especially in the spaceflight environment. While 3D clinostats are not novel, their use in microbial studies is uncommon; these are to my knowledge the first microbial studies using 3D clinorotation. Most simulated microgravity experiments use LFS SM systems such as rotating wall vessels, or 2D clinostats when agar plates are used [43,77]. This is likely due to the focus on microbial behaviors independent of hosts, and high commercial costs for microgravity simulation devices, which is why I recruited a team to build one for these studies.

As the scope of this type of work expands to other phenotypes, many liquid media-based protocols will have to be adapted, such as with the indole production assay here. These efforts will allow screening of these phenotypes under a more relevant *in situ* environment, refining our predictions of how PGP phenotypes may change in SM with plants. For example, given the observed reduction in indole

production by *Pa* in Figure 3.16, I am able to hypothesize that the increased growth observed in Figure 4.3 will be reduced or negated in inoculated plants grown in SM.

Observed phenotypic differences between assay types (i.e. solid media vs liquid culture) either in regular or microgravity are not semantic but may well point to significant differences in microbial behavior with broader impacts on plant-microbial associations. This is exemplified by the differences in the results of my siderophore production assays with those published by Golaz et al. [85]. As a result, the phenotypes selected here lay a foundation for studying PGP microbes in SM in a more relevant environment. Unfortunately, such direct comparisons such as this cannot be made for other PGP related phenotypes. This is because these phenotypes have not been previously, to the best of my knowledge, analyzed in a microgravity environment. Importantly, these findings support the observations of species-specific reactions to microgravity observed in other phenotypes. While these findings do not directly elucidate the background, likely transcriptomic, level alterations common to these observed changes to microgravity, it is my hope that these investigations may provide pieces to the proverbial puzzle.

5.5 Future Directions

These experiments are the first to establish a testing methodology to study ISSd isolates with the ultimate goals of understanding and improving their role in SF plant growth. However, further work on these isolates is warranted. For example, despite its problematic behavior, *Bp* is still of significant interest as it is implicated in chapter 2 to work as fungal biocontrol, which is of great interest for ISS and other BLSS systems [23,91]. If it is only an issue of dosage, and other bacteria could help to effectively "tame" *Bp*, it could be a boon to the hosts' ability to resist actual pathogens. To this end, future studies should also include disease resistance assessments by exposing inoculated plants to potential pathogens, such as ISSd *Fusarium oxysporum* samples. It is also possible that each bacteria has an optimal inoculum dose, and this dose may even vary by host, creating a greater level of complexity in understanding these holobiont communities.

The next step in continuing this work is to investigate more isolates. For example, *Pantoea vagans* is established in use for biocontrol of fire blight [164]. *Enterbacter cancerogenus* is a well characterized plant growth stimulant [165]. Potentially most interesting, *Cupriavidus metallidurans* is a common contaminant in ISS systems and has only been studied for plant interactions since 2021 but has been implicated in confering heavy metal resistance to host plants [166–169]. This would be especially beneficial in BLSS on the Moon or Mars where it has been

proposed to use regolith as a substrate, however the regoliths can be high in heavy metals such as chromium or aluminum.

However, while continual screening is an important consideration for the identification of new PGPB in different plants across multiple ISS missions, the long-term goals of this project beyond my own work will be to determine if we can develop methodology which leverages the plant microbiome to successfully combat disease and improve yield. This likely occurs though one of two methods, discussed briefly here. Method I involves biasing the existing microbiome of plants in the SF environment to favor PGPB. Given the stochastic nature with which PGPB are distributed among different plants or even different missions, Method I seems unlikely as it is too dependent on knowing the existing constituents of the microbiome.

A second method (Method II), would focus on the development of a consortium of PGPB introduced to host plants that will improve growth. Such an application would likely occur on seeds and could be applied prior to launch. Alternatively, PGPB could be introduced into the growth media at later stages of growth. Indeed, it may be possible in this approach to consider the application of different cohorts of microbes based on the life stage of host the host plant. For example, siderophore producing microbes maybe especially helpful at later stages of growth when iron can frequently become limiting. Conversely, ethylene

reduction via ACC deaminase producing bacteria in early stages could accelerate growth or in the case of legumes improve nodulation efficiency. Meanwhile, indole production could have benefits during early root development, late stage vegetative growth and even fruiting.

If we consider Method 2 a viable option, then improved strategies to evaluate the potential co-culture of different PGPB at quantities where these phenotypes are likely to translate into significant gains in crop yield and viability is crucial. This would also necessitate the evaluation of a minimum number of strains required for improving plant growth. However, this question is again driven by the host and our goals with that plant. Indeed, for legumes a single strain of Rhizobia is sufficient to provide a stable nitrogen source and arguably serves as the most significant mutualism throughout the host plant's life cycle. However this accounts for only 30% of land plants, with the remainder relying on mixtures.

Lastly, true microgravity experiments, aboard the International Space Staton, on inoculated plants will be crucial for verifying these effects in SF. In certain cases, such as *Bp* and *Cf*, while the gravitational environment may not cause wide-spread changes in behavior, these experiments have shown that nutrient content plays a large role in the behavior of these microbes. The primary source of carbohydrates and nutrients for these microbial symbionts is the metabolites generated by the host plant. This metabolic profile has been shown to change in

microgravity environments, which likely causes alterations to the makeup of the microbiome. It is also well established that microgravity has effects on microbial virulence, which could be another explanation for my observations of *Bp*'s opportunistic behavior, despite being isolated from healthy plants.

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