# MICROBIAL COMMUNICATION WITH EXTRACELLULAR VESICLES IN INTERSPECIES AND INTERKINGDOM INTERACTIONS

A Thesis
by
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## ABSTRACT

MICROBIAL COMMUNICATION WITH EXTRACELLULAR VESICLES IN INTERSPECIES AND INTERKINGDOM INTERACTIONS

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Microbiomes of the soil and gut form a complex community of bacteria, fungi, viruses, and host cells. Housing many times more genetic material than the host genome and a vast array of bioactive compounds. Understanding the microbiome could prove useful in improving agricultural practices and human health (1). Microbes communicate to one another and with the host in response to changes in their environment. Transportation of bacterial molecules to more distant cells, tissues, and locations to influence host functions has been attributed to extracellular vesicles (EVs) (2, 3). EVs consist of small molecules, RNAs, and proteins secreted by all kinds of cells and bound by the same outer membrane (4). They act as cellular "packages", housing and transporting compounds for interactions between cells and host structures. EV-mediated changes to host and bacterial functions in both gut and plant models is not well-characterized. The objectives of this research are to 1: isolate EVs from *Pseudomonas fluorescens* and *Saccharomyces cerevisiae,* 2: to understand their impact on the growth and soil microbiome composition of *Arabidopsis thaliana*, and 3: to quantify

changes in yield of EVs secreted by *Escherichia coli (E. coli)* and *Enterococcus*. The results will help elucidate the role of EVs in cellular communication by bacteria in gut and soil microbiomes.

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## Dedication

This thesis is dedicated to Zoe Clay, the most wonderful example of joy, independence, and goofiness I have ever witnessed. Zoe passed on October 8, 2023, mere weeks before I was able to finish this work. Rest in peace, your memory will always light the hearts of the countless souls you touched.

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## List of Abbreviations

**EV:** Extracellular Vesicle

**RNA:** Ribonucleic Acid

**UN:** United Nations

**GMO:** Genetically Modified Organism

**PGPR:** Plant Growth-Promoting Rhizobacteria

**ABA:** abscisic acid

**PAMP:** Pathogen-Associated Molecular Patterns

**AIEC:** Adherent Invasive Escherichia Coli

**OMV:** Outer Membrane Vesicles

**BFI**: Bacterial-Fungal Interactions

**GI:** Gastrointestinal

**IBD:** Irritable Bowel Disease

**CD:** Crohn's Disease

**KB:** King's Broth

**UTI:** Urinary Tract Infection

**LAB:** Lactic Acid Bacterium

**YPD:** Yeast Peptone Dextrose

**LB:** Luria-Bertani

**PBS:** Phosphate Buffered Saline

**ICS1:** Isochorismate synthase 1

**PGPB:** Plant Growth Promoting Bacteria

# INTRODUCTION

## Agricultural Practices

Agriculture is a constantly evolving technology. The transition from nomadic, hunter-gatherer societies to communities and cultures centered around farming has allowed for many advancements in human technology and a generally improved standard of living. Agriculture has enabled large societies to inhabit the same condensed area, by reducing competition for resources and facilitating advancement in other areas of interest. Populations have grown rapidly since this cultural shift. At the current population and rate of growth, there will be a necessity for major increases in food production in agriculture by 2030 (5). However, recent changes in fertility rates and reduced cultural focus on reproduction has seen a new leveling trend in many areas (6). Modern agriculture practices have resulted in a global system mostly centered around large farming operations.

Agriculture is a fiercely competitive industry that favors the scalability of large farm operations that tend to outcompete smaller operations in efficiency or operations and mass acquisition of resources. Attention to changing global climate conditions, combined with focus on reducing pollution and other human environmental impacts has targeted corporate practices, including those of the agricultural industry. Many organizations emphasize sustainability as a key marker for environmental health and reduction of adverse impacts. A majority of the UN's sustainable development goals, a key blueprint for the best practices concerning human environmental interactions, can be related to agricultural techniques and practices (7). To increase crop yields, the industry has started modifying plant species to implement more complex and effective pesticides and fertilizers. This technique produces crops called transgenic organisms, known commonly as genetically modified organisms

(GMOs). GMO production requires a large investment to research and develop and consequently, is inaccessible to many small-scale operations, providing demand for other methods to increase agricultural productivity. GMOs are also highly controversial as the long-term effects of consumption are disputed and have been a focus of the media. They have attracted public attention due to the uncertain outcomes associated with genetic modification(8). These crops are still often consumed by humans and used as feed in animal agriculture where efficiency tends to be a higher priority, and use of GMOs is less apparent to the end consumer.

New trends to implement robotics in agricultural techniques may lead to another shift in global practice that focuses on smaller farming operations by easing access to sustainable and efficient techniques, allowing competition in price from small-scale farming operations, (9). The shift from large industry to organic, sustainable, non-GMO, grass-fed, free range, etc. has followed consumer trends of increased demand for products made with sustainable agricultural practices. Small farms with an emphasis on low to no chemical pesticide and fertilizer use have experienced growing demand for product. The best methods to meet growing demand will be through sustainable agriculture and advancing technology to increase crop yields while focusing on sustainability and environmental conservation (10). Therefore, effective farming methods with fewer environmental impacts are being utilized more frequently by both small and large operations and will continue to grow in popularity.

**Soil Microbiome**

The interactions between plants and their environment, specifically soil, is crucial for plant health and growth. Soil contains a wide range of compounds used by the plant as a

main source of nutrients for growth and immune function. The soil ecosystem houses many organisms; small mammals, insects, worms, reptiles, amphibians, viruses, and bacteria exist in the layers of decaying organic material and minerals. Of the many microorganisms, bacteria, yeast, and fungi are key components of the soil microbiome. The soil microbiome impacts soil composition by affecting the cycling of nutrients, plant productivity, chemistry, and structure (11). Plants interact with the soil primarily through their root system, where nutrients, chemicals, and biological molecules are transported into the plant. This zone of root and surrounding soil is called the rhizosphere. It houses a concentrated community of bacteria that produce metabolites with abilities to affect plant growth and immune response(12, 13). Modern agricultural practices such as plowing, fertilizing, and mono-cropping have a lasting effect on both the soil microbiome and composition; however, these impacts are largely unexplored (11). Effective methods of fertilization that avoid negative impacts on soil microbiome and composition are necessary in the scope of sustainable practice. Crop rotation and traditional manure fertilizers can have a profound effect on soil composition, with a possible result of degradation of soil microbial communities (11). Shifting away from methods that lead to soil degradation is important for the longevity, productivity and sustainability of farms and prevention of environmental degradation.

Bacterial and fungal communities in the soil vary significantly, responding to environmental factors that can promote growth of some species and reduce the prevalence of others. Bacteria and fungi can be particular in their growth conditions, favoring environments with acidity, nutrient composition, temperature, and moisture suitable for growth. In the microbiome, a constant battle for resources exists, as nutrients and space foster competition between organisms. The microorganisms in the soil communicate between one another

through compounds including proteins, lipids, genetic material, and carbohydrates that they emit directly into the soil. Communication between species can influence soil composition by promoting growth of biofilms, transferring organic material, and promoting growth. The microbial community in the rhizosphere hosts a unique composition of microbes due to the interaction between plants and soil, this microbiome can provide functional traits that benefit plant health (14). The rhizosphere hosts an array of organisms, all competing for limited resources in a scarce environment, and all responsible for a particular function in the soil ecosystem (Table 1) (15). Understanding and modifying the composition of the rhizosphere's microbiome through EV treatments could have significant effects on plant growth and health.

| | Nutrient cycling | Soil structure |
|---|---|---|
| Microflora e.g., bacteria, fungi | Catabolize organic matter<br>Mineralize and immobilize nutrients | Produce organic compounds that bind aggregates<br>Hyphae entangle particles into aggregates |
| Microfanua e.g., protozoans, nematodes | Regulate bacterial and fungal populations<br>Alter nutrient turnover | May affect aggregate structure through interactions with microflora |
| Mesofauna e.g., mites, collembola | Regulate fungal and microfanual populations<br>Alter nutrient turnover<br>Fragment plant residues | Produce fecal pellets<br>Create biopores<br>Promote humification |
| Macrofauna e.g., amphipods, centipedes, earthworms | Fragment plant residues<br>Stimulate microbial activity | Mix organic and mineral particles<br>Redistribute organic matter and microorganisms<br>Create biopores<br>Promote humification<br>Produce fecal pellets |

*Table 1: Influences of soil biota on soil processes in ecosystem* (15)

Organisms in the rhizosphere play a crucial role in processing organic materials, converting them into simpler molecules and byproducts of unique microbial metabolic functions. Bacteria and fungi can help assimilate some nutrients in complex soil residue into new cell biomass. This simultaneously mineralizes and releases other stored nutrients into inorganic forms that provide a crucial source of nutrients for plant growth. Select species of fungi and bacteria also serve as catalyzers in Nitrogen, Phosphorus and Sulfur cycles(15). Bacterial and fungal communities, and their roles in soil composition alteration are greatly

affected by agricultural practices, which can result in decreased microbial density, function, and nutrient ratios in the soil (16).

### *Pseudomonas fluorescens*

*Pseudomonas fluorescens* is a nonpathogenic bacterium that thrives in soil, water and on plant surfaces. This rod-shaped, gram negative, flagellate is an obligate aerobe: requiring oxygen as an electron acceptor. However, some strains can utilize $NO_3$ as an alternative electron acceptor (17). Specific strains can be utilized as agents for biocontrol, protecting plants from fungal infection and promoting plant growth contributed to specific bacterial byproducts(18–20). *P. fluorescens* are rapid colonizers and can out compete pathogenic bacteria in the rhizosphere (21).

*P. fluorescens* is an important member of the plant rhizosphere, known as plant growth-promoting rhizobacteria, (PGPRs) are responsible for several environmental modifications that benefit plant growth. *P. fluorescens* release toxins, antibiotics, and siderophores (22). Understanding the complex interactions between PGPRs and the plant host has been approached through many different methods, as implementation of *P. fluorescens* in field experiments can be inconsistent (23). Mekureyaw et al. found that root inoculation with *P. fluorescens* significantly improved tomato growth (24). They also found drought-stressed plants showed higher drought related mitigation mechanisms; increase leaf chlorophyll, abscisic acid (ABA) content and stomatal closure when inoculated with *P. fluorescens* (25–27)

E xposure to pathogen-associated molecular patterns (PAMPs) was found to protect plants from subsequent pathogen challenges (28). Mcmillan et al used *P. fluorescens (Pf)*

outer membrane vesicles (OMVs) to illicit plant immune response and found exposure to *Pf* OMVs protected *Hyaloperonospora arabidopsidis, Solanum lycopersicum* and *Solanum tuberosum* from bacterial and oomycete challenge by *Pseudomonas syringae* and *Phytophthora infestans*, through complete rescue from leaf yellowing and reduction of *P. syringae* growth. *Pf* OMVs demonstrated structural stability when biochemically disrupted and can elicit plant immune response (3).

### *Saccharomyces cerevisiae*

Yeasts are a single celled fungal organism, they have been used for fermentation, baking, and production of nutritional yeast, a food product emerging in popularity. Yeast is regarded as possibly the earliest domesticated organism, and much of early scientific understanding revolves around its agricultural use (29). Soils were often considered a reservoir for yeasts that were not significantly active until emerging from the soil environment, but studies performed in the early nineteenth century found yeasts present and active in the soil (30). Yeasts residing in the soil must adapt to survive in a harsh and vast range of conditions, and yeast communities in soils are diverse and different from those above (30) .

*Saccharomyces cerevisiae*, a particular species of yeast, is often found in and around agricultural environments. Believed to have been discovered on the skin of grapes in the early nineteenth century and defined later in that century, *S. cerevisiae* is closely linked with the development of human agriculture and civilization (31). *S. cerevisiae* is broadly used in fermentation of alcoholic beverages such as beer and wine, and in baking of breads as a leavening agent. Commonly known as brewer's yeast, the species was historically overlooked as a key player in the phylosphere and rhizosphere, research instead being

focused on its food processing abilities. Recently, a few species, selected for their high production of yeast oils, have been applied in agriculture as potential defenses against soil-borne plant pathogens and as promoters of plant growth (30). *S. cerevisiae* is often used in research settings for its ease of production of biological materials on a large scale, specifically it can produce high concentrations of mRNA, one of the key functional components of EVs (29, 32).

**Bacterial-Fungal Interactions**

Bacteria and fungi coexist in a variety of conditions and environments, interactions between these two microorganisms can be significant as the role of both organisms independently are tied to the health of plant and animal systems (33). A recent focus of multi-disciplinary research has been on the complex nature of Bacterial-Fungal interactions (BFI) and the role of these interactions in environmental science, medicine, and biotechnology (33). Bacteria and fungi have shared microhabitats throughout their evolutionary history, co-existing, they have evolved direct and indirect mechanisms of communication and defense against and between cells. Medical sciences have been using these bacterial and fungal products throughout the history of medicine, using compounds excreted by these organisms in antibiotics and other therapies (34).

BFI innately affect the behavior of one or all the organisms involved, these effects are difficult to predict solely using current understanding of the biology of isolated species grown in lab cultures. The specificity of BFI ranges in degree and level based on a variety of factors, biophysical and metabolic interactions during which bacterial and fungi interdependently develop and evolve may be much more pronounced and apparent. Concurrently, somewhat random presence of two species could be the result of microbial

community shifting and mixing not due to co-evolution or interdependence. The interactions between fungal and bacterial species may be very simple, highly refined or absent depending on a range of mechanisms and environmental factors (35).

Bacteria and fungi are involved in plant and soil health and growth. Fungi may also play a symbiotic host to soil bacteria as providers of growth-promoting environments. The mycosphere, the zone in which fungal hyphae extend into the soil, much like the rhizosphere, is a zone associated with increased bacterial cells (36). Select bacteria have adapted to selection pressures found in the soil environment by acquired capabilities in their evolution that increase their survivability and prevalence in the fungal hyphae. Haq et al. composed a list of known relationships in bacterial and fungal hosts, and the hosts they often accompany (36). Notably, the *Pseudomonas* genus is associated with more fungal hosts than any other genus, providing relevance to further fungal interactions such as an association between *Saccharomyces* and *Pseudomonas.* This known behavior of *Pseudomonas* increases likelihood that the genus developed mechanisms and behaviors to communicate with fungal species.

**Human Microbiome**

Human health is an intricate balance of systems and processes, and the body is in a constant state of exchange with the environment. Many mechanisms and systems in the body are focused on defense and symbiosis with our surroundings. One of the most influential and constant states of exposure the human body experiences occurs in the digestive tract. An expansive surface area, the organs involved in digestion are specialized to process wide varieties of food into energy that the body can utilize. The gut is involved in more than energy production; it is the site of many immune responses and home to a vast array of

bacteria, fungi, and viruses. These microorganisms in the gut are collectively known as the gut microbiome. The gut microbiome houses 150 times more genes, and roughly the same number of cells as the rest of the human body (37, 38). The many roles played by the gut microbiota make it key in understanding links between human health and nutritional behaviors.

**Crohn's Disease, Inflammatory Bowel Disease**

Characterized by chronic inflammation of the gastro-intestinal (GI) tract, irritable bowel disease (IBD) such as Crohn's disease (CD) affects millions of people (39). Those with CD may experience symptoms varying in severity including diarrhea, abdominal cramps and pain, constipation, and possible bleeding of the rectum (40). CD, like its associated microbial species, tends to remain fixed in the GI tract, with the ileocolic area most frequently affected (41). Severity of the disease and response to treatment varies between patients. Roughly 40-50% of patients diagnosed with CD can be treated, entering a state of remission (42, 43). However, a large portion of patients diagnosed with CD have chronic symptoms and complications (44). These persisting symptoms have a broad spectrum of therapeutic remedies ranging from steroids and biologics. However, when these treatments are not effective, surgical intervention is a common alternative (45, 46).

**Adherent Invasive *Escherichia coli***

Growth of specific strains of bacteria such as adherent invasive *E. coli* (AIEC); distinguished by their increased adhesion and invasion in intestinal epithelial cells and replication in macrophages, are associated with the pathogenesis of CD (47, 48). AIECs trigger an inflammatory response in the intestine through several key strategies (49). AIECs

are resistant to antimicrobial defenses in the gut, which enables adherence to the epithelial cells of the intestine and increases colonization of the gut mucosa. AIECs modulate tight junction complexes between intestinal epithelial cells, allowing for permeability and bacterial invasion through the epithelial barrier (50). Furthermore, AIECs follow these initial steps with the colonization of epithelial submucosal compartments. In response, macrophages engulf the invading AIEC cells; however, AIECs can survive and replicate inside of the macrophages (51). This sequence of events, combined with host immune deficiencies, can be a substantial contributor to intestinal inflammation, a leading symptom of CD (50).

**Enterococcus**

*Enterococcus* is a bacterial species commonly responsible for food spoilage and utilized in some fermentation processes. *Enterococcus* is commonly found in the body and has been used as a probiotic for humans and animals. It has been associated with some virulence delivery, as problematic lineages are associated with immune response in humans. Many *Enterococcus* strains are, however, linked with beneficial effects such as lower instances of diarrhea, irritable bowel disease, and lower cholesterol levels (Franz et al., 2011). *Enterococci* are often considered a commensal bacterium in the human gastrointestinal (GI) tract. Increasingly, *Enterococcus* has been linked to nosocomial infections (53, 54). Some strains can lead to bacteremia, endocarditis, and some urinary tract infections (UTI). Many pathogenic strains display antibiotic resistances and virulence factors including hemolysin, adhesins, and invasins (52, 55).

*Enterococcus* is a genus of Gram-positive, with a cell membrane covered by a waxy peptidoglycan layer. It is a “cocci” shaped bacterium, sometimes forming chains. *Enterococci* are facultative anaerobes, preferring aerobic respiration. However, it can use

anaerobic fermentation when oxygen is scarce. First documented in the late 1800s, *Enterococcus* was originally found in the intestinal tract, one of its more common locations (56). It was also associated with endocarditis, an inflammation of the inner layer of the heart(57). *Enterococci* are opportunistic pathogens, associated with some host vulnerability and resulting overgrowth of the bacteria, a condition common to Crohn's disease, to become pathogenic.

A main precursor for severe infection by *Enterococcus* is colonization in the GI tract, where the bacteria translocate through the gut. This requires survival of gastric pH, intestinal colonization, epithelial phagocytosis, and resistance to macrophage killing (58) Virulence of *Enterococcus* is often associated with use of antibiotics and other drugs that lower host defenses and gut microbiota diversity. Use of antibiotics is also linked to increased growth of strains associated with unique mechanisms, such as Vancomycin-resistant *Enterococcus* (59). A mechanism of regulation often implemented by *Enterococci* is secretion of an antibiotic substance called bacteriocin, which increase bacterial competition in a dense microbial environment by targeting other like bacteria. Bacteriocins in *Enterococcus* are secreted and tend to target other gram-positive organisms that may compete for space and similar resources in the gut (60). Overgrowth of *Enterococcus* in the colon often leads to the side-effect of bacterial translocation into lymph tissue and subsequent distribution throughout the body. This pathogenic behavior of *Enterococci* to translocate and cause bacteremia and other complications is not due to any singular mechanism, rather it is a combined secondary effect of the bacteria's durability in combination with its opportunistic overgrowth capabilities. *Enterococcus faecalis,* a common species associated with pathogenicity is durable enough to survive up to 72 hours in macrophages, this is sufficient time to result in distribution of the

bacteria when there is sufficient overgrowth (61). *E. faecalis* can follow this translocation with an ability to localize and persist within distal tissues such as the lymph nodes, liver, and spleen (62).

*Enterococcus* is an emerging and important opportunistic bacterial pathogen. Associated with 14% of hospital-acquired infections in the United States between 2011 and 2014, *Enterococci* were the third most common nosocomial pathogen during this period (63). The bacteria are linked to many adverse health outcomes including bacteremia, sepsis, endocarditis, and others. The pathogenic behavior of *Enterococcus* is often caused by some form of host defense inadequacy. Recent understanding of the dangers of hospital-derived strains and their increased ability to cause adverse effects, as well as having unique antibiotic resistance abilities has bought focus to this bacterium as a potential emerging pathogen. Increasing rates of microbial dysbiosis in the human population as well as diseases associated with this condition, including Irritable Bowel Disease (IBD) and Crohn's Disease (CD) is leading to more and more cases of *Enterococcus*-associated complications. IBD and CD have both been linked to increased *Enterococcus* abundance, and the rise of microbiome research and subsequent focus on gut composition will undoubtedly lead to greater understanding of the role of *Enterococcus* in CD and other related diseases (64).

**Microbial Interactions in Crohn's Disease**

Diagnosis of CD is not a straightforward process; a comprehensive analysis of symptoms and fecal microbiota composition is used to diagnose the disease (65). The gut microbiome plays a large role in the pathogenesis and progression of IBD (66). CD patients have a microbiome that differs in composition from a healthy gut, and there are multiple strains of bacteria associated with CD (67). Specifically, *E. coli* and *Enterococcus* are known

to grow together in association with inflammatory bowel disease (47, 65). The specifics of this interaction are largely unexplored. To characterize the communication between species in the CD microbiome, these specific bacterial strains *(E. coli* and *Enterococcus)* will be examined. Underlying interactions between these bacteria could be crucial to understanding the relationships between CD, gut inflammation, and the microbiome.

**Extracellular Vesicles**

In the soil, gut, and other environments, bacteria often make and utilize small packages called vesicles for many of their functions. Vesicles have many different names, as they have been observed playing a wide range of roles in bacterial processes. When emitted into the environment they are often called extracellular vesicles (EVs) or outer membrane vesicles (OMVs). EVs are membrane-bound packages ranging from 50-200nm in size and often contain molecules like proteins, RNAs, and lipids (Cho et al., 2021). They are secreted by a parent cell and surrounded by a small portion of the same parent cell membrane that provides protection for bioactive materials inside (32). EVs can house and transport these protected compounds to other cells as a method of non-specific interkingdom and interspecies communication. They can be translocated through the gut wall and into distal tissues of the human body (Fig. 1) (Bittel et al., 2021). EVs are also known to provide nutrients for further bacterial infection and promote interkingdom transfer of material (68, 69).

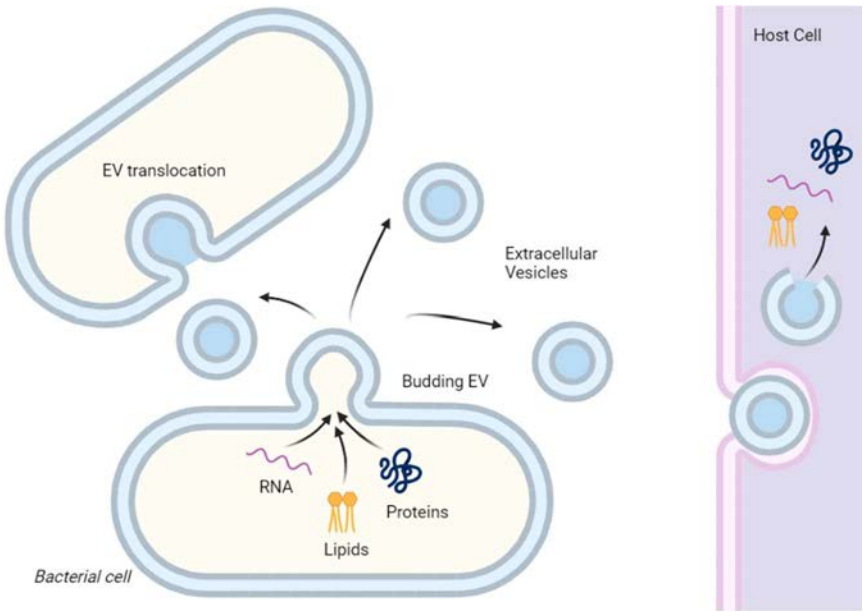


*Figure 1: Representation of EV-mediated protein, lipid, and RNA transfer from parent bacterial cell to host and surrounding bacterial cell targets- created with BioRender*

**Comparing and Contrasting Vesicles in Bacteria, Plant Fungi**

Fungal vesicles are similar in function to those of bacteria. Production of vesicles in fungi occurs at the outer membrane. Like bacterial vesicles, the cargo of fungal vesicles varies and can contain different combinations of proteins, lipids, RNAs, polysaccharides, and toxins (70). Fungal vesicles are produced in a similar condition as those of bacteria, often a result of cell metabolism and budding of the cell wall. Vesicles can alternatively form as a response to environmental stress, defense against pathogens, and resistance to other fungi (70). Fungal vesicles emitted into the soil are also able to elicit a plant immune response. Plant cells, like fungi and many bacteria, are surrounded by cell walls, these would theoretically prevent the formation and secretion of vesicles. However, it was recently discovered that plants do indeed produce extracellular vesicles as a pathogen defense mechanism. EVs are utilized by plants in intercellular transport of multiple materials and were found to contribute to plant growth, defense, and plant-microbe symbiosis (71).

Similarities between plant, fungal and bacterial vesicles are their composition, being that of the parent cell membrane, and EV's function as transportation methods in intercellular and interkingdom interactions. The cargo of EV's, and surface markers are likely to differ between the three domains, as composition of cellular metabolites and byproducts would differ.

**Extracellular Vesicles in Agricultural Practices**

Vesicles have a unique ability to protect bioactive molecules in transportation through the environment. This function allows for potential use of vesicles in the delivery of specific molecules to targets in agricultural practices. Vesicles can activate innate immune response in plants allowing for the protection of plants from bacterial infection (3). Use of EVs to induce a transcriptional shift in Arabidopsis and resulting upregulation of many immune pathways, could facilitate resistance to infection in agricultural practices (72). Plant EVs also contain RNAs and proteins that may be absorbed by fungi, resulting in reduced virulence from fungal pathogens (73). The effectiveness of vesicles as a nutrient delivery vehicle in a human model has been introduced, but its application as fertilizer is under investigation as well as implications for modifying the rhizosphere microbial communities (74). The particle's ability to transport material with high nitrogen content and other materials correlated with growth shows promise for the use of EVs as a fertilizer. Understanding of the movement of EVs, which is mainly associated with water, in different soils will help identify proper applications of them in agriculture. Research associated with vesicle-mediated pathogen protection and immune response present EVs as a device with applications like a vaccine, with ability to prepare inoculated plants for pathogen encounters. Further applications could include soil microbiome modification and subsequent shifts in soil health

and presence of bacteria associated with nitrogen fixation, conversion of heavy metals, and soil detoxification to prevent common environmental impacts of modern agricultural practices including mono-crops and long-term soil turn-over. Understanding the exact mechanisms and interactions between plants, EVs, and the soil microbiome could provide the agricultural industry with a new tool that can be sustainably sourced and distributed to provide plants with nutrients necessary for growth, protection from pathogens, and positive soil microbial change with few environmental impacts.

**EVs in Co-culture and Significance in CD**

Methods of communication used by microbial strains within the gut are still largely unknown. Interspecies microbial communication through secreted material is known as an effective method in which microbiota alter genetic expression and protein secretion (75, 76). However, the gut environment is harsh and contains a wide range of metabolic biproducts, and compounds consumed by the host. This diverse environment can quickly deactivate and dilute bioactive materials and secreted molecules. Probiotic molecules and species have greater effects in modulation of the gut microbiome; possibly due to persistence and concentration in the gut (77). EVs have a membrane that protects them from the harsh gut environment and are an effective nutrient and genetic material transfer device in transkingdom interactions (2, 78, 79). This unique ability to transfer protected material makes EVs a promising method in which CD-associated *E. coli* and *Enterococcus* may communicate. Furthermore, AIEC may offer a more influential platform in invasion of gut epithelial cells that could shuttle EVs further into host systems (2). Investigation into this mechanism of communication may provide insight into the CD gut microbiome and its differing composition from a healthy gut (65, 67).

## OBJECTIVES

1. Isolate EVs from *S. cerevisiae and P. fluorescens* and comprehensively characterize EV impact on plant growth and other plant parameters.
2. Characterize changes in soil microbiome of *A. thaliana* when treated with isolated EVs from *S. cerevisiae and P. fluorescens.*
3. Isolate EVs from *E. coli* and *Enterococcus faecalis* and characterize the changes in EV yield in conditioned media to determine the role of EVs in species interaction in Crohn's disease.

## METHODS

### Bacterial strains and culture conditions

***Pseudomonas fluorescens*** strain ATCC 13525 (a gift from H. McMillan, Duke University, Durham, NC) was inoculated from frozen glycerol stocks onto King's Broth (KB) agar plates [2% proteose peptone, 8.6 mM K2HPO4, 1.4% glycerol, 6 mM MgSO4, 1.5% agar] and grown for two days at 28 $^{o}$C. Colonies were used to inoculate 50 mL liquid KB media [2% proteose peptone, 8.6 mM K2HPO4, 1.4% glycerol, 6 mM MgSO4] and incubated overnight at 28 $^{o}$C with constant shaking. 1 mL of this overnight culture was used to inoculate 1-2 L cultures of KB media and incubated at 28 $^{o}$C with constant shaking for 17 h.

***Saccharomyces cerevisiae*** strain YEF473 (a gift from D. Lew, Duke University, Durham, NC) was inoculated from frozen glycerol stocks onto Yeast Peptone Dextrose (YPD) agar plates [10g/L yeast extract, 20g/L Bacto-Peptone, 20g/L Dextrose, 1.5% agar] and grown for two days at 30 $^{o}$C. Colonies were used to inoculate 50 mL liquid YPD media [10g/L yeast extract, 20g/L Bacto-Peptone, 20g/L Dextrose] for overnight incubation at 30 $^{o}$C with constant shaking. 1 mL of this overnight culture was used to inoculate 1-2 L cultures of KB media and incubated at 30 $^{o}$C with constant shaking for 17 h.

***Escherichia coli*** strains were inoculated from frozen glycerol stocks onto three Luria-Bertani (LB) agar plates [20g/L LB Broth, 1.5% agar] and grown for two days at 30 ºC. Separate colonies were used to inoculate three 50 mL liquid LB media [20g/L LB broth] (or conditioned media) cultures and incubated overnight at 30 ºC with constant shaking. 1 mL of each overnight culture was used to inoculate two 250mL cultures of LB media (or conditioned media), for a final volume of six, 250ml cultures of biological triplicates and technical duplicates incubated at 30 ºC with constant shaking for 17 h.

***Enterococcus faecalis*** strain JA0187 (a gift from Janelle Arthyr, UNC Chapel Hill) was inoculated from frozen glycerol stocks onto a Luria-Bertani (LB) agar plate [20g/L LB Broth, 1.5% agar], grown for two days at 30 ºC. Colonies were used to inoculate a 50 mL liquid LB media [20g/L LB broth] culture and incubated overnight at 30 ºC with constant shaking. 1 mL of this overnight culture was used to inoculate two 500mL cultures of LB media and incubated at 30 ºC with constant shaking for 17 h.

**Vesicle Preparations and Isolation**

*P. fluorescens, S. cerevisiae,* and *E. coli* vesicles were isolated using modifications to published protocols (3, 80). Cells were pelleted from cultures in a Sorvall RC 6+ centrifuge (2011; F14-6x250y rotor; 10,000 x g; 10 min), cell-free supernatant was collected, and vacuum filtered (0.45 μm HV, Millipore Durapore). Vesicles were pelleted from cell-free supernatant in a Sorvall RC 6+ centrifuge (2011; F14-6x250y rotor; 30,000 x g; 3 h) and resuspended in 1 mL PBS for 1 h at 4 ºC. Vesicles were then filtered in an Eppendorf centrifuge 5420 (2021; FA-24x2 rotor; 9,000xg, 2 min) (0.45 mm HV, Millipore Durapore spin tubes), before pelleting in a Sorvall MTX 150 micro-ultracentrifuge (2011; S55-A2

rotor; 91,000 x g; 1 h). The vesicle pellet was resuspended in 1 mL Phosphate Buffered Saline (PBS) (overnight; 4 $^{o}$C) before protein quantitation.

Protein concentration was determined with a Coomassie (Bradford) Protein Assay Reagent kit (Fisher Scientific, Loughborough, UK; 23200), prepared with the standard microplate protocol. Concentrated *P. fluorescens* and *E. coli* OMVs were diluted 4 times in $dH_2O$. Concentrated *Saccharomyces cerevisiae* vesicles were measured as concentrate and 4X dilution. 5 µL of each vesicle dilution was added to 150 µL Coomassie reagent. Vesicles were stored at 4 $^{o}$C. Samples were measured in duplicate at an absorbance of 595 nm and compared to a standard protein dilution curve to quantify protein concentration.

**Soil Experiments**

Vesicles were diluted in sterile, $dH_2O$, analyzed using the Bradford Assay, and used in rhizosphere experiments. Vesicle concentration for use in experiments were determined using previous Isochorismate synthase 1 (ICS1) expression assays (3). *Pf* vesicle concentrations were at 5X concentration (15 µg/mL) of protein for "high concentration" and 2.5X (7.5 µg/mL) for "low concentration". *Sc* vesicles were diluted based on yield relative to *Pf* to 5X (1.18 µg/mL) and 2.5X (.6 µg/mL) respectively.

*A. thaliana* were treated in seven groups containing four replicates each. They were separated into control (containing no EVs), *Pf* (high, 15 g/mL), *Pf* (low, 7.5 g/mL), *Sc* (high, 1.18 g/mL), *Sc* (low, 0.6 g/mL), *Pf-Sc* (high, 15 g/mL *Pf* and 1.18 g/mL *Sc*), and *Pf-Sc* (low, 7.5 g/mL *Pf* and 0.6 g/mL *Sc*).

Professional growing mix soil was prepared for experiments through autoclave sterilization. *Arabidopsis thaliana* Col-0 seeds were transferred into four-inch plastic pots

containing the sterilized soil. The pots were placed in large, covered tubs for cold stratification at 4C for 3 days, then germination (Figure 2). The tubs were then incubated under cyclical light and temperature conditions (Table 3) for 16 weeks, with consistent watering to maintain moisture and addition of EVs, according to treatment conditions, every two weeks after the first two weeks of incubation. Samples were harvested on May 13, 2022, and the following metrics of morpho-physiologic effects were analyzed by the Ogwu lab; photosynthesis rate, stomatal conductance, substomatal $CO_2$, transpiration rate, water use efficiency, chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll a:b ratio, and carotenoid concentration.

*Figure 2: A. thaliana* immediately after seed transfer into plastic pots and growth tubs prior to growth chamber placement (courtesy of Dr. Matthew Ogwu, Appalachian State University).

| Conditions | Temp. | Light level | Time |
|---|---|---|---|
| Long Day (16 h) | 21°C | dark | 00:00 |
| Normal light | 23°C | 150 µmol/$m^2$/sec | 06:00 |
| | 21°C | dark | 22:00 |

*Table 2:* Growth conditions in chamber for *A.thaliana* during 16 week growth period (courtesy of Dr. Matthew Ogwu, Appalachian State University).

**Conditioned Media Cultures**

*E. faecalis* cultures were grown according to methods stated previously. They were split into two groups, one 500ml culture in which cells were be pelleted in a Sorvall RC 6+ centrifuge (2011; F14-6x250y rotor; 10,000 x g; 10 min), cell-free supernatant was collected, and vacuum filtered (0.45 μm HV, Millipore Durapore), then used as a vesicle-present conditioned media. The other 500ml culture continue through the above steps, upon which vesicles were pelleted from cell-free supernatant in a Sorvall RC 6+ centrifuge (2011; F14-6x250y rotor; 30,000 x g; 3 h) and the supernatant was collected as a vesicle-free conditioned media. Both 500mL conditioned media were diluted and fresh LB broth added for a final volume of 1.5L for use as conditioned media for *E. coli* cultures with further vesicle isolation/quantification as described previously.

**Data analysis**

Raw absorbance readings collected from Bradford assays were analyzed using excel and standards plots included with the Bradford assay. Calculations for all raw data were performed in Microsoft Excel and Jamovi. Graphs and statistical analysis of data using Students T-test and Mann-Whitney U tests were performed in Jamovi(81). Data were considered significantly different with a P value less than 0.05.

Alpha diversity was calculated using Shannon diversity index and the differences in Shannon diversity were analyzed with Kruskal-Wallace test and visualized with a whisker plot. Bray-Curtis dissimilarity between samples were calculated using normalized abundance of genera and visualized using Principal Coordinate Analysis (PCoA). The differences in

microbial composition between groups were analyzed by Novogene Corporation (Sacramento, CA). P-values of <0.05 were considered significant.

**DNA extraction and sequencing**

After soil experiments, DNA was extracted from Phylosphere samples using the ThermoFisher Scientific MagMax (A32549 Isolation Plant DNA Kit) from duplicates of 150mg soil samples from each treatment group to a 75 µl final volume in elution buffer. Rhizosphere samples were collected, and DNA was extracted to a final volume of 75 µl in elution buffer using the Omega E.Z.N.A.® Soil DNA Kit (D5625-01). In total, DNA extraction produced 120 samples from 30 groups, separated into rhizosphere and phylosphere and duplicated for sequencing. The samples were analyzed for concentration using a Nanodrop Spectrophotometer (Thermo Scientific Nanodrop 2000), measurement mean of samples was 80 ng/µl concentration in 75µl samples. DNA Sequencing was done by Novogene Corporation (Sacramento, CA). Raw sequencing reads were processed and analyzed as described in a previous section.

## RESULTS

**RB057 Conditioned media increases production of vesicles in NC101 cultures**

Understandings of Crohn's disease have been limited to uncovering bacteria associated with symptoms of the disease and those diagnosed with inflammation of the bowels. The mechanisms underlying the chronic nature of the disease have not been greatly understood, as the causes of gut inflammation can be multi-faceted and evasive. Investigating particular mechanisms associated with known species in the CD model can help further understanding of the disease as a whole and improve treatment and diagnosis procedures.

Investigation of communication between bacterial species associated with CD and subsequent increases in similar inflammation associated species can add context to the progression of the disease. To understand one aspect of interspecies communication in CD, we examined the role of vesicles in interkingdom relationships to uncover the importance of these packages in communication and transfer of material including RNA, which can have powerful effects on surrounding cells and hosts(2, 32, 69, 78).

To investigate the role of EVs in communication between known CD-associated bacteria, *E. coli* NC101 and *E. faecalis* RB057 were tested in a conditioned media model where NC101 was grown in *E. faecalis* conditioned media containing and lacking EV's. To evaluate changes in EV production of NC101, Bradford assays were used to quantify protein concentrations in vesicles isolated in staged centrifugation. Trial one revealed promising results, indicating a statistically significant difference in concentration of EVs under the conditioned media culture with a mean difference of 15.75 µg/mL including the groups of conditioned media still containing *E. faecalis* vesicles. Concentration for trial 1, when *E. faecalis* EV concentrations were subtracted using the known concentrations found in vesicle removed conditioned media was 14.67 µg/mL more compared to standard LB broth cultures.

The second trial of conditioned media experiments backed up the original findings, indicating a trend of NC101 to increase EV production in growth media containing products from RB057 growth. Trial two indicated this trend with elevated vesicle production (SE=3.38µg/mL) in the conditioned media groups ($t(10)=1.6$, $p=0.134$), increase in vesicle production was still present (SE=2.42 µg/mL) when the groups were adjusted for RB057 vesicles ($t(10)=1.2$,$P=0.258$). When results from both trials were combined (Figure 3) results showed increased vesicle production in conditioned media experiments containing RB057

EV's (Fig.3A) and when adjusted for EVs from RB057 conditioned media containing EVs, with slightly less statistical significance when adjusted (Fig. 3B).

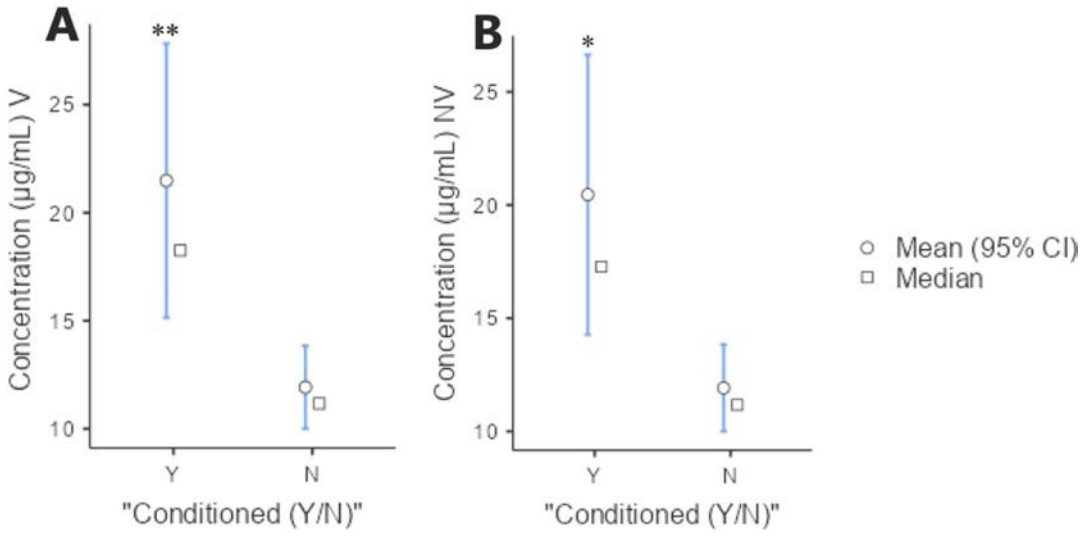


*Figure 3: Combined Trial Bradford Assay Protein Concentrations A) Concentration (µg/mL) in samples not adjusted for RB057 vesicle concentration. B) Concentration adjusted for RB057 vesicle concentration. All data shown as means SE, n=12 representative of two experiments in biological triplicates and technical duplicates. Statistical analysis was performed with a Mann-Whitney U test *p<0.05, **p<0.01, indicates a statistically significant difference between groups* (81).

### Growth of *A. thaliana was regulated in groups treated with vesicles from P. fluorescence*

Extracellular vesicles from *Pseudomonas fluorescence* have been associated with upregulation in immune response and subsequent increase in protective mechanisms and growth of *A. thaliana* (3). Addition of organic compounds into soil is a known method to increase plant growth markers, yet more nuanced and targeted methods have not extended into the field of EVs. During soil experiments, phylosphere samples were taken and morpho physiologic effects were examined using a collection of markers associated with plant growth. Statistically significant increases in EV treated groups chlorophyll a (F(3,6)=[3.64] p=0.015), chlorophyll *b* (F(3,6)=[3.79] p=0.013), and subsequently total chlorophyll (F(3,6)=[3.89] p=0.012) were found. The "Pf-low" group, where plants were dosed with 7.5

μg/mL of *P. fluorescence* EVs saw the greatest increase in chlorophyll *a* (Figure 4A), chlorophyll *b* (Figure 4B), and total chlorophyll (Figure 4C).

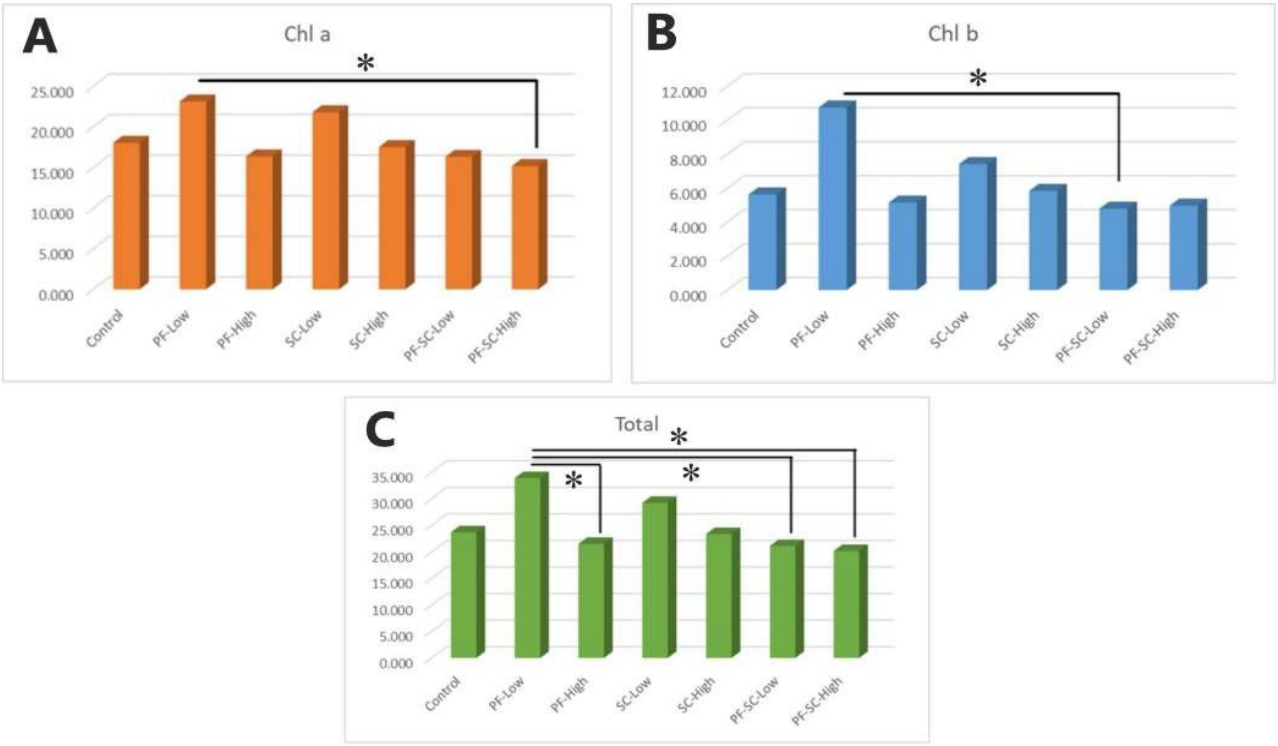


*Figure 4: Chlorophyll readings in μg/mL A) chlorophyll* a *B) chlorophyll* b *C) total chlorophyll. All data shown as means SE, n=4 phylosphere samples per group. Statistical analysis was performed with a Tukey pairwise post hoc comparison. *p<0.05, **p<0.01, ***p<0.001, indicates a statistically significant difference between groups. Data collected and interpreted by Dr. Matthew Ogwu (ASU, Boone, NC).*

**EVs increase specificity of microbial communities in *A. thaliana* rhizosphere**

Rhizosphere microbial communities can vary between plants and the environments they inhabit. Bacterial communities can be specific to region, agricultural history, organic compounds, and other soil conditions. To examine the effects of EV treatment on the soil of our experiments, we looked at sequencing data examining alpha and beta diversity of bacteria in the soil (Figure 5). The mean diversity of bacterial species in each treatment, or alpha diversity, was significantly lower in the Pf-low treatment condition compared to control and the remining EV treatments (Fig. 5A). This reduction in alpha diversity indicates fewer

acterial communities in the PF-Low treatment, which is often considered detrimental to microbiome health, however, could also indicate reduction in bacterial species that have pathogenic properties and an increase in more beneficial bacteria. Increases in diversity in the Sc-Low and PfSc-Low could implicates lower concentrations of *S. cerevisiae* EVs in increasing diversity of bacterial communities, specifically over Pf-low treatments. The specific bacterial taxonomy, and further implications of diversity change is investigated in taxonomy data (Fig. 6). The weighted comparison of communities among the treatments, or beta diversity, indicated a significant specification in bacterial communities from both high and low PF treatment groups (Fig. 5B). These findings show *P. fluorescence* EVs have significant impact on bacterial diversity in the soil.

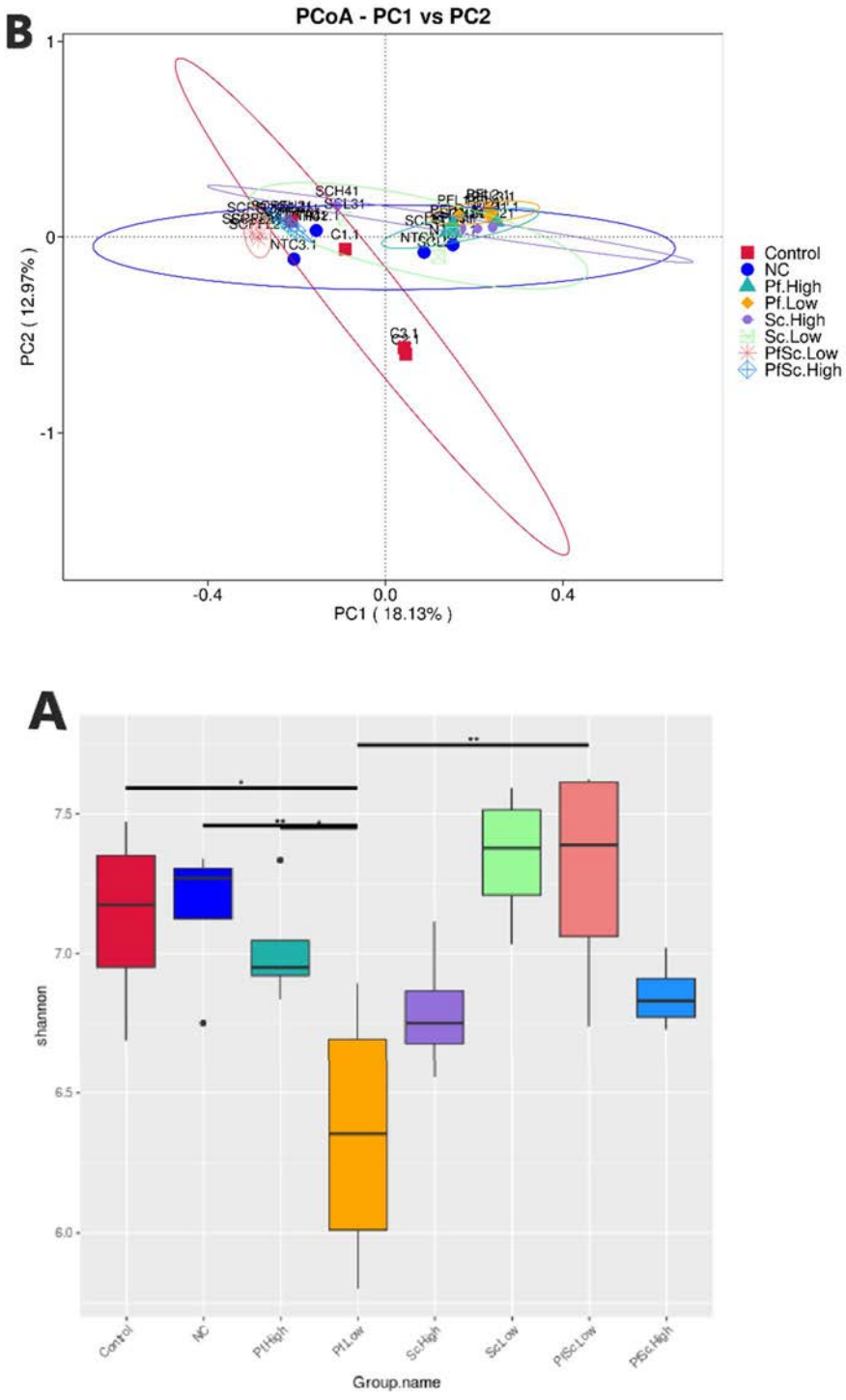


*Figure 5: Diversity of Bacterial Communities in the Rhizosphere A) Alpha diversity between treatment groups. B) Beta diversity between groups. Alpha diversity was shown with the Shannon diversity index Statistical analysis was done using the Kruskal-Wallace test . Beta Diversity is shown as a PCoA, statistics analysis was via Bray-Curtis dissimilarity, *p<0.05, **p<0.01, ***p<0.001, indicates a statistically significant difference between groups. Data interpreted by Novogene corporation (Sacramento, CA)*

**Extracellular Vesicles alter bacterial community composition in differing magnitudes with respect to treatment and origin.**

The rhizosphere is a hotbed for bacterial interactions with plant biology. This zone hosts an array of bacteria, all in proximity and with capability to influence plant health, growth, and behavior through changes in soil properties. Rhizosphere microbes influence soil composition and plant health through many methods, and specific bacterial presence can be associated with pathogenicity or as beneficial for plant growth and protection. To investigate the specific changes of the bacterial communities, present in each EV treatment, we investigated the taxonomy of the microbial communities (Figure 6).

Beginning at the phylum level of taxonomy, changes in the PF-High group indicate increased communities of the phylum *Actinobacteriota*, a known group of Plant Growth Promoting Bacteria (PGPB) (Fig. 6A) (82). The combined PfSc -High and -Low groups were associated with an increase in the phylum *Bacteriodota* an important indicator of soil quality in context of studies investigating the soil biological degradation process (83). Investigating further, into the class level of taxonomy (Fig. 6B) which indicates an increase in PfSc groups of *Alphaproteobacteria*, a class that harbors an array of plant symbionts (*Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium*(84)), and *Bacteroidia* which are associated with less agriculturally processed soil (85) and a decrease in these groups of *Gammaproteobacteria*, which is commonly associated with immune response (Fig. 6B) (86).

Taxonomy by order is consistent with class taxonomy results with notable variations in PfSc-Low and -High groups, with increased populations of *Rhizobiales*, a well-known

associate of plants that provide beneficial functions for their hosts by providing nutrients, phytohormones, and plant metabolite precursors (87). Pf-Low also indicated a higher level of order *Xanthomondales* than any other Treatment, (Fig. 6C). This order encompasses a wide range of pathogens with virulence factors associated with pathogenicity and fitness in plants (88). In Figure 6D, taxonomy by family indicates an increase in concentration of the family *Rhodobacteraceae* in the PF-Low group. This family is associated with sodium chloride transport, mercury detoxification, CO oxidation, vitamin-B12 production and transport of nutrients in the soil (89). *Burkholderiaceae,* a family known as a source of antibiotics, bioactive secondary metabolites, and promotion of plant growth was also increased in the PF-Low group (90). PfSc-Low and -High treatments correlated with an increase in the *Sphingomonadaceae* family, which take on various roles as helper bacteria by assisting their host plants in survival in contaminated environments (Fig. 6D)(91).

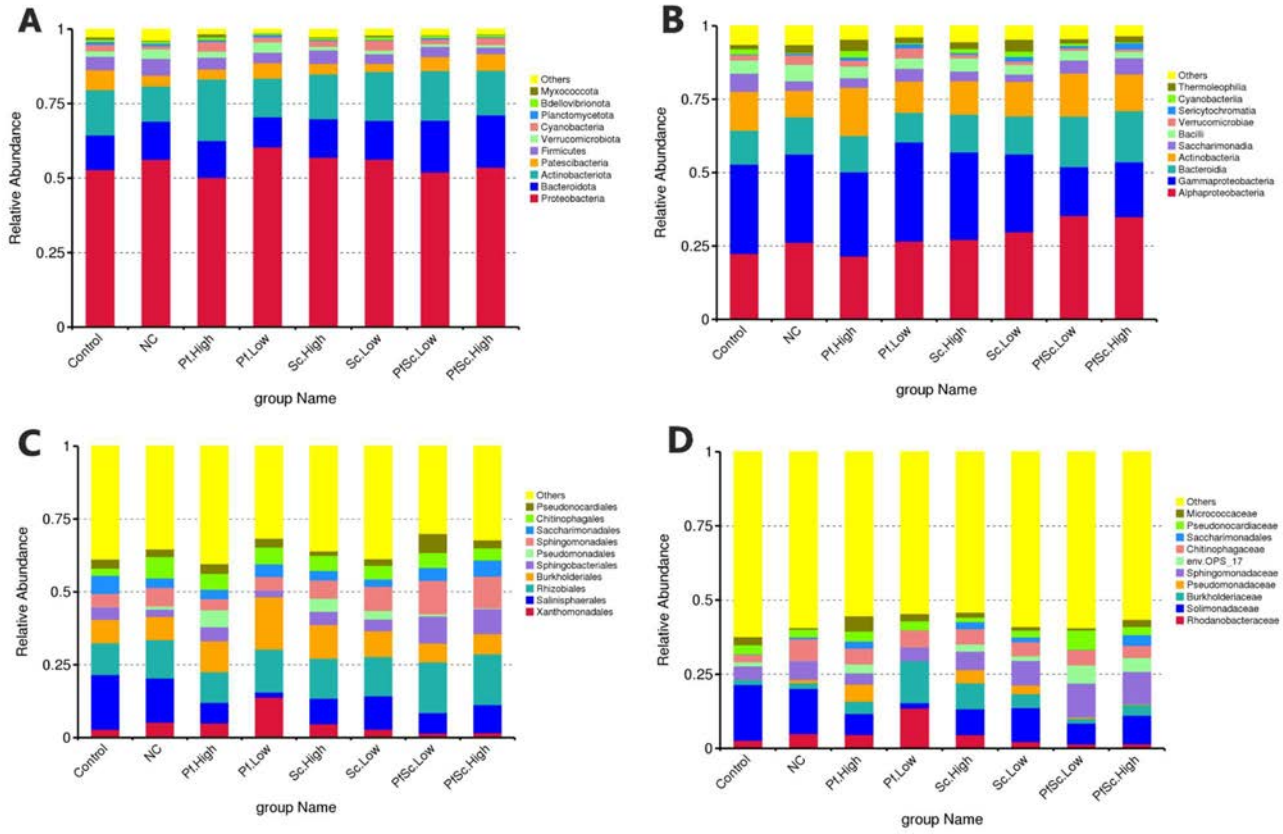


*Figure 6: Taxonomy Bar Plot of Rhizosphere Samples Separated by EV treatment A) Taxonomy by Phylum B) Taxonomy by Class C) Taxonomy by Order D) Taxonomy by Family. Plots show top ten most abundant of each respective taxonomical group, the remaining groups are categorized as "other" sequencing and interpretation by Novogene corporation (Sacramento, CA).*

## DISCUSSION

Bacteria and Fungi are present in great numbers both in the human body, where bacterial cells outnumber human cells by nearly 8 trillion (92) and in the soil, where total estimated prokaryotic organisms are estimated at $1.2 \times 10^{29}$ (93). These organisms are associated with a wide range of both beneficial and pathogenic interactions with their human and plant hosts. Furthering understanding the prevalence of these organisms in the microbiome of their hosts, along with communication and regulation of species prevalence in

these areas of increased host- microbe interaction is crucial to improving gut and plant health in multiple applications. To investigate this area of interaction, we approached these microbiomes and their modulation by examining the effects of EVs on the soil microbiome and their role in signaling between two gut microbes. In this study, we have expanded on the impacts of EVs on the gut microbiome as a marker for communication in bacteria associated with the CD model, and a role of EVs from *P. fluorescence* and *S. cerevisiae,* in a concentration and combination dependent manner, as a modulator of rhizosphere microbial diversity and composition (Figure 5,6). We indicated EVs as a potential marker of communication in the gut through an in vitro experiment where increased EV production of murine gut-isolated *E. coli* NC101 was observed when grown in media conditioned by murine gut-isolated *E. faecalis* RB057 compared to standard growth medium (Figure 3). Soil experiments revealed an EV concentration and make-up dependent change in *A. thaliana* rhizosphere microbial diversity and composition, particularly in treatments of *P. fluorescence* low dose and combined *P. fluorescence* and *S. cerevisiae* vesicles of both high and low concentrations (Figure 5,6). These alterations of microbial composition may lead to changes in plant growth and pathogen defense capabilities of the host plant.

EVs have been shown capable of transporting genetic material, cellular metabolites, and other components to other cells, through the gut wall, and even to distal organs of the human body (2, 32, 69, 94, 95). The role of EVs in disease models and interspecies bacterial communication in the gut microbiome has not been fully investigated. To discover a role of EVs in the CD model we examined the relationship between the presence of byproducts of cellular metabolism from *E. faecalis* RB057 and the production of EVs by *E. coli* NC101, both strains associated with the CD, by conditioning media and examining the change in

vesicle quantity of *E. coli*. We found a significant increase in vesicle quantity when in conditioned media compared to media in standard broth at the same growth medium level. This result was independent of the presence of *E. faecalis* vesicles indicating a cell-metabolite linked communication between RB057 and NC101 that results in vesicle production and ties these two species together as related components of the CD model. EVs have been shown capable of transporting mRNAs from trees into the human body via ingestion of EV-containing honey, resulting in anti-inflammatory effects in the host (2). EVs have also been associated with transfer of genetic material from the gut microbiota to distal host organs and have been shown to penetrate the gut wall (32, 69). The interspecies mechanism shown here between two known bacteria commonly found in the microbiomes of CD patients implicates vesicles in cell-cell communication in the gut microbiome.

Further studies focusing on the link between bacteria found in the CD gut microbiome and the specific role of EVs will help to clarify EVs as a possible contributor to or indicator of CD. Our data indicates EVs as a possible cargo molecule for transportation of inflammatory metabolites and mRNAs of both NC101 and RB057 past the gut wall, and to the host as a mechanism that can be linked to the chronic inflammation found in CD. These results also lead to questions of the types of cargo carried by these EVs, how the EVs are influencing the inflammatory potential of these strains, and how the EVs may be interacting with the host in a more comprehensive CD model. Increased production of *E. coli* EVs and potential inflammatory cargo, could play a role in the progression and persistence of CD.

EVs in the soil possess similar communication and genetic material transfer potential as those in the gut microbiome, with similar proximity to the nutrient acquisition members of their plant hosts. The rhizosphere microbial community interacts similarly to the plant host as

that of the gut microbiome in human hosts. This area of interaction in the rhizosphere which similarly to the intestinal tract, revolves around nutrient and water acquisition, is selected by plants and bacteria for acidity, salinity, and nutrient composition among other factors. Agricultural practices can have an impact on the microbial community composition of the soil, subsequently changing the diversity and taxonomy of the community. Methods such as crop rotation and fertilization attempt to restore bacterial and nutrient compositions of soil to maximize growth and health of the crop and increase agricultural profitability. Changes to the soil microbiome can be slow, and the cultivation of crops is often not enough to restore bacterial populations, resulting in lower nutrient and secondary metabolite concentrations received from symbiotic bacteria. Certain species of soil bacteria are also associated with pathogen protection, not only in the rhizosphere, but also in the phylosphere (3, 80). We investigated the effects of treatments from *P. fluorescence* and *S. cerevisiae* EVs separately and combined in two concentrations to uncover the effects of EV inoculation of the rhizosphere on *A. thaliana* growth and health. Compared to control, initial testing revealed EV-treated plants had higher levels of chlorophyll (Figure 4), a powerful indicator of overall plant health and condition, and a pigment of chief importance to photosynthesis and growth (96). Further investigation of treatment showed chlorophyll levels of Pf-Low treated soil was significantly higher than the chlorophyll levels of Pf-High and PfSc -High and -Low soil. We followed initial tests to analyze rhizosphere bacterial communities through DNA extraction and sequencing. This study showed that low *P. flouresens* EV treatment resulted in lower alpha diversity, an indicator that they may be recruiting more specific bacterial communities in the soil compared to the control or low treatments of *S. cerevisiae* and ScPf, which significantly increased alpha diversity over Pf low (Figure 5). Investigations into the

taxonomy of these changes on several different taxonomic levels showed an increase in phylum and class levels of PGPB bacteria in Pf-High treatments as well as combined vesicle treatments (Figure 6A) (82, 83). These groups also increased *Bacteriodota,* which are a known indicator of soil quality, and a reduced prevalence of *Gammaproteobacteria* that are known to cause plant immune response (Figure 6B) (85, 86). Investigation into lower taxonomic levels enforced the trend in combined ScPf groups as promotors of growth to PGPB (Figure 6C,D). Notably, the order level taxonomy of the Pf-Low treatment showed an increase in *Xanthomondales,* an order associated with many plant pathogens (Figure 6C) (88). Family level taxonomy of the Pf-Low group counteracted this, with an increased level of *Burkholderiaceae,* a family known as a source of antibiotics (Figure 6D) (90). These findings enforce combined EV treatment, in high and low concentrations, and low concentrations of *P. fluorescence* EVs as possible means of altering soil composition to promote host plant growth and positively affect rhizosphere bacterial communities.

Further investigation of the cargo of these EVs and the mechanisms in which EV treatment alters bacterial communities could open the way for use of EVs in agriculture. Investigation into other beneficial bacteria, which may produce higher levels of EVs could increase the viability of this treatment on a large scale. EVs could serve as a viable treatment, in combination with current agricultural practices, to increase agricultural output, as well as plant and soil health.

## CONCLUSION

The soil and gut host complex microbiomes that interact with host plant and human systems, these systems can be altered through many actions. EVs act as a transportation molecule with unique properties that allow for protection of their cargo and passage into areas other molecules could not reach. In this study, we investigated the production of EVs by CD-associated bacteria, indicating a role of EVs in microbe-microbe interactions. Our data suggests that *E. coli* modulates EV production as a response to metabolites of *E. faecalis*, both common microbes in CD patients. We also investigated the role of EVs in rhizosphere microbial communities and the effects of EV treatment on *A. thaliana* growth. This data indicates EV treatment, specifically treatment with low concentrations of *P. fluorescence* EVs, recruits specific rhizosphere bacterial communities. Both *P. fluorescence* low and *S. cerevisiae* and *P. fluorescence* combined treatments were associated with increased PGPB. These results add further evidence to the many roles of EVs in the soil and gut microbiome and subsequent plant and human host health.

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# VITA

William Robert Hardin was born in Winston-Salem, North Carolina in 1998 to Thomas and Beth Hardin. He graduated from Ronald Reagan High School in Pfafftown in June 2016. During high school, Will earned the rank of Eagle Scout in the Boy Scouts of America and found a passion for biology through his anatomy classes. The following fall, he entered Lee's McRae College on a combined cycling athletic and academic scholarship to study Biology, and in May 2020 he was awarded the Bachelor of Science degree with a minor in mathematics and cycling. In Fall of 2021, he accepted a research assistantship at Appalachian State University and began study toward a Master of Science degree in cell and molecular biology as a part of the Bleich lab. Will has worked closely with the multi-disciplined VESICLE research group and has been awarded multiple university sponsored research grants to fund his project. Will has aided as lecture support in Biology. He has also trained several students in lab techniques.

Will is a professional cyclist, currently riding with the veteran's non-profit Project Echelon, where he volunteers in veteran onboarding. In 2024, he will be moving to the Miami Nights, a team in the National Cycling League, where he will continue to race professionally and coach children and adults in cycling. Will hopes to work as a research scientist in microbiology, regenerative medicine, or medical device development.