PAPER AS A SIMPLE TOOL TO STUDY BACTERIAL ECOLOGY

by

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Dedicated to my father, mother and brother

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LIST OF ABBREVIATIONS

BEF	Biodiversity and Ecosystem Functioning
LAB	Lactic Acid-producing Bacteria
ATCC	American Type Culture Collection
OD_{600}	Optical Density at 600 nm wavelength
PBS	Phosphate Buffered Saline
MRS	de Man, Rogosa & Sharpe
GUI	Graphical User Interface
DPI	Dots Per Inch
PPI	Pixels Per Inch
TIFF	Tagged Image File Format
RGB	Red Green Blue
CMYK	Cyan Magenta Yellow black
HSI	Hue Saturation Intensity
qPCR	quantitative Polymerase Chain Reaction

ABSTRACT

Community interactions based on various parameters in defined niches have been studied to understand their influence on bacterial life. Yet there currently are no models that can depict how spatial interactions control the complex combinatorics of different microbial communities. Biodiversity influences the ecosystem properties of bacterial communities, but the relationship between bacterial biodiversity and function remains to be understood entirely. Here, the focus is on developing a simple and effective platform to study neighbourhood interactions between different species of lactic acid bacteria by controlling two metrics – distance and composition. Using this simple platform, I explore 1) how spatial and temporal arrangement between different bacteria affect their interaction in a high throughput manner, 2) how biodiversity can be manipulated in terms of its starting population, the number of species, and species identity.

1. INTRODUCTION

1.1 Background and significance

Traditionally, biodiversity research is concerned with understanding the abiotic and biotic drivers of organisms' diversity in an ecosystem (Chesson 2000; Paul et al. 2020). But in the last two decades, there has been a growing interest in the issue of how important a diverse biotic population is to the functioning of an ecosystem (Cardinale et al. 2007; Hooper et al. 2005; Loreau et al. 2001; Schulze and Mooney 1994). Most of the experimental evidence for testing the effect of biodiversity on ecosystem function has resulted from mostly terrestrial ecosystems, particularly grasslands (Hector et al. 1999; Naeem et al. 1994; Tilman et al. 1997; Weisser et al. 2017). These studies have shown that functional characteristics of individual species are of paramount importance in defining communities. For example, in different trophic levels of plant populations, a loss of diversity from a system will evoke changes in the structure at all trophic levels (for e.g., loss of predators) (Duffy 2003; Hairston, Smith, and Slobodkin 1960). Understanding the relationship between biodiversity and ecosystem function is essential as it can help predict, for example, how ecosystems and communities respond to environmental change. It also helps understand how declining diversity can have an impact on ecological services on which humans depend (Duffy 2003). This knowledge will pave the way to the sustainable management of biodiversity (e.g., biodiversity conservation), reinforcement of ecosystem performance and resilience (e.g., maintenance of soil fertility, water purification, pollination and many others) (Mace 2014)

Microbial communities underpin major ecological processes that sustain both the animal and plant kingdom (Stocker 2012; Widder et al. 2016). The biodiversity of these communities affects their function in the ecosystems in which they occur. The relationship between biodiversity and ecosystem function (BEF) is maintained and expressed through various ecological processes. Among bacteria, these processes occur over a spatial range, i.e. within microscale aggregates of ~100 μm (Cordero and Datta 2016). Spatial patterns are key determinants of the function and resilience of bacterial metacommunities (Kerr et al. 2002; Hassell, Comins, and May 1994). The distribution of interacting microbial communities across different physiochemical

heterogeneities—spatially and temporally—strongly influence the behavior of the microbes and the function and resilience of the community as a whole

BEF relationships in plant and animal communities have been tested extensively (Langenheder et al. 2010). Still, challenges surrounding the need to study communities with control and replication constrain our ability to determine quantitative relationships between species richness and ecosystem function (Naeem 2008). Given the enormous importance of bacterial assemblages and processes (e.g., wastewater treatment, industrial chemical and pharmaceutical production, bioremediation) for ecosystem sustainability (e.g., biogeochemical cycles) and overall function, there is a need to test BEF relationships in bacterial assemblages (Prosser et al. 2007). Studies exploring microbial BEF relationships associated with abiotic and biotic factors (Godbold, Solan, and Killham 2009; Petchey et al. 1999; Solan et al. 2004; Worm et al. 2002) and their variation over time (Cardinale et al. 2007) are limited. Studies that have investigated BEF relationships in bacteria (Bell et al. 2005; Plas 2019; Venail and Vives 2013) usually confirm a positive effect of species richness on ecosystem processes, but not always (Langenheder et al. 2010). Functionally equivalent species showed signs of saturation at low species richness levels (Wohl, Arora, and Gladstone 2004), and negative sampling effects, where species offering scarce contributions to the functioning of the ecosystem seem to pervade in species-rich communities (Jiang 2007). It can, therefore, be theorized that the function of an ecosystem function increases with greater species richness due to stronger complementarity effects. Higher species richness might reduce competition between species owing to partition and differentiation in resources and an increase in species diversity might support facilitative interactions like cross-feeding on metabolic by-products among similar species (Langenheder et al. 2010).

Chip-based technology, microfluidics, encapsulation techniques etc. have emerged to find their applications in microbial community ecology and high-throughput screens of beyond pairwise microbial compositions (Kehe et al. 2019). While they have set a precedent for providing exciting insights into quantitative measurements of community dynamics, the use of microfluidics to assesses BEF relationships has not been explored widely (Hol, Whitesides, and Dekker 2019). Even in seemingly simple populations, the complexity of microbial interactions and environmental dependencies (Ghoul and Mitri 2016; Sanchez-Gorostiaga et al. 2019) may lead to unpredictable behaviours, presenting a challenge to consortium design. The logical complexity of constructing strain combinations and studying them in a deterministic manner is challenging (Kehe et al. 2019).

While microfluidics provides the potential to assess interactions between hundreds and thousands of microbial communities, they are limited in their approach to studying how space affects microbial interactions in a high throughput manner (Hol, Whitesides, and Dekker 2019; Li, Ballerini, and Shen 2012; Widder et al. 2016). A better understanding of the microbial world and its interactions will depend on improved cultivation techniques that permit the study of axenic and mixed cultures in combinations (Nai and Meyer 2018). To provide an additional empirical tool for experimental analysis of microbial BEF relationship (BEFR), I demonstrate the use of paper as a multi-purpose platform to study how spatial arrangement and distribution of select bacteria of interest affect their interactions.

I will describe a novel experimental design in which filter paper is used as a tool for a co-cultivation screen of lactic acid bacteria (LAB). Paper, an inexpensive, disposable, and widely used commodity contain microscopic pores (several tens of micrometers) that are similar in scale to most bacteria. They can be patterned (using wax, laser-cutting technology, or lithography) and stratified into many layers (for combinatorial studies of bacteria). The use of paper permits both spatial and compositional control of the microbial community of interest (Hol, Whitesides, and Dekker 2019; Martinez, Phillips, and Whitesides 2008). Additionally, the capillary action of paper distributes fluid without the need for external pumps (Verma et al. 2018). A paper-based approach is amenable to quantitative assays that can evaluate the relationship of biodiversity to the functional diversity of microbial communities under a range of environmental conditions.

1.2 Research Objectives

Experiments involving microbial communities present a great potential for understanding BEFR because conditions to grow and manipulate bacterial communities in a laboratory setting can be achieved easily. Although the structure, composition and interactions among bacterial communities have been extensively studied and reviewed, there is a general lack of experimental tools that can systematically define how space and composition drive the population dynamics and function of microbial communities. To address the major gap in our understanding of BEFR, this research was aimed at testing the hypothesis that increasing the number of interacting bacteria increases the function. The number and type of bacteria are used as surrogates to biodiversity, and biomass is used as the surrogate to ecosystem function. I used paper and an image acquisition platform to develop a simple platform to control and study bacterial interactions. I made use of the

substantial benefits that microorganisms offer as model systems to test theories of ecology. My goal was to examine whether bacterial species diversity determines total system biomass over time. The primary emphasis was to develop and standardize a simple platform to test how ecosystem function changes over time.

1.3 Thesis Outline

- Chapter 1 is an introduction to the thesis, explaining the research problem and specific objectives.
- Chapter 2 reviews the current literature on the use of bacterial communities to study the BEF relationship.
- Chapter 3 utilizes knowledge from the literature review to develop a simple platform to study bacterial ecology. This chapter describes the methods I used to study the interactions among three lactic acid bacteria in a controlled environment.
- Chapter 4 explains the results of the experiments in detail.
- Chapter 5 discusses the results and their implications, draws conclusions, and outlines recommendations for future work.

2. LITERATURE REVIEW

2.1 Summary

Under this section, I first review the fundamental concepts of bacterial interactions required to understand the dynamics of microbial communities in general. There are three parameters that define bacterial interactions: interaction range, interaction strength, and community context. Then, I focus on reviewing current technologies and microbiological tools in play that study community behavior. Based on the theoretical understanding of bacterial interactions and an awareness of the shortcomings of current microbiological tools, I describe my research on the development and standardization of a simple yet high throughput and cost-effective platform to test the theories of microbial ecology through combinatorial studies.

2.2 Introduction

How and to what extent does biodiversity influence the function of natural ecosystems? The answer to this fundamental ecological question holds the key to our future, as protecting species and their habitats are not only a means of conserving biodiversity but could also preserve the productivity and services of ecosystems for future generations (Cardinale et al. 2012; Naeem, Duffy, and Zavaleta 2012). Despite mounting evidence that ecosystem functioning is affected by the loss of biodiversity (Cardinale et al. 2012), the mechanism behind biodiversity-ecosystem functioning (BEF) relationships and the extent to which biodiversity broadly contributes to ecosystem functioning have remained a subject of debate. Ecosystems worldwide are rapidly losing biodiversity (Naeem, Duffy, and Zavaleta 2012) and biodiversity's role in the function of ecosystems has been under intensive investigation for the past two decades.

The debate has been concentrating on whether it is the sampling effect or the complementarity effect that shapes BEFR. It is believed by some (Cardinale 2011; Gillman and Wright 2006; Hector et al. 1999; Hector 2011; Naeem et al. 1994; 1996; Naeem and Li 1997; Tilman et al. 2001; 1997; Tilman, Lehman, and Thomson 1997; Tilman 1994) that biodiversity has a direct, positive, and causative impact on ecosystem function through niche complementarity (Tilman, Lehman, and Thomson 1997) and efficiency (Liang et al. 2015). Niche complementarity contributes to positive BEF relationships through increased total resource use, through resource

partitioning, or positive interactions between species (Loreau and Hector 2001). Studies in support of this view draw their data mostly from small-scale controlled experiments on grassland ecosystems. Other authors (Berendse 1998; Cardinale, Ives, and Inchausti 2004; Iii et al. 2000; Grime 1997; 1998; Hooper and Vitousek 1997; Huston 1997; Laakso and Setälä 1999; Mikola and Setälä 1998; Schulze and Mooney 1994; Whittaker 2010) argue that ecosystem function is not necessarily driven by biodiversity *per se*, and that the results to the contrary were confounded by other factors (Huston 1997; Wardle et al. 1997) such as a sampling effect.

Microbial communities are vital and influence all ecosystems on Earth. They play an essential role in - human health and ecosystem sustainability. Synthetic microbes are leveraged in industrial processes as biotechnological tools and are deployed in bioprocess and bioremediation techniques as well. Given the wide range of applications, several methods have described microbial communities and their interactions (Kehe et al. 2019a; Tshikantwa et al. 2018; Zaccaria, Dedrick, and Momeni 2017). The complexity of bacterial interactions and their environmental influences may lead to unpredictable behavior even in simple bacterial communities, making it a challenge to design and study these consortia. Tackling this challenge will require an integration of multiple approaches that may include the development of engineering strategies (Harcombe et al. 2014) and screening experimentally constructed synthetic combinations of strains to identify desired properties of the consortia (Bell et al. 2005; Friedman, Higgins, and Gore 2017). But before working on developing tools to advance in the field of microbial ecology, it is essential to understand the general rules of microbial interactions.

A fundamental goal of community ecology is to comprehend the rules and dynamics of species interactions because of their direct influence on community structure and species biodiversity. Studies in microbial community behavior have become central in the fields of ecology and microbiology (Konopka 2009). Advances in microbial community behavior have important implications for food production, disease control, climate change mitigation, and biodiversity conservation, to name a few (Bodelier 2011; Cavicchioli et al. 2019; Gilbert et al. 2018).

Bacteria are among the most researched microorganisms in microbial community ecology, particularly for the experimental assessments of microbial interactions (Konopka 2009). Nevertheless, we lack consensus on how bacterial species network and control the community structure. Researchers face several critical challenges when studying bacterial interactions: - 1)

defining the spatial and temporal scale at which bacteria interact, 2) quantifying the strength of their interactions, 3) the need to study microbial interactions under anaerobic conditions, and finally, 4) the unpredictable behavior of consortia undergoing rapid evolution and changes in bacterial abundance (Prosser and Martiny 2020; Zhang et al. 2020).

In the following sections, I will focus on studies that attempt to provide insights into BEFR via three key concepts – 1) range of interactions, 2) strength of interactions and 3) community context. Additionally, I will describe the microbiological tools and techniques used in co-cultivation studies.

2.3 Understanding bacterial interactions

Microbial ecology can be made more predictive by applying general rules of microbial interactions to the study of community diversity, functioning, and evolution. Studying interaction range (short and long), strength (weak and strong) and community context (low and high diversity) will improve our understanding of how microbes typically network (Zhang et al. 2020). Range of interaction is the spatial domain in which individuals of the same species can interact with one another or with individuals of different species. The interaction range between bacterial cells is usually limited to their immediate surroundings and is on the order of micrometers. The size of the organisms also determines the smallest range or ecological context over which organisms interact. The intricate spatial scale of the interaction within microbial habitats has a decisive control on the populations they support. Experiments carried out by Dal Co and group (Dal Co et al. 2020) demonstrated that the spatial scale of biotic interaction plays a central role in the ecological dynamics of communities. They worked with two genotypes of *Escherichia coli* that exchange metabolites for growth. They found that bacterial cells only interact with other bacterial cells in the immediate neighborhood. The study highlights how space facilitates coexistence and how short-range interactions govern the functioning of microbial communities.

Studies designed to understand how physical and chemical heterogeneities across space result in the formation of complex microbial communities are difficult because laboratory infrastructure is limited and expensive, and because it is difficult to produce the micro landscapes needed to evaluate combinatorial interactions between bacteria within defined spaces (Hol, Whitesides, and Dekker 2019; Zhang et al. 2020). As a consequence, how microbial consortia interact over the spatial domain remains to be understood. To overcome this barrier, Hol *et al.* (Hol,

Whitesides, and Dekker 2019) demonstrated the use of paper scaffolds as a platform to study bacterial communities in spatially structured environments at relevant microscopic scales. They studied range expansions in branching and non-branching landscapes and explored the island biogeography colonization of bacteria. This research stands as a model in establishing a versatile, low-cost alternative to genetic tools to test ecological concepts in microscale habitats

Theoretical and experimental research suggests that biodiversity and the stability of ecosystems are determined by the strength of species interaction. These interactions are not always positive and do not always lead to an increase in diversity or system stability or productivity. Ratzke and colleagues (Ratzke, Barrere, and Gore 2020) experimented with soil bacteria to show how concentrations of available nutrients affected the strength of interactions between bacteria. They found that increased availability of nutrients supported the ability of soil bacterial species to alter their environment's pH. Changes in pH subsequently resulted in magnifying the strong negative interactions between the soil bacteria, excluding more species from the community. This negative interaction impeded species coexistence, thereby resulting in biodiversity loss. A decrease in system stability caused by the exclusion of species provides a mechanistic link between community context, interaction strength and the overall stability of microbial systems.

An experiment by Scheuerl and colleagues (Scheuerl et al. 2020) demonstrated that community context alters evolutionary responses. The authors showed that low diversity pools of bacterial species growing in rainwater in tree holes had more significant evolutionary responses to environmental changes (low pH) than axenic control cultures. They attributed the strong evolutionary response of the rainwater samples to weaker competitive interactions. They also showed that strong competitive interactions decreased resource degradation, which presumably limits the capacity of bacterial species to evolve (Scheuerl et al. 2020). These results, therefore, highlight that 'community context' is a key predictor of microbial evolutionary dynamics.

Collectively, these independent studies on interaction range, interaction strength and biodiversity improve our understanding of the dynamics of microbial communities. It may be beneficial to test whether the rules that govern bacterial interactions also apply to inter-kingdom interactions (e.g., bacteria with fungi, protists, etc.).

2.4 Technological advances in microbiological tools

Conventional techniques involving pipettes and liquid handlers to construct combinations in multiwell plates are inadequate for combinatorial studies in space because they would require a large number of liquid handling steps and sample replicates (Kehe et al. 2019). It is essential to explore applications that facilitate rapid construction and high throughput screening of microbial interactions beyond binary combinations. Fortunately, alongside the recent exponential-like increase in microbial co-cultivation studies, there has been a steady development in technologies to study microbial interactions in co-cultivation studies.

2.4.1 Microfluidic devices

Tools like microfluidic devices have the potential to assist researchers who wish to study combinatorial studies in spatially structured habitats. Microfluidic devices are designed using computer-aided design software and constructed by moulding, engraving or micromachining materials like – ceramics, silicones or acrylic glasses. These devices handle fluid interfaces at millimeter (mm) to nanometer (nm) scales and are also referred to as 'Lab-on-a-chip'. The advantages of using microfluidic devices are that they allow for miniaturizing and permitting single scale and parallel experiments; these features are potentially useful to those who wish to carry out combinatorial studies of microbes. Different approaches to using microfluidics such as gel microdroplets, microfluidic chips, capillary flow, porous metallic membranes, etc., vary in terms of scale and construction but share a common functional characteristic. In all, the microbes are physically trapped, and nutrients are provided via diffusion across a trapping barrier or via direct channels. Optical transparency of the materials further facilitates observations under a microscope.

Keymer and group (Keymer et al. 2006) constructed a linear array of coupled microscale chambers connected by corridors to mimic microscale patches of habitat. Upon inoculating *E. coli* cultures into the device, they observed the emergence of metapopulations. The study highlighted how biochemical interactions between bacterial cells modulate spatiotemporal population dynamics. Their result was reinforced in a study of physically separated but chemically coupled populations by Park *et al.* (Park et al. 2003) who developed a microfluidic maze to mimic structured habitats to study *E. coli* cultures. They found that bacterial populations organized

themselves into complex patterns despite the lack of patchiness in the habitat in which they were allowed to grow. The formation of the patterns was due to nutrient depletion within the closed system and not a result of colonization and extinction events, as observed in metapopulations. In conclusion, these experiments show the potential use of microfluidic devices in testing metapopulation theories. In other investigations conducted by Hol *et al*. (Hol et al. 2013; 2016) and Seymour et al. (Seymour, Marcos, and Stocker 2009), microfluidics was used as a tool to study interspecies interactions in spatially structured habitats. Hesselman and colleagues (Hesselman et al. 2012) constructed a two-compartment reusable device separated by micro-sieves to review interkingdom interactions between *E. coli* and *C. elegans*. A suspended microfluidic platform designed by Casavant *et al.* (2013) was useful in investigating chemotaxis in eukaryotic cells.

Bacterial populations often form dense, surface-associated communities called biofilms in response to stress. Microfluidics is being used to study cell-cell interactions of biofilms in a controlled manner. Research into the biochemical signals that regulate biofilm production will have important implications for understanding bacterial infections. Kim *et al.* (Kim et al. 2008) showed how high throughput microfluidic devices assist in screening compounds that promote or inhibit the formation of pathogenic bacterial biofilms.

2.4.2 Encapsulation techniques

Encapsulation technology is a distinct form of microfluidics where droplets form using a continuous and dispersed phase (e.g., water-oil-water). Microcompartments are generated by microdroplets which confine cells within aqueous, polymer or gel-based emulsions. These microdroplets are semi-permeable and allow diffusion of molecules. Culturing of axenic and mixed cultures is permitted by encapsulating cells in microdroplets that are physically isolated but chemically connected (Nai and Meyer 2018). Droplet-based approaches showed to be useful in probing the effect of community size on the composition of microbes (Cao, Hafermann, and Köhler 2017; Park et al. 2011). Park and group (Park et al. 2011) found that encapsulation proved useful in exploring synergistic effects in bipartite and tripartite assays of microbes. Kehe and colleagues (Kehe et al. 2019) used kChip, a droplets-based platform to screen synthetic microbial communities. The authors claim that the kChip platform will be useful in screening the functional capabilities of microbial consortia beneficial in applied microbial ecology, biofuel production, or

environmental remediation. SlipChip is a mode of encapsulating where cells grow in thousands of microcompartments split into two wells. It can be used to produce thousands of miniaturized reactions without the need for bulky equipment. The splitting protocol allows single-cell inoculation and replica plating simultaneously and could potentially be useful for co-cultivation experiments (Ma et al. 2014).

2.4.3 3D printing

Connell and colleagues (Connell et al. 2013) described a strategy for 3D printing bacterial communities to gain insight into how geometry influences bacterial pathogenicity. Using this approach, they showed that the resistance of a single pathogenic species to the treatment of antibiotics enhanced the resistance of the adjacent species. This micro-3D printing strategy builds different geometries with micrometer resolution to create complex microbial communities. They used bacterial microcolonies organized under dynamic orientations to understand social behaviors such as resource competition, resilience to stress, and symbiosis. This versatile fabrication technique can be used to explore the intricate mechanisms that permit bacteria to acclimatize and thrive in heterogeneous environments in nature. The major drawback associated with this micro 3D-printing method was the relatively high price of the specialized apparatus. Besides, it is not well equipped for high-throughput analyses.

The flexibility of designs and the ability to control small populations in microenvironments make microfluidics a potent platform to study bacterial dynamics, but it has high operating costs, its protocols are expensive to standardize, it is difficult to scale up, and it requires peripheral lab equipment such as external pumps, tubing and connectors, which add complexity and expense. To overcome the above-mentioned disadvantages, my research was focused on developing a simple, paper-based platform to study microbial interactions. The following sections describe the methodology of the research and the results.

3. MATERIALS AND METHODS

3.1 Identification and selection of bacteria of study

To study bacterial interactions, three closely related species were selected among the lactic acid bacteria (LAB) as a model community - *Lactobacillus brevis (L. brevis), Lactococcus lactis cremoris (L. cremoris),* and *Pediococcus pentosaceus (P. pentosaceus).* The reasons to choose LAB as a model community were: 1) these cultures are easily grown under optimized laboratory conditions, 2) LAB are ubiquitous bacterial species (e.g. they are found in soil, food, human gastrointestinal tract) (Ruiz Rodríguez et al. 2019), 3) LAB are dynamic in their interactions with other domains of life (George et al. 2018; Morgan, Darling, and Eisen 2010).

3.2 Fabrication of stamps

Triangular molds were designed, and 3D printed using Autodesk® Fusion 360™ software and RAISE3D Pro2 Plus (Raise 3D Technologies, Inc., Irvine, CA) respectively. Two molds were designed, and 3D printed – a triangular hollow frame and a negative stamp mold. To fabricate the stamps, the triangular hollow frame was placed on top of the negative mold and sealed using Dragon Skin™ 10 FAST silicone rubber. Dragon Skin™ 30 silicones were mixed in equal ratios (1A:1B by volume of approximately 100 mL each), degassed under vacuum to remove air bubbles and poured onto the stamp mold. The rubber was allowed to cure in room temperature for approximately 10 hrs. Before pouring the Dragon Skin™ 30 silicone mixture, the sealed molds were sprayed with Ease ReleaseTM 200 release agent. The stamps were removed from the molds upon curing and autoclaved at 121℃. Figure 3.1 shows a schematic representation of how the stamps were constructed using the molds.

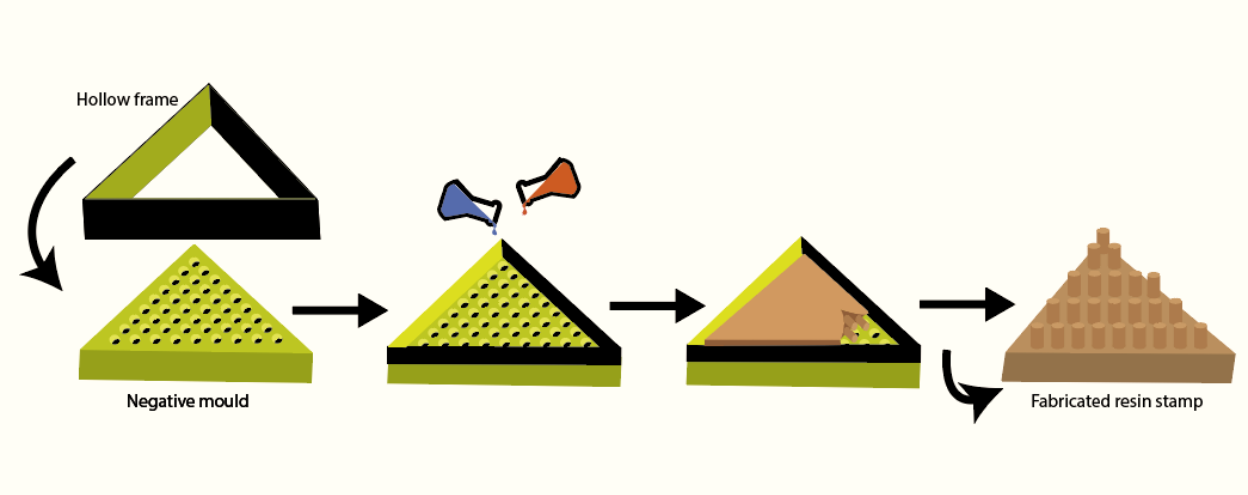


Figure 3.1 Fabrication of stamp. To fabricate a resin stamp, a hollow frame was placed on top of a 3D printed mold and tightly sealed with Dragon Skin™ 10 FAST silicone rubber. Equal ratios of Dragon Skin™ 30 silicones were poured after degassing in a vacuum. The silicone mixture was allowed to solidify at room temperature and peeled to get the stamp

3.3 Spatial and compositional control of bacteria in the study

The shape of the negative mold of the stamp was an equilateral triangle to accommodate interaction studies of three bacterial species of interest. The length of the side of the triangle is 9.5 cm, and the total surface area of this equilateral triangle is 39.08 cm^2. Within the triangular framework were circular wells of depth 1.5 cm and diameter of 0.5 cm. The distance between adjacent circular wells in the triangular stamp was 0.5 cm. Using a diameter and distance of 0.5 cm helped in accommodating four stamps onto a square agar plate (which allows us to test four different conditions simultaneously). The diameter of circular wells determined how much bacterial suspension was transferred onto the nutritional medium and the distance between circular wells controlled the spatial distribution of the transferred bacteria. Once the resin was poured and cured into the 3D printed negative mold, the result was a triangular stamp with fifty-five pegs protruding outwards. The height of each peg was 1.5 cm, each peg had a diameter of 0.5 cm, and the distance between each of the fifty-five pegs was 0.5 cm. Future designs can be modified by varying the distance between the pegs and changing the diameter of the pegs to study bacteria interactions at different spatial and compositional scales.

I used paper to control the composition and spatial distribution of bacteria in the study. The bacterial solutions added at the edges were carried towards the center of the paper by capillary

action. Paper stratification permitted conjunctional studies of bacteria in mixed cultures. The stamping technique (Fig 3.2) allowed the easy and effective transfer of bacteria onto a nutritional agar medium and further facilitated spatial control on a millimeter scale. Using the same stamp for a series of controlled replicates ensured that variability from stamp to stamp could be removed as a random effect, but the stamps had to be sterilized at the beginning of each experiment. Since silicone-based resin stamps can withstand high temperatures without losing their structural integrity, these stamps were autoclaved repeatedly between experiments to avoid any issue of contamination

3.4 Inoculation of bacteria using paper and culture media

Lactic acid bacterial KWIK STIK™ strains of *Lactobacillus brevis* (ATCC 14869), *Lactococcus lactis cremoris* (ATCC 393) and *Pediococcus pentosaceus* (ATCC 33316) were purchased from Microbiologics® (Microbiologics, Inc., St Cloud, MN) and were grown overnight in De Man, Rogosa and Sharpe (MRS; CRITERION™, Hardy Diagnostics, Santa Maria, CA) selective culture medium at room temperature. Once they were grown, the monoculture bacterial solutions were normalized to an optical density (OD_{600} ~1) by diluting them with phosphate-buffered saline (PBS) solution (1X with pH of 7.2). Eight serial log dilutions of the three bacterial solutions (1 mL) were carried out in individual test tubes (15 ml, Falcon) containing 9 mL PBS with dilutions ranging from 1:10 to $1:10^8$.

Whatman® qualitative filter paper, Grade 1 (180 µm thick) was cut in a triangular design using a Cricut Maker (Cricut, Inc., South Jordan, UT). Three Whatman Grade 1 filter paper triangles were sandwiched between two transparency sheets so that one of the edges of each paper protruded out (Fig.3.2). To each of these sandwiched filter papers, 600 µL of the prepared bacterial solution of *L brevis* (in PBS with OD600 ~ 1) was added to the vertex (Fig 3.2). The bacterial solution of *L. brevis* was allowed to spread uniformly on the paper under capillary action. Once spread uniformly, each of these three filter papers was stacked and pressed (using a roller) onto another Whatman Grade 1 filter paper (coated with agar). They were pressed along the three vertices individually using a PlateSeal™ Roller (Thomas Scientific, Inc., Swedesboro, NJ) to obtain a uniform monoculture spread of *L. brevis*. The same procedure was used for *L. cremoris* and *P. pentosaceus* bacterial solutions, respectively. Subsequently, to another set of three Grade 1 filter paper triangles, also sandwiched between the transparency sheets, 1 mL of *L. brevis*,

bacterial solution (OD_{600}~1) was added to each of the three vertices of the filter paper individually. Each of the grade 1 filter papers soaked with the bacterial solution was stacked and pressed using a roller onto a single agar coated paper across each of the three vertices separately. The filter paper triangles were coated with agar by dipping them in MRS-agar solution and allowing the coated papers to cool. The fabricated silicone rubber stamp was first stamped onto the agar coated paper and then onto the MRS-Agar medium (55 g of MRS and 15 g of agar in 1L of de-ionized water, autoclaved at 121℃). Subsequently, 2 µL of each of the eight serially logged dilutions of the three lactic acid bacterial solutions were added onto the MRS-Agar medium to serve as controls for the stamped monocultures. The method of producing monospecific and mixed-species stamps was similar (Fig. 3.2). The bacterial stamps after growth on the MRS-Agar medium represent three monocultures (*L. brevis*, *L. cremoris* and *P. pentosaceus*) and a mixed culture of the three lactic acid bacteria (Fig 3.3)

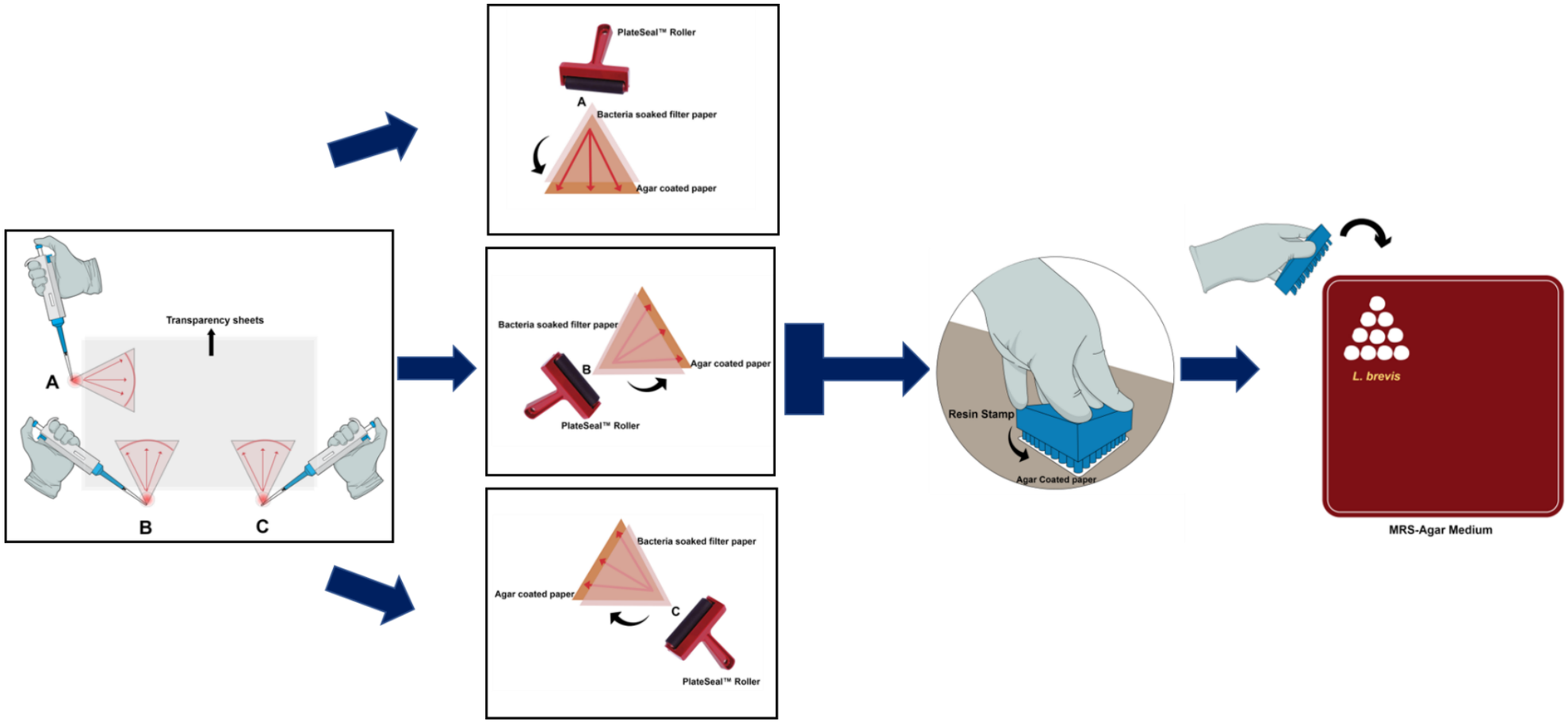


Figure 3.2 Using paper and stamp to pattern bacteria

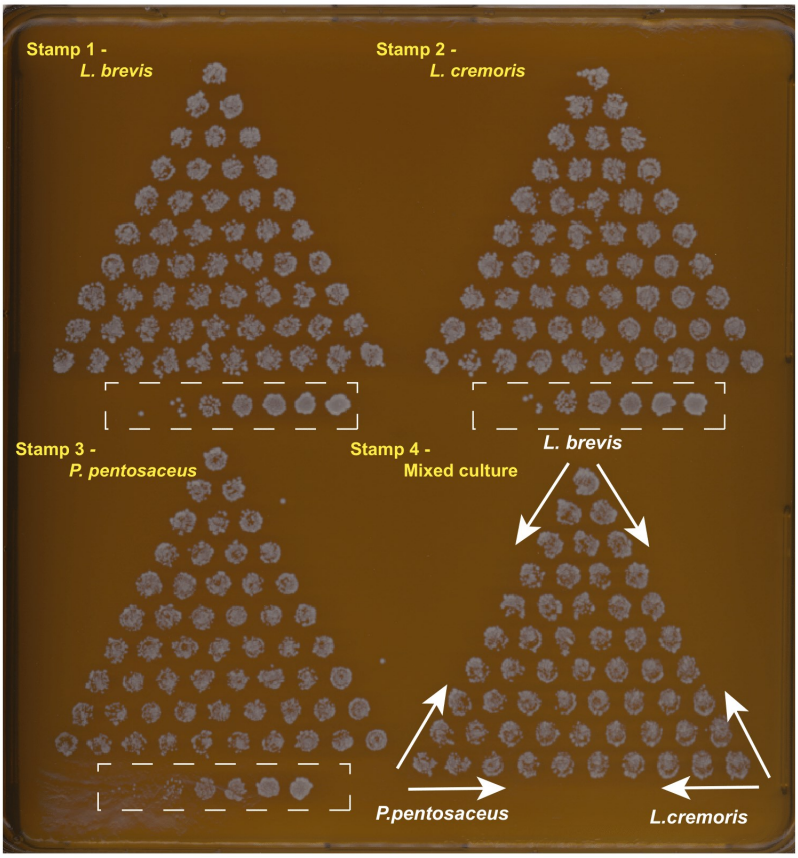


Figure 3.3 Scanned image of all the stamped monocultures and mixed culture. The boxed regions represent growth of individual monocultures at serial dilutions in the increasing ratio of 1:10 to $1:10^8$.

3.5 Troubleshooting the experimental design

Prior to using a 3D designed resin stamp, transfer of bacteria onto a solid medium was carried out using transparency sheets as a template. The transparency sheets were cut in a triangular design with holes of 0.5 cm diameter within the geometric framework. The circular holes were spaced by 0.5 cm. (Figure A.1). The triangular transparency sheets were sterilized with 70 % alcohol prior to placing them onto the MRS-agar medium. The filter grade papers soaked with bacteria were then placed onto the transparency sheets and pressed with a roller. Bacterial growth was observed in regions where there was a contact of the paper with the nutrient medium. This experimental design was inefficient because poor contact between the paper and the solid agar medium resulted in little or no bacterial growth. Further, pressing the paper by using a PlateSeal™ Roller onto the transparency sheet allowed the bacterial solutions to spread and grow inconsistently across the MRS-agar medium. A comparison study using resin stamps and a transparency sheet was carried

out and evaluated based on the bacterial printing and growth (Figure A.2). The disadvantages of using transparency sheets were overcome with the use of a 3D designed stamp.

The second challenge of the design was the use of filter paper as the medium to transfer and pattern bacteria. Transfer of the bacteria was limited by the wicking capability of the grade 1 filter paper which produced non-uniform colony growth. This challenge was overcome by dipping the paper in agar, giving it an even coating (Figure A.3). The soft bedding of agar encouraged transfer and adherence of the bacteria. Paper versus agar coated paper were compared based on bacterial growth (Figure A.4). The agar coated paper (Figure A.5) was enclosed in a box and refrigerated at 4 °C to prevent drying.

3.6 Data acquisition and image analysis

To measure bacterial growth, the Square Bioassay Dishes (Corning™, Corning, NY) containing the MRS-Agar medium onto which the bacterial had been stamped were inverted onto a flatbed scanner (Epson Perfection V800 Photo Color Scanner) and scanned every 15 mins using VueScan (Hamrick software) (Table A.6.). The images were scanned at 1200 dpi in the flatbed mode, and the images were saved as TIFF with 48 bits RGB. The bacterial colonies on the resulting images were individually identified using custom code in Python that created a GUI (Graphic User Interface) where image files could be uploaded and analyzed. The raw image of growth at the final time point (t ~ 168 hours) was first denoised using the scanned image at t=0 as a reference to remove the background (nutrient-agar). The denoising step was essential to exclude any values that might be extracted from the color of the nutrient medium. The python code recognized controls (monocultures) and the mixed culture as individual stamps. Within each stamp, the fifty-five growing colonies were identified by setting a circular boundary around them. Once the boundary constraints were defined, the average intensities of the colonies were derived as grey values using the RGB color system. These average intensities were then normalized (rescaled to the range between 0 to1) for each stamp using the formula –

$$X_{norm} = \frac{X - X_{min}}{X_{max} - X_{min}}$$

where X_{norm} = normalized value

X = original mean intensity value

X_{min} = minimum mean intensity value in the data

X_{max} = maximum mean intensity value in the data

From the images, cartesian co-ordinates of each circular grown sites across the four stamps were identified, and distance was measured from the centroid of the triangular stamp. Distance between two points was calculated using the formula:

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

where d = distance

(x_1, y_1) = co-ordinates of the first point

(x_2, y_2) = co-ordinates of the second point

Distances were calculated from the cartesian co-ordinates for the bacterial colonies separately for each of the four stamps. Normalized mean intensities derived from the images were used to represent bacterial biomass.

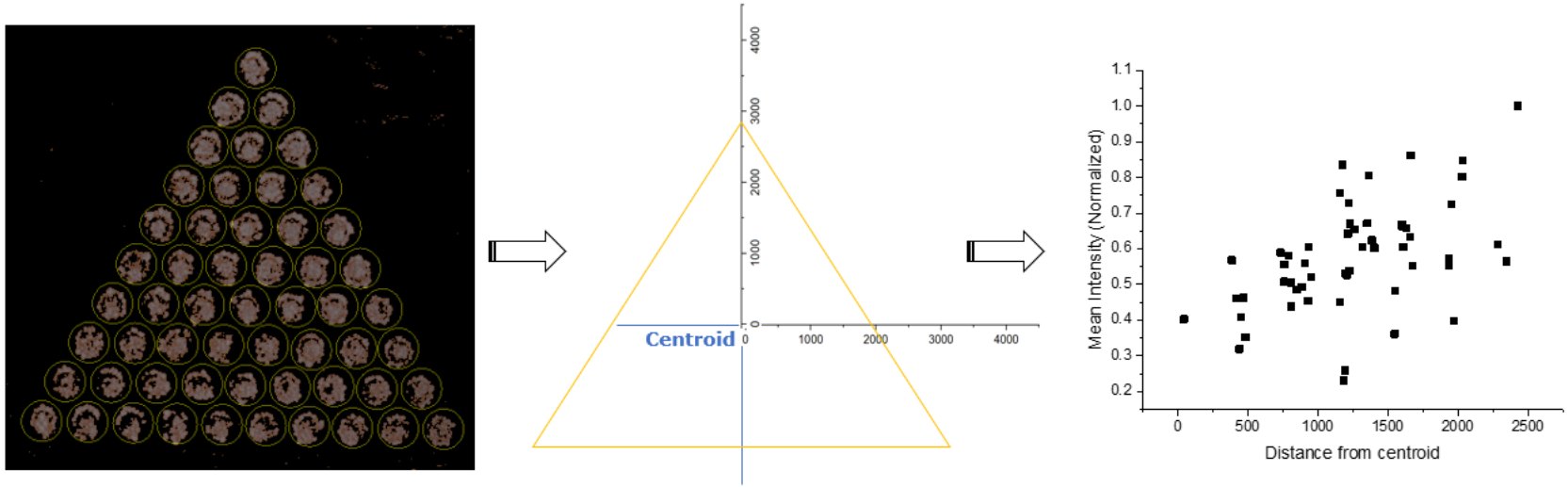


Figure 3. 4 Image analysis of the stamped region. The denoised image represents a single stamp. The individual colonies within the denoised image were identified. Using the python code, the co-ordinates, and the grey mean intensities of the identified colonies were extracted. Using the cartesian co-ordinates, the distances from the centroid were calculated for each colony growth and plotted against their normalized mean intensity value.

3.7 Hill Number

The Hill number is expressed as -

$$^{q}D \equiv \left(\sum_{i=1}^{S} p_i^{q}\right)^{1/(1-q)}$$

for i from 1 to S, where S is the number of species, p_i is the proportion of species i and q is the Hill order. The proportion of species is the relative abundance of species, and the hill order (q) defines the sensitivity of the diversity value to either rare or abundant species by modifying how the weighted mean of the species proportional abundances is obtained (Jost 2006).

The initial diversity (effective number of species) in the mixed culture (stamp 4) can be predicted by calculating Hill number at the individual fifty-five circular sites of the stamped region. The hill number for each of these circular sites is represented using Shannon's entropy index (x) which is expressed as –

$$x \equiv -\sum_{i=1}^{S} p_i \ln p_i$$

where p_i represents the probability of the effective number of species (i = 1 to 3) at the n^{th} circular site of the mixed culture (stamp 4) where n = 1 to 55. Using the controls (e.g., stamp 1 when only hydrated from one spot), we can predict which of the 55 circular sites will receive *L. brevis* (depending on how far the liquid spreads on the triangle) in the mixed culture, i.e., in stamp 4. Similarly, we can estimate which of the fifty-five circular sites will receive *L. cremoris* and *P. pentosaceus* in the mixed culture based on stamp 2 and stamp 3, respectively. Not all the circular sites in the mixed culture (stamp 4) will receive all species. Using this method, we determined the effective number of species at the p_1, p_2 and $p_3$values for each of the 55 circles by taking the exponential of Shannon's entropy index values, i.e., exp(x). By definition, circular sites with only one species (e.g., at the vertices) had a Shannon-Index of 0. Circular sites with two species and three species had a Shannon-Index of 0.69 and 1.09 respectively.

3.8 Statistical analysis

The data generated from all the technical and biological replicates were used to carry out a linear regression analysis, and the r^2 and p-value were used to analyze the data.

4. RESULTS AND DISCUSSION

Images that were taken at 1200 dpi (dots per inch), 48 bits RGB with a flatbed scanner enabled high-resolution visualization of growing colonies on the solid nutritional agar medium. Since the bacterial growth estimation was captured using image processing software, the accuracy of the measurements depended directly on the count of available data points. Images stored as TIFF files retain more PPI (pixels per inch) than other file formats and are better than other formats for maintaining the integrity and clarity of the scanned images. In the experimental system I describe here, the growth of more than one bacterial species was monitored in a single image. RGB (Red Green Blue), CMYK (Cyan Magenta Yellow blacK) and HSI (Hue Saturation Intensity) color models were also explored for processing the images and extracting bacterial growth information. Because the chromatic characteristics of the bacteria I grew were close to white, the RGB color model was chosen to extract mean intensity values.

Bacterial biomass (used as a surrogate for ecosystem function) was measured as the average grey intensity value extracted at the final time point of growth for all four stamped bacteria. The experiment was designed so that when mixed cultures were stamped together, the central region of the stamp should have the highest biodiversity and the vertices have the least biodiversity. Therefore, drawing from the hypothesis, we should observe greater biomass (function) at the center of the stamped mixed culture where biodiversity was greatest.

The normalized mean intensity values were plotted against distance from the centroid of the triangular stamp (Fig.3.5). Two technical replicates were carried out within each day, and three biological replicates were carried out over three different days. Looking at the scatter plots (see Fig 4.1 – Panels [a-c]) of the monocultures – *L. brevis*, *L. cremoris* and *P. pentosaceus*, there was a positive relationship between the mean intensities and the distance from the centroid of the triangular frameworks of growth. As the distance from the centroid increased, the mean intensity values increased. This positive correlation indicated greater growth (biomass) at the edges than in the central region. The same positive correlation was observed for the mixed culture. This observation appears to contradict my hypothesis. Linear regression analysis (Fig.4.2) carried out on all six replicates (two technical replicates and three biological replicates) for all the four stamped bacterial cultures showed that the slope of the regression was significantly different from zero at the 0.05 level, indicating a positive correlation between the dependent and independent

variables - *L. brevis* (r^2=0.050, p<0.001, y=7.4798E-5 ± 1.74377E-5x), *L. cremoris* (r^2=0.072, p<0.001, y= 8.72027E-5 ± 1.68885E-5x), *P. pentosaceus* (r^2=0.036, p<0.001, y= 6.6442E-5 ± 1.83336E-5x and mixed culture (r^2=0.047, p<0.001, y= 7.28666E-5 ± 1.76256E-5x). The low r^2 values showed that the data points were widely scattered about the mean values and that diversity had little influence over colony intensity in these experiments.

The considerable variation in mean intensity within a stamp and apparent randomness in the distribution of the data can be attributed to the physical nature of the stamp. The surface roughness varied across each peg of the stamp, which affected the transfer of bacteria from the agar-coated paper to the nutritional- agar medium. The result was an inconsistent and unreliable transfer of bacteria and high within-experiment variability in bacterial growth across individual circular regions. While the roughness of certain pegs allowed better adherence of bacteria, certain surfaces of the pegs which were smooth failed to adhere to bacteria efficiently, thereby resulting in non-uniform circular growth patterns across each stamp. The issue of uneven adherence of bacteria to the pegs of the stamp was tackled by designing a 3D stamp with uniform surface roughness. Experiments performed using the new stamp seemed to show that variability in bacterial adherence to the pegs was reduced, and consistency in the intensity of the colonies spotted by each of the pegs was improved (Fig 4.3).

If there were an effect of biodiversity on colony intensity, we would have observed a trend in the mixed culture where higher mean intensity values (bacterial growth) should have been observed at the center as opposed to the peripheral regions of the stamp. This would mean that as the distance from the centroid increases, the mean intensity values would dip. What we observed instead was a positive correlation between mean intensities and the distance from the centroid. This counterintuitive outcome might have been a function of the experimental design and not the effect of biodiversity. Since the experimental protocol involved loading the bacterial solutions at the edges of the filter paper, only a few of the bacterial cells seemed to traverse to the central region (areas around the centroid of the stamp). The effect of the design can be observed in the controls (monocultures) where there was a greater growth at the edges than in the region around the centroid of the stamp (Fig 4.1 a-c).

A positive BEFR is usually explained as a consequence of complementarity or sampling effects (Loreau et al. 2001; Loreau, Mouquet, and Gonzalez 2003; Loreau and Hector 2001) and the existing data suggest that a combination of complementarity and sampling is probable to be

operational (Cardinale et al. 2007). Complementarity denotes to increased resource consumption through a positive interaction or segmentation. It proposes that biodiversity affects ecosystem function by means of the ecological interactions among species. These interactions can either be positive (i.e., facilitation/cooperation) or negative (i.e., interference/competition). Sampling effect denotes to the effect of dominance by certain species with specific traits on ecosystem processes. Therefore, the ecological capacitance of species – e.g. good or bad competitors, low or high producers, etc. drive the function of the system. Variation in how species predominate might result from how species densities vary (i.e., population dynamics) and/or variation in the rates of species contribution to the functioning of the ecosystem (Venail and Vives 2013). The experimental design implemented here curtailed changes in the relative abundance of each species with time but did not eliminate the possibility for sampling effects through differences in metabolic rates. Thus, in this experiment, we cannot rule out that sampling effects could have masked complementarity effects.

The current design of the experiment helps in predicting regions of initial diversity in the mixed culture. Diversity in this experiment was represented using Shannon's entropy index. Hill numbers offer a general statistical framework that is amply robust to address scientific questions that researchers often try to answer via measurement, estimation, and comparison of diversities (Chao, Chiu, and Jost 2010; Jost 2006; Tuomisto 2010). Higher values of Shannon's entropy index indicate greater diversity of the bacterial species at the individual sites of the stamp. In the future, this framework of "mean intensity vs Hill number" can be used to predict both the biomass and biodiversity of species in a mixed culture environment (Fig 4.4).

The BEF relationship has implications for the function of natural systems, human health, and industrial processes. Future research using the system I describe could be productively directed at experiments with a lower concentration of bacteria (i.e., fewer bacterial cells) to observe the effect of an increased number of different types of species on their biomass production. Additionally, the relationship between the biodiversity of microbial communities and their function can be tested by measuring the resilience of communities to external perturbations. These external perturbations can be introduced by changing, for example, the nutritional availability of the growth medium or by changing the pH of the solid nutritional medium. The current experimental protocol is for image acquisition at regular intervals of time. This method of data

collection should enable a time-dependent analysis in which growth rates of different bacteria can be observed and measured.

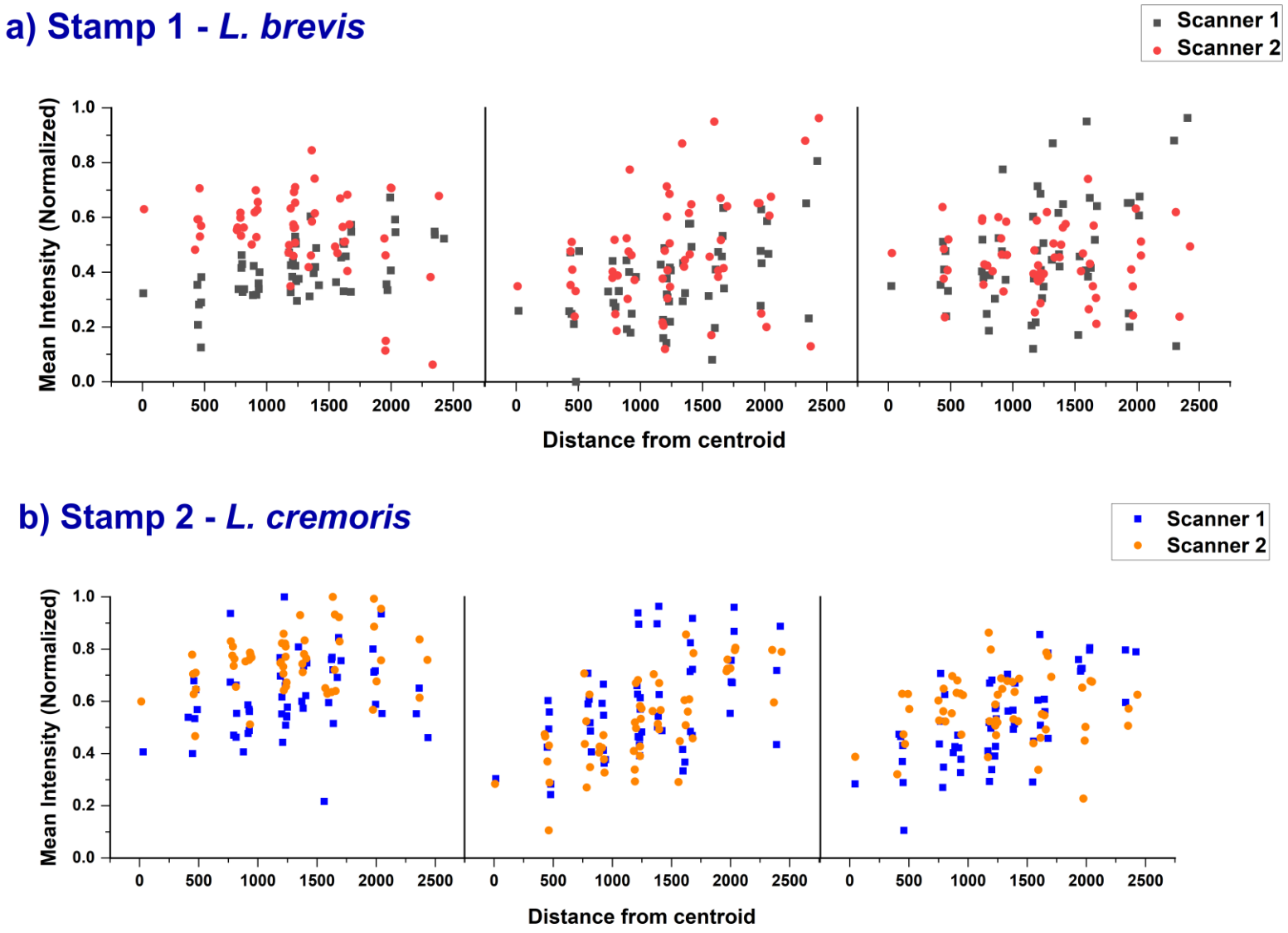
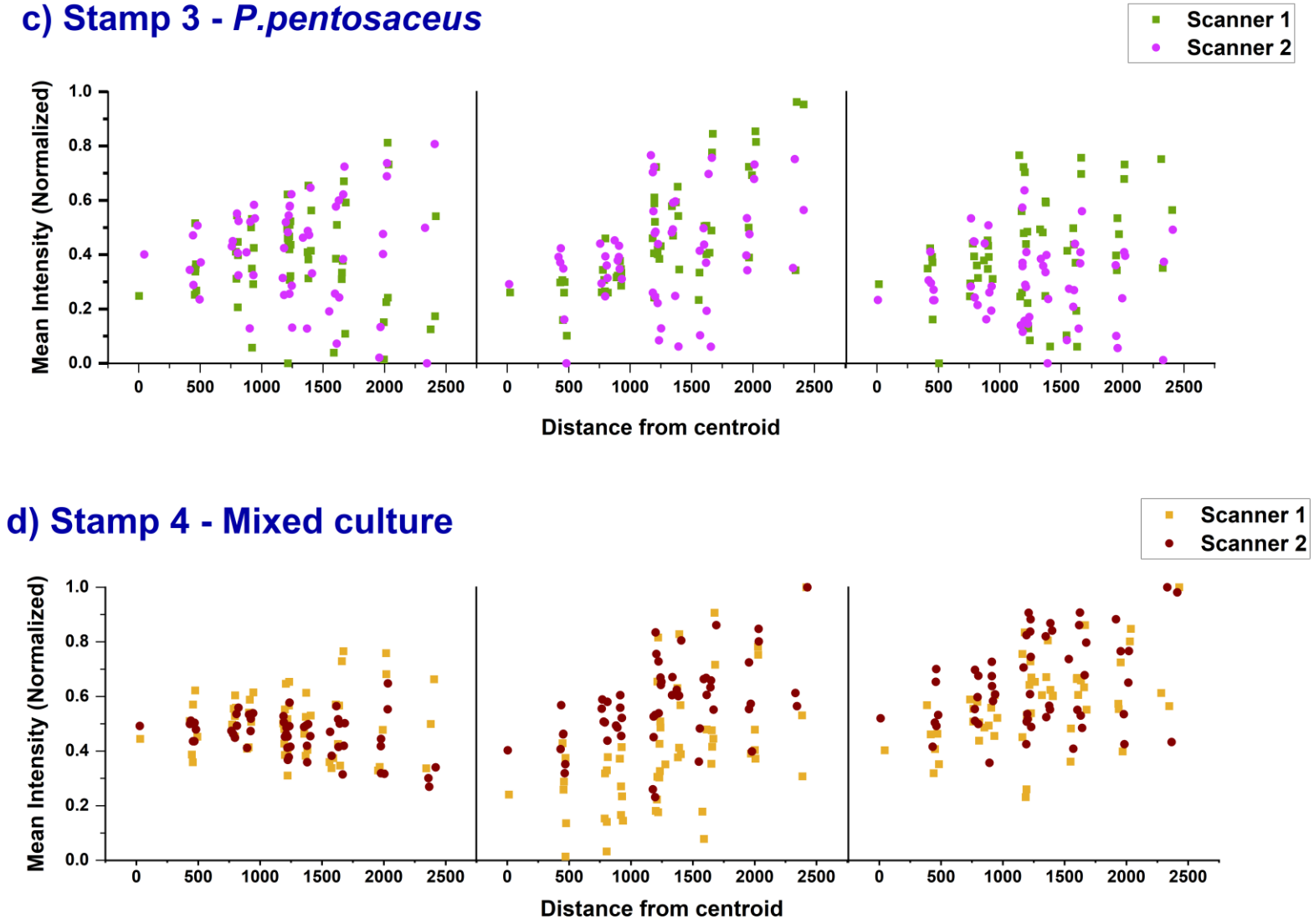


Figure 4.1 Graphs of normalized mean intensity versus the distance from the centroid of the four triangular stamps.

Figure 4.1 continued



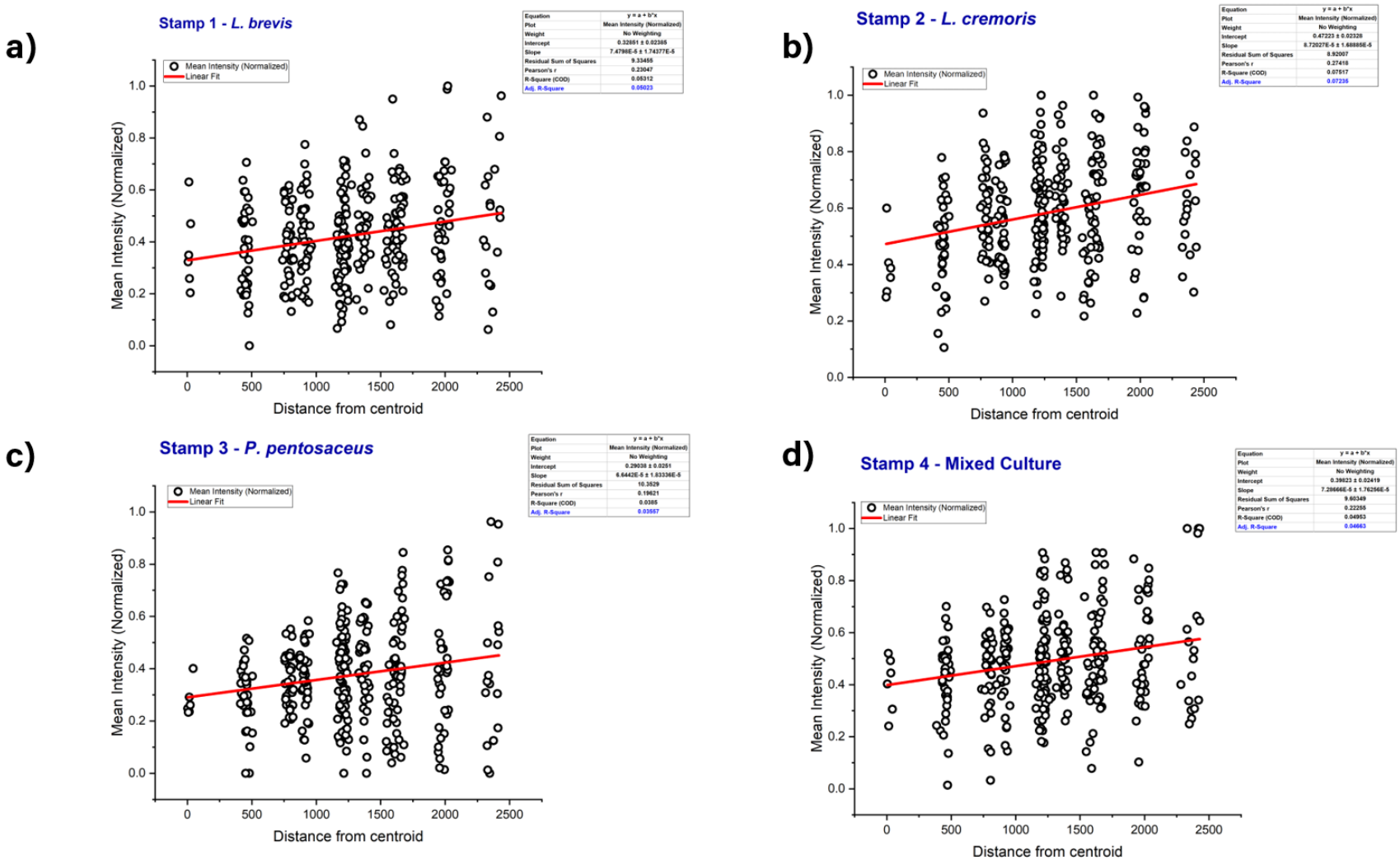


Figure 4.2 Linear fit of all replicates across all the four panels showed that slope is significantly different from zero at the 0.05 level, indicating a positive correlation between the dependent and independent variables. a) For *L. brevis*: r^2=0.050, p<0.001, y=7.4798E-5 ± 1.74377E-5x. b) For *L. cremoris*: r^2=0.072, p<0.001, y=8.72027E-5 ± 1.68885E-5x. c) For *P. pentosaceus*: r^2=0.036, p<0.001, y=6.6442E-5 ± 1.83336E-5x d) For mixed culture: r^2=0.047, p<0.001, y=7.28666E-5 ± 1.76256E-5x

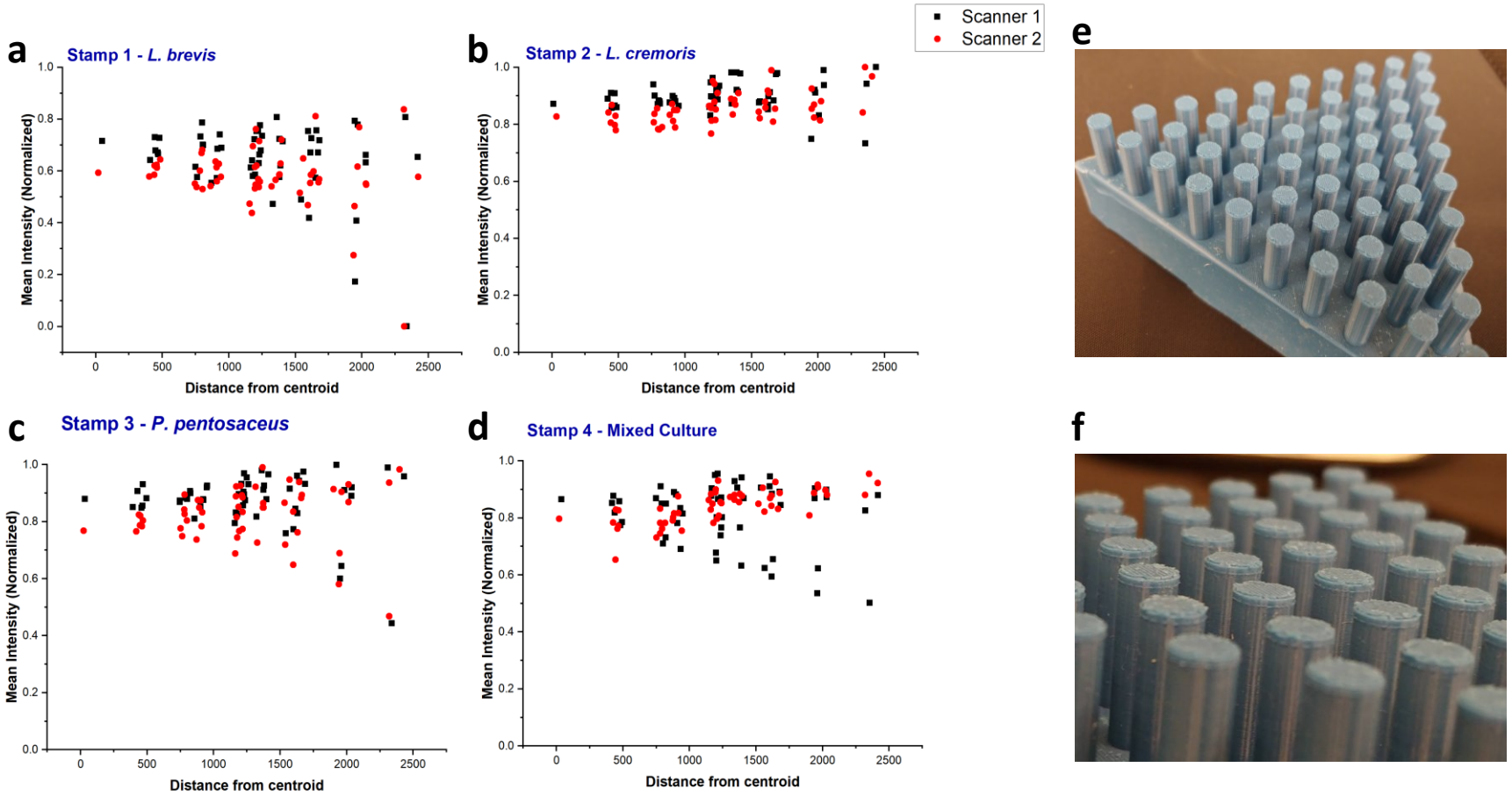


Figure 4.3 Panels (a-d) – Graphs of mean intensity vs distance from centroid of the stamps. The data points represented show mean intensity values for technical replicates carried out using an improved stamp. The design of the stamp contributed to a uniform distribution in datapoints. Panels (e & f) show the new stamp and a zoomed view of the peg surfaces.

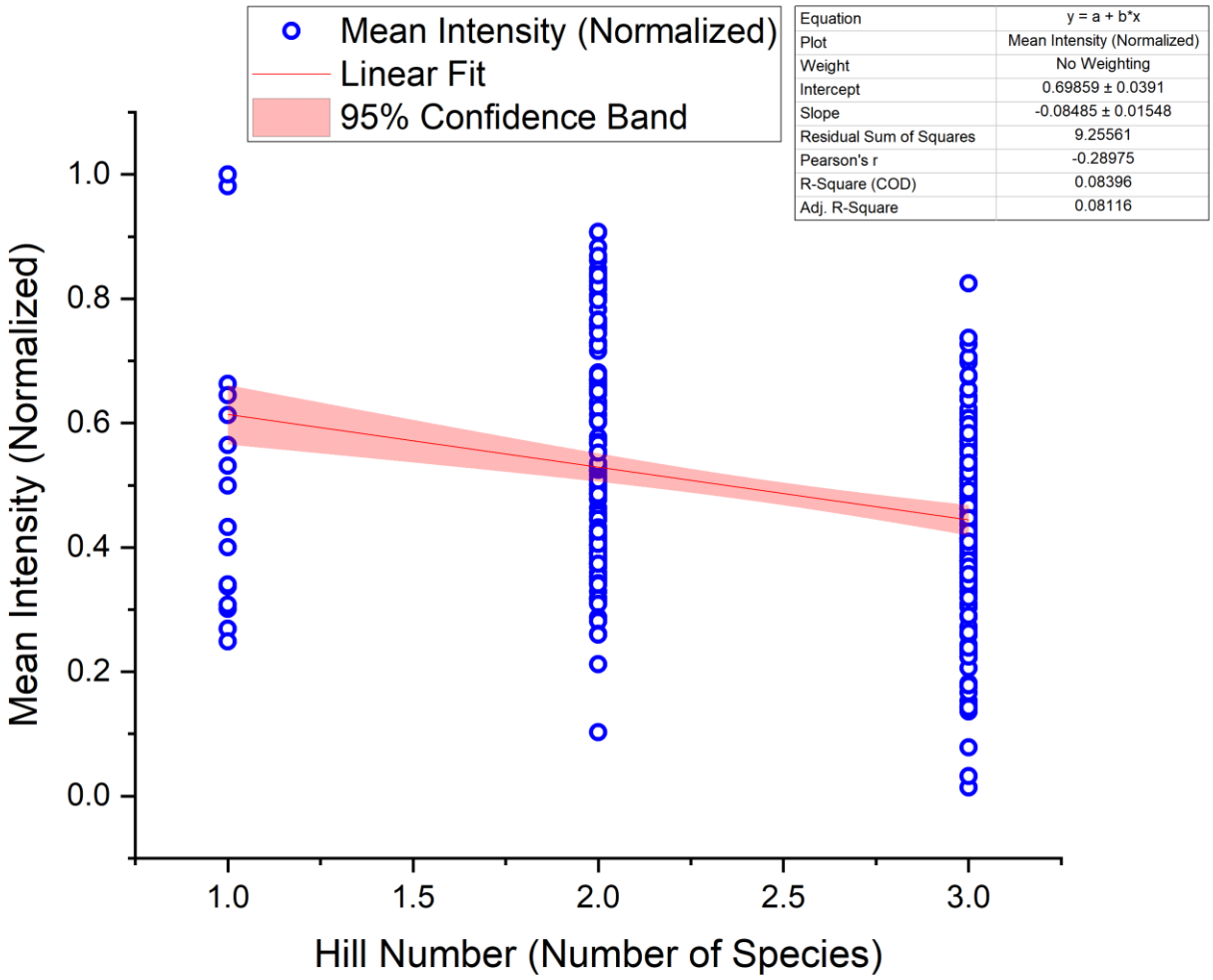


Figure 4.4 Graph of mean intensity vs hill number represented by Shannon's entropy index. 95% CI [0.42, 0.66]

5. CONCLUSIONS AND FUTURE WORK

In this work, I developed and standardized a simple platform to study bacterial interactions through 1) spatial control, 2) compositional control. The ease with which paper can be patterned and stacked and the ease with which resin stamps can be designed and 3D printed reduces the cost and difficulty of studies to understand spatially defined microbial interactions. Paper and resin stamps can be cut and redesigned to variable geometric shapes to accommodate an increasing number and types of bacterial interactions. This humbler version of microfluidics provides a low-cost, easy-to-use alternative to test the theories of bacterial ecology.

A major limitation of this platform, as described, is its unsuitability for the study of interactions between motile microbes. By fluorescently tagging the bacteria of interest, however, and performing the image analysis as described, this limitation might be overcome. Fluorescent labels with different fluorophores could be used to track the location and number of interacting bacterial species.

The effects of environmental attributes on microbial communities and their interactions can be tested using the paper and stamp system described here. Perturbations of factors such as – 1) nutritional availability (changing the concentration of the growth medium), 2) the pH of the growth medium, 3) temperatures during incubation periods, etc., are likely to have a dramatic effect on community behavior and BEFR. The distribution of bacteria in mixed cultures can be confirmed using qPCR analysis. Image analysis of interactions can be improved by using species of bacteria that inherently exhibit color or by fluorescently tagging them. The usefulness of the platform might also be extended through the study of trans-kingdom interactions (e.g., bacteria and fungi).

APPENDIX. A

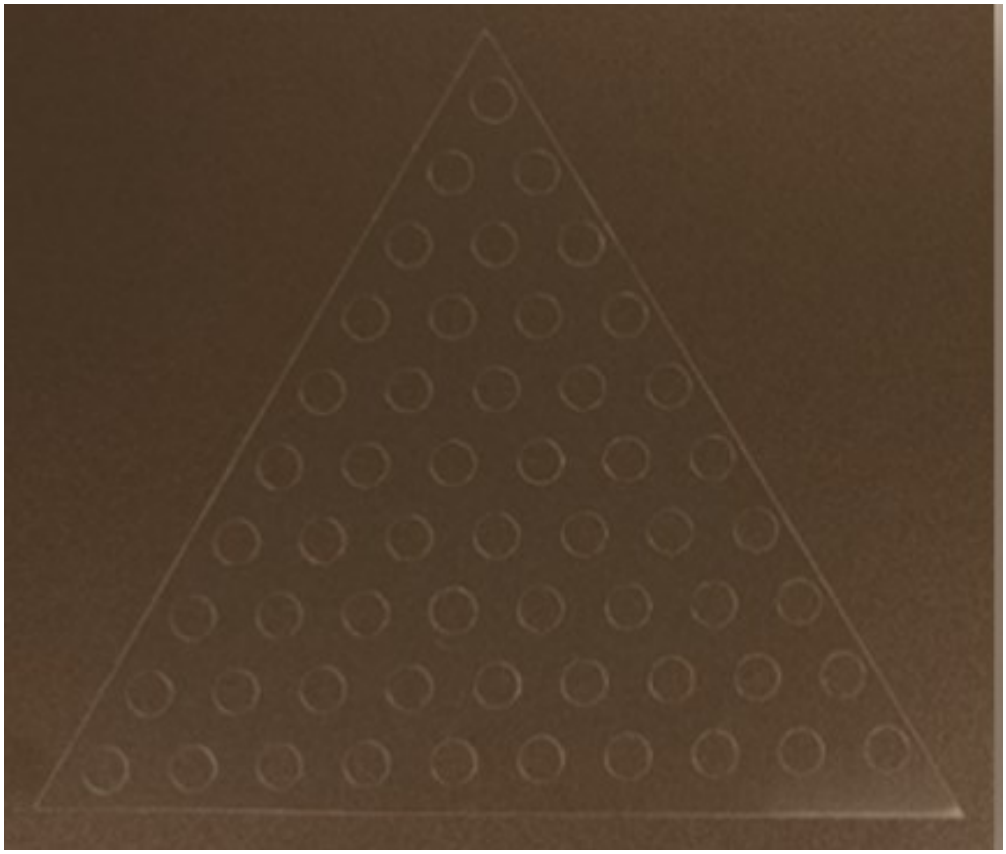


Figure A.1 Transparency sheet cut, designed, sterilized and placed on MRS-agar medium.

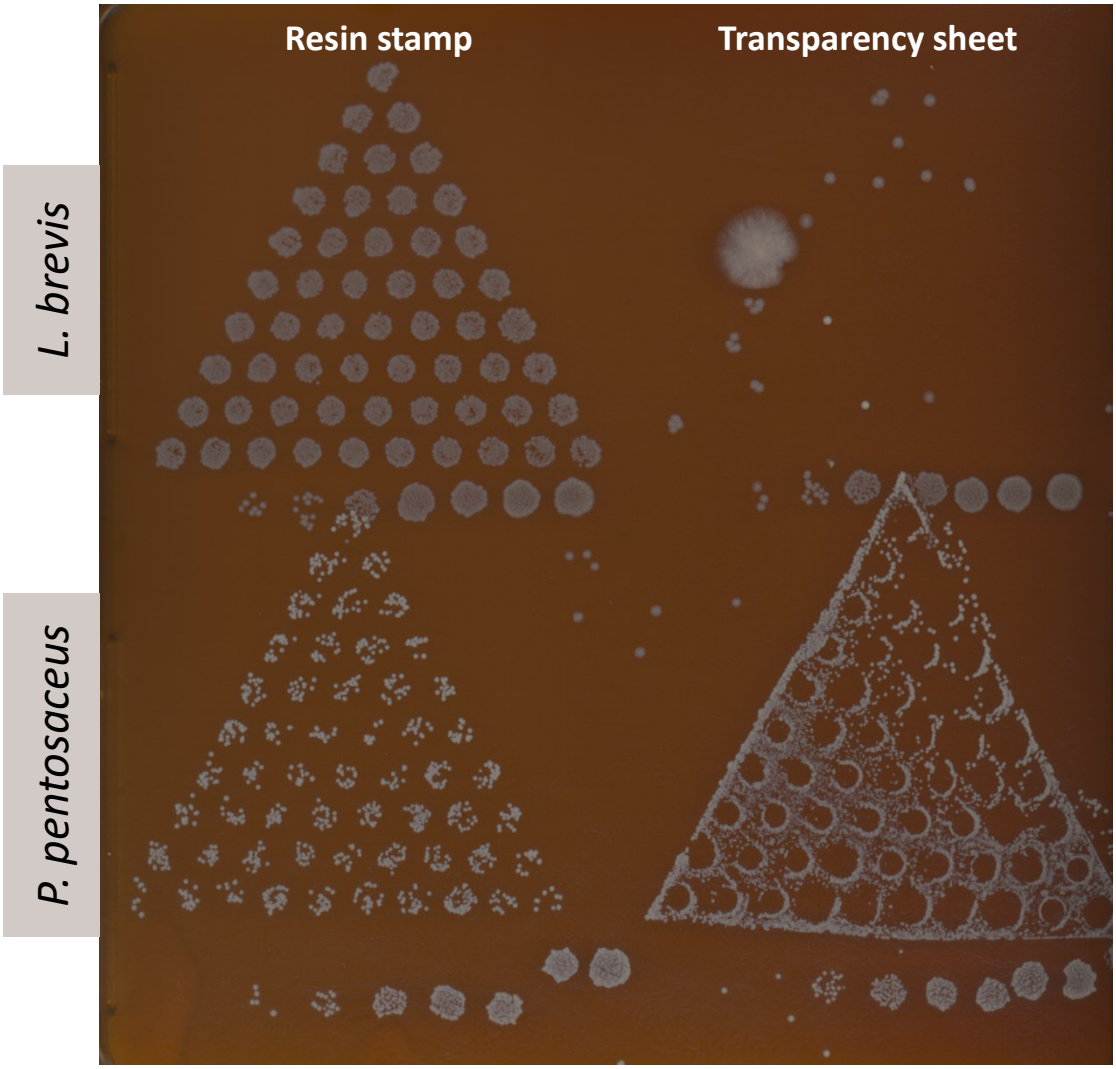


Figure A.2 Comparison of the transfer and the growth of bacteria using a 3D resin stamp and a transparency sheet. The resin stamp seemed to effectively transfer bacterial colonies whereas using a transparency sheet as a template resulted in improper bacterial printing and growth.

Figure A.3 Image of filter papers coated with agar.

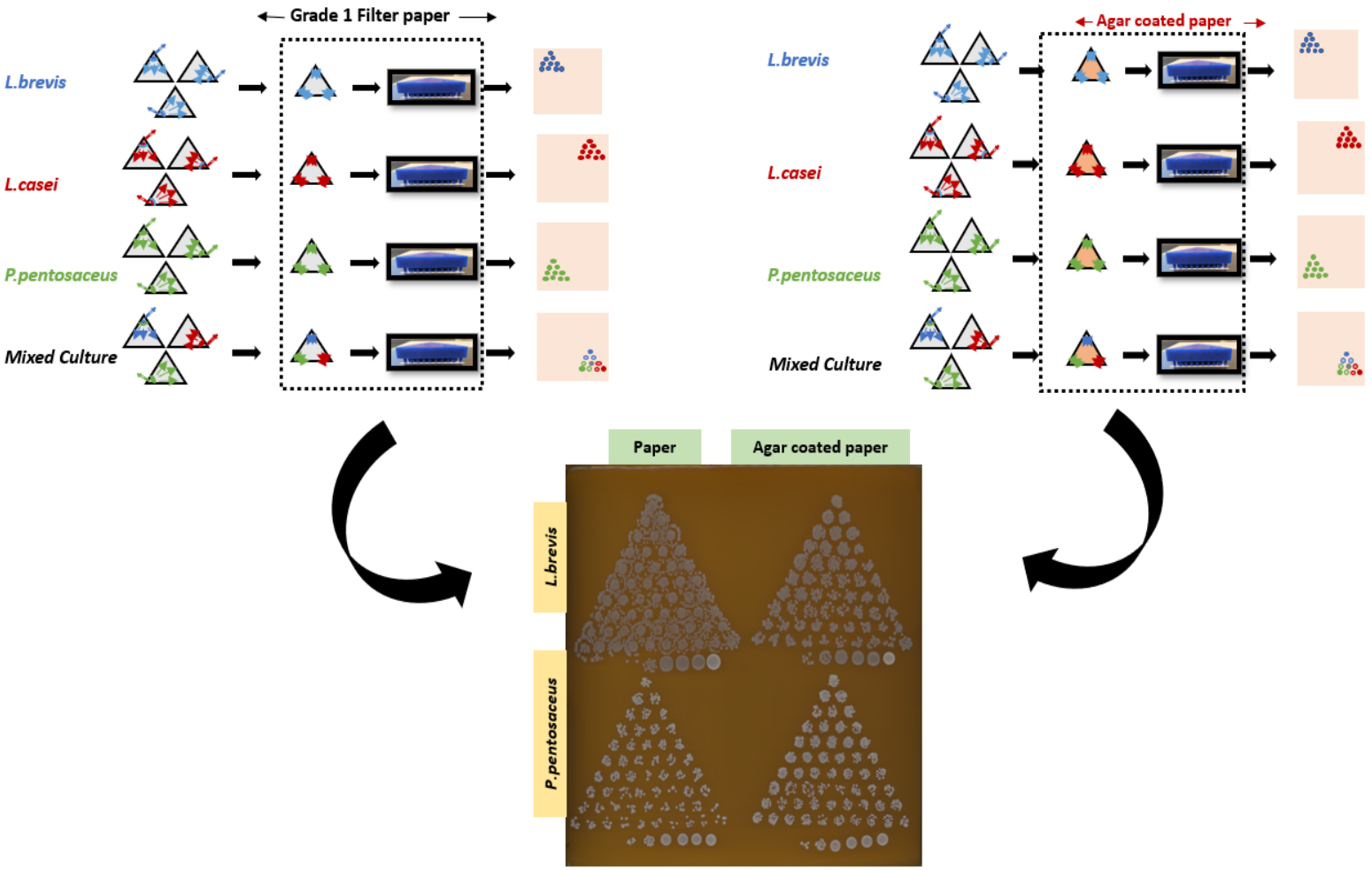


Figure A.4 Bacterial transfer using paper versus paper coated with agar (steps represented in dashed boxes). Paper coated with agar allowed for efficient transfer of bacteria which later was stamped on to the solid nutritional medium. Growth of *P.pentosaceus* indicated uniform bacterial transfer using agar coated paper.

Figure A.5 Image shows drying of agar coated papers. The drying up of the agar coated papers posed a challenge when patterning or transferring bacterial solutions. This challenge was overcome by storing them at 4°C

TABLE A.1 Scanner settings within the Vue Scanner software

VueScanner settings	
Input	
Options	*Professional*
Mode	*Flatbed*
Media	*Colour*
Bits per pixel	*48 bit RGB*
Scan resolution	*1200 dpi*
Auto-repeat	*15 mins*
Output	
File format	*TIFF*
TIFF file type	*48 bit RGB*

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