From phyllosphere to ferment:
Linking patterns to processes in microbial community assembly.

A dissertation submitted by

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In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biology

Tufts University

May 2020

Advisor:

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Abstract

As improvements in sequencing techniques continue to elucidate patterns in microbiomes, we need to develop research that can experimentally explore the processes that structure these communities. The research in this dissertation uses a new model system - the Napa cabbage microbiome - to link patterns with processes in microbiome assembly. Fermented vegetables such as sauerkraut and kimchi, are spontaneously fermented by lactic acid bacteria (LAB) found on the surfaces of raw ingredients. These LAB are essential for vegetable fermentation, and while their role in fermentation is well-studied, their ecology in the vegetable environment is unknown. Here, I describe a novel approach that utilizes gnotobiotic Napa cabbages together with sterile vegetable extract, that can be used to explore ecological processes in fermented vegetable microbiomes. Together with a field survey I show that LAB are surprisingly low in abundance in the Napa cabbage phyllosphere. This rarity may be due to their low abundance in the environment and their limited ability to grow and establish in the Napa cabbage phyllosphere. Using metagenomic sequencing I also explored the diversity of North American fermented vegetable products. Similar LAB species were identified in all vegetable ferments suggesting that a core microbiome exists. In addition to LAB, I identified yeast in many fermented vegetable products. Using *in vitro* ferments, I found the presence of yeast was influenced by the temperature of incubation during fermentation. Finally, I review the role of dispersal as an ecological process structuring fermented food microbiomes. To study dispersal I propose a propagule limitation framework for food producers to use to consider all aspects of dispersal in their food system. Quality of fermented foods can be impacted by dispersal as producers aim to increase dispersal of desirable microbes into the food system to the exclusion of spoilage or pathogenic organisms.

This Napa cabbage system is an easy model for microbial ecologists to use, in order to address how patterns in microbiomes link with ecological processes of microbial community assemble.

Acknowledgements

I would like to thank everyone who has helped me on this journey. I have met so many amazing, inspiring people while living in the US, and you have encouraged me in so many ways.

To Dr Benjamin Wolfe, thank you so much for accepting me into your lab. I love my cabbage system and have really appreciated having the freedom to explore ideas in your lab. ‘Team Cabbage’ has changed a lot, and I would like to thank all who worked on this system, with particular thanks to Jonah Schwartz O’Mara.

My fellow graduate students have helped to stay focused, motivated, and excited by science - it’s been great to be part of the Tufts Biology Community! Drs Colin Orians and Elizabeth Crone, thank you for being on my committee and for your advice and ideas. I am also grateful to Dr Jenny Bhatnagar for serving as my external committee member, I am looking forward to discussing my work with you.

To my family, I would like to thank you for your patience, understanding, and support in all my plans. Zoë, I couldn’t do this if you weren’t always a WhatsApp call away. Finally, I would like to thank Dr Ryan Harrison for always challenging me.

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List of abbreviations

LAB	Lactic acid bacteria
SVE	Sterile vegetable extract
MS	Murashige and Skoog Basal Medium
MRS	deMan, Rogosa, and Sharpe agar
TS / TSA	Tryptic soy and tryptic soy agar
YPD	yeast peptone dextrose
PBS	Phosphate buffered saline
NCP	Napa cabbage phyllosphere
SPC	Synthetic phyllosphere community
RA	Relative abundance
CFU	Colony forming units
NCBI	National Center for Biotechnology Information

From phyllosphere to ferment:
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Chapter One

Introduction

Microbiomes have important roles in many natural, agricultural, biomedical, and industrial systems. For instance, soil microbiomes enable nutrient cycling in agricultural systems (Tully and Ryals 2017) and our gut microbiome helps us digest foods (Walter and Ley 2011). The specific composition of a microbiome, including the types of microbial species present and their relative abundances, can have important consequences for the functions of that microbiome. For example, microbiomes enriched in some microbial taxa can have detrimental impacts on associated hosts (He et al. 2017; Klein et al. 2013; Morgan et al. 2012). Understanding how microbiomes are structured is essential for microbiologists aiming to manipulate the function of microbiomes. While scientists can use next-generation sequencing techniques to understand patterns of microbiome diversity, the ecological and evolutionary processes that drive microbiome structuring are not well understood (Wolfe 2018; Nemergut et al. 2013; Hanson et al. 2012).

The best way to identify the dominant ecological processes that influence microbiome structure is through experimental manipulation of microbial communities. However, the complexity and limited culturability of many environmental microbiomes make them challenging to manipulate. Model microbiomes such as fermented foods strike a balance between unrealistic simple lab communities and something as complex as the human gut microbiome (Wolfe and Dutton 2015). Most fermented foods are composed of species that can be cultured on standard lab media making them easy to replicate in lab settings (Wolfe and Dutton 2015; Zalán et al. 2009; Treco and Lundblad 1993).

Spontaneously fermented foods such as fermented vegetable products, are created by bacteria that are naturally present on the vegetable surface, also known as the phyllosphere (Di Cagno et al. 2013; Tamang et al. 2020). Morphologically distinct bacteria representing all of the core phyllosphere families can be cultured from plant samples (Humphrey et al. 2014; Williams and Marco 2014). Fermented vegetable products typically have a low bacterial diversity with ~ 10 species of lactic acid bacteria (LAB), LAB can also be cultured on standard lab media (Corry et al. 2003; Treco and Lundblad 1993). The tractability of phyllosphere and vegetable microbiomes allows for the creation of synthetic phyllosphere and ferment communities. These synthetic communities can then be used to experimentally address how the phyllosphere and ferment microbiomes are structured (Miller et al. 2019).

Microbial ecologists have recently argued that microbiomes are structured by the same evolutionary and ecological processes - dispersal, selection, diversification, and drift - that shape the communities of plants and animals macro-ecologists have studied for many years (Nemergut et al. 2013; Hanson et al. 2012). Microbes are easily dispersed long distances due to their small size (Fenchel and Finlay 2004; Finlay 2002; Smith et al. 2013). This ease with which microbes can spread led Baas-Becking, a microbiologist working with milk microbes in the 1930s, to state that ‘everything is everywhere but the environment selects' (O'Malley 2007). Based on this theory, many studies have focused on the role of environmental selection in shaping microbiomes. While many studies have demonstrated that everything is not everywhere in microbial ecology (Talbot et al. 2014; Hedlund and Staley 2004; Adams et al. 2013); the impact of dispersal in structuring microbiomes is still understudied.

Scope of Dissertation

The body of work presented within this dissertation uses Napa cabbage as a model system to address how patterns of assembly seen in phyllosphere and ferment microbiomes, can be linked to processes. We first present the model system gnotobiotic Napa cabbage and sterile vegetable extract (Chapter Two). After noticing that LAB are in low abundance in the Napa cabbage phyllosphere microbiome we used a field survey and lab experiments to explore the processes that explain this pattern. We found that LAB are source-limited, (absent or rare in communities that could be reservoirs for LAB) in two environmental species pools, soil and leaves, across farms and community gardens in the northeastern United States. Using lab experiments we investigate the role of establishment limitation (the inability of LAB to thrive in the phyllosphere), in explaining the prevalence of LAB in the Napa cabbage phyllosphere (Chapter Three). Next, we present a survey describing the diversity of LAB in fermented vegetable products from North America. The unexpected abundance of yeast in some products was further explored using *in vitro* fermentation experiments (Chapter Four). In a synthetic review, we present an overview of dispersal in fermented foods which dissects our current knowledge of dispersal in fermented foods into a framework - the propagule limitation framework - which can be used for anchoring research (Chapter Five). Finally, we discuss the implications of this work and the future directions (Chapter Six).

Summary of chapters

Chapter Two outlines the methods we developed to set-up a model system for studying microbial community assembly in the phyllosphere. Our methods show how to grow gnotobiotic Napa cabbage in the lab and include protocols for inoculating these plants with specific microbial communities in order to study microbiome assembly. To complement experiments carried out in the phyllosphere we have created a sterile vegetable extract (SVE) which can be used to make *in vitro* ferments that replicate the fermentation environment. Together these two protocols enable researchers to track microbial community assembly in the phyllosphere and the subsequent microbiome restructuring that occurs as a result of spontaneous fermentation. This methods paper has been accepted at *The Journal of Visualized Experiments* (Miller et al. 2020). This manuscript will be supplemented with an online video demonstrating the described methods.

Chapter Three investigates how dispersal processes influence the abundance of LAB in the Napa cabbage phyllosphere. Plate counts of culturable bacteria and metagenomic sequencing showed that LAB are in low abundance in the Napa cabbage phyllosphere. To determine the processes that explain this pattern we explored the field ecology of LAB by sampling soil and leaves from 51 sites across the northeastern United States. We found that LAB are also in low abundance in two environmental species pools: soil and leaves. Using field and lab experiments we showed LAB are establishment limited and do not persist in the Napa cabbage phyllosphere even when inoculated at high levels. This work has been published in *Applied and Environmental Microbiology* (Miller et al. 2019).

Chapter Four examines the microbial diversity of North American fermented vegetable products using metagenomic sequencing and culture-dependent methods. Despite the growing popularity of fermented vegetables in the United States, surprisingly little is known about which bacteria and fungi live in these products. We found that North American fermented products contain many of the LAB species also detected in sauerkraut and kimchi communities produced worldwide. We did not find a significant difference in the number of LAB species per product which was surprising as the literature suggests that kimchi might be fermented by a greater number of LAB species than sauerkraut. After unexpectedly detecting high levels of yeast within these products, we investigated how abiotic selection might influence the abundance of yeast in vegetable ferments using *in vitro* fermentations.

Chapter Five provides a review of how dispersal can influence the structure of fermented foods. We propose using the propagule limitation framework initially discussed in Chapter three to anchor future dispersal research in fermented foods.

Chapter Six summarizes the findings of this dissertation, considers broader implications of this work, and suggests further directions for research in this field.

Chapter Two

"I'm just as happy growing cabbages as the rarest plant in the world."

Monty Don

A Gnotobiotic System for Studying Microbiome Assembly in the Phyllosphere and in Vegetable Fermentation

The Journal of Visualized Experiments 2020

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Keywords:

phyllosphere, cabbage, fermentation, germ-free, gnotobiotic, sterile vegetable extract, microbiome, microbial community

Summary

A method of growing germ-free Napa cabbages has been developed which enables researchers to evaluate how single microbial species or multispecies microbial communities interact on cabbage leaf surfaces. A sterile vegetable extract is also presented which can be used to measure shifts in community composition during vegetable fermentation.

Abstract

The phyllosphere, the above ground portion of the plant that can be colonized by microbes, is a useful model system to identify processes of microbial community assembly. This protocol outlines a system for studying microbial community dynamics in the phyllosphere of Napa cabbage plants. It describes how to grow germ-free plants in test tubes with a calcined clay and nutrient broth substrate. Inoculation of germ-free plants with specific microbial cultures provides opportunities to measure microbial growth and community dynamics in the phyllosphere. Through the use of sterile vegetable extract produced from cabbages shifts in microbial communities that occur during fermentation can also be assessed. This system is relatively simple and inexpensive to set up in the lab and can be used to address key ecological questions in microbial community assembly. It also provides opportunities to understand how phyllosphere community composition can impact the microbial diversity and quality of vegetable fermentations. This approach for developing gnotobiotic cabbage phyllosphere communities could be applied to other wild and agricultural plant species.

Introduction

Microbial diversity of the phyllosphere plays an important role in maintaining plant health and can also influence the ability of plants to withstand environmental stress (Grady et al. 2019; Pii et al. 2015; Berendsen, et al. 2012; Bai et al. 2015; Bulgarelli et al. 2013). In turn, the health of crops directly impacts food safety and quality (Dinu and Bach 2011; Heaton and Jones 2008). Plants play a role in ecosystem functioning and their associated microbiomes both affect the ability of plants to carry out these activities as well as directly influencing the environment themselves (Bringel and Couée 2015). While scientists have begun to decipher the function and composition of the phyllosphere, the ecological processes that influence phyllosphere microbial community assembly are not fully understood (Maignien et al. 2014; Carlström et al. 2019). The phyllosphere microbiome is an excellent experimental system for studying the ecology of microbiomes (Meyer and Leveau 2012). These communities are relatively simple and many of the community members can be grown on standard lab media (Carlström et al. 2019; Humphrey et al. 2014; Williams and Marco 2014).

Fermented vegetables are one system where the community structure of the phyllosphere has important consequences. In both sauerkraut and kimchi, the microbes that naturally occur on vegetable leaves (the phyllosphere of *Brassica* species) serves as the inoculum for fermentation (Di Cagno et al. 2013; Köberl et al. 2019). Lactic acid bacteria (LAB) are considered ubiquitous members of vegetable microbiomes, however they can be in low abundance in the phyllosphere (Yu, et al. 2019). Strong abiotic selection during fermentation drives a shift in microbial community composition enabling lactic acid bacteria to increase in abundance. As LAB grow, they produce lactic acid which creates the acidic environment of fermented vegetable products (Miller et al. 2019). The link between the phyllosphere and the ferment provides an opportunity to use vegetables as a model to understand how microbiomes are structured.

We have developed methods to grow germ-free Napa cabbages and to inoculate them with specific microbial communities using spray bottles. This is an inexpensive and reliable method of evenly inoculating the cabbage with either individual microbes or mixed communities. A sterile vegetable extract (SVE) has also been developed from three different cabbage types/varieties: red and green cabbage (*Brassica oleracea*) and Napa cabbage (*B. rapa*). The addition of salt to these SVEs replicates the fermentation environment and allows for small-scale and relatively high-throughput experimental studies of fermentation microbiome assembly. These methods can be used to study microbial community assembly in the phyllosphere and how microbial community dynamics in the phyllosphere can be linked to the success of vegetable fermentation.

Protocol

1. Growing germ-free cabbages

1.1. Preparing equipment for growing germ-free cabbages

1.1.1. Cleaning the calcined clay to remove fine dust particles

1.1.1.1. Rinse calcined clay (**Table 2.2**) at least 3x with tap water; drain off water.

CAUTION: Calcined clay produces very fine dust and it is recommended to wear a protective mask (**Table 2.2**) when washing.

1.1.1.2. Spread calcined clay out as a thin layer (~4 cm) into an autoclave tray and autoclave on a dry cycle (121 °C heating for 20 min and 20 min drying time) to sterilize.

1.1.1.3. Allow the calcined clay to fully dry prior to use by spreading out on trays and placing in a warm incubator (30−37 °C) for at least a week. Stir to mix every 3 days to fully dry the calcined clay so that it will absorb an even amount of Murashige and Skoog (MS) nutrient broth (Section 1.2).

NOTE: Drying also helps to keep the volume of calcined clay even when it is weighed into tubes. Drying by other means, such as a drying oven, would also be suitable.

1.1.2. Cleaning the glassware for growing germ-free cabbages

1.1.2.1. Thoroughly clean and sterilize the glass tubes (**Table 2.2**) between each use. Soak tubes for 30 min in 30% bleach solution and rinse well with tap water before cleaning in an acid wash on a bacteriology setting. Acid-wash two-way test tube caps (**Table 2.2**) between uses.

1.1.3. Surface sterilizing cabbage seeds

1.1.3.1. Place up to 100 Napa cabbage (*B. rapa var pekinensis*) seeds in a 1.5 mL microcentrifuge tube.

NOTE: Adding more than 100 seeds to one microcentrifuge tube or changing the size of the tube may affect germination rates of the seeds due to lack of seed coat removal.

1.1.3.2. Add 1 mL of 70% ethanol to the seeds and vortex for 5 min. Discard the ethanol using a pipette.

1.1.3.3. Add 1 mL of 50% bleach and vortex for 5 min. Discard the bleach solution using a pipette.

1.1.3.4. Add 1 mL of autoclaved deionized water and vortex for 5 min. Discard the deionized water using a pipette.

1.1.3.5. Repeat step 1.1.3.4 3x to rinse off all bleach. Soak the seeds in sterile deionized water for 2−8 h prior to planting to soften the seed coat.

1.2. Growing germ-free cabbages

NOTE: Napa cabbages (*B. rapa var pekinensis*) are grown in glass tubes (15 cm x 2.5 cm) containing calcined clay soaked in Murashige and Skoog (MS) nutrient broth (**Figure 2.1**).

1.2.1. Weigh 10 g of clean calcined clay into a clean glass tube (15 cm x 2.5 cm).

1.2.2. Prepare MS nutrient broth by dissolving 4.4 g of MS medium in 1 L of deionized water. Add MS nutrient broth (~9 mL) to each glass tube to cover the calcined clay using a pipette.

NOTE: Standing liquid in the tube will prevent the seed from germinating so it might be necessary to add slightly less MS broth to some tubes.

1.2.3. Loosely cap glass tubes with 22 mm two-way test tube caps and autoclave (121 °C for 60 min). When removing them from the autoclave, push caps onto the glass tubes to seal them. Cool tubes to room temperature before use.

1.2.4. Gently place one sterile cabbage seed into the center of each tube using sterile, extra-long (25.4 cm) forceps. Place the tubes in a 7-way tray then place under light racks (full-spectrum T5 fluorescent bulbs or other illumination setup for plant growth) with a 16 h light cycle at 24 °C.

NOTE: Seeds germinate overnight and develop their first true leaf after 5 days. A true leaf is the first vascular leaf after the cotyledons have formed. It has a more wrinkled edge and in *Brassica rapa* is covered in trichomes.

1.3. Testing for sterility of germ-free cabbages

NOTE: To test whether the cabbages are germ-free, select a few (5−10) cabbages from each batch and plate out to determine whether any culturable colonies are present.

1.3.1. Gently remove the cabbage from the glass tubes by gripping the base of the plant with sterilized forceps and pulling it out. Before removing the cabbage fully from the tube, carefully cut off the roots using sterilized dissection scissors. Compact the cabbage leaves into a 1.5 mL microcentrifuge tube.

NOTE: Larger cabbages might require removing one or two of the larger leaves while the cabbage is still in the tube to make it easier to get the cabbage into the 1.5 mL microcentrifuge tube. These larger leaves can be added to the 1.5 mL tube after the rest of the cabbage has been placed into the tube if the entire cabbage is required.

1.3.2. Add 400 μL of 1x phosphate buffered saline (PBS) to each 1.5 mL microcentrifuge tube. Using a sterile micropestle, homogenize the cabbage by pestling 30x.

1.3.3. Plate 100 μL of the cabbage homogenate onto agar plates to determine whether there are any contaminants present in the sample. Most bacteria found in the phyllosphere will grow on tryptic soy (TS) agar plates. Use wide orifice pipette tips when plating cabbage homogenate as the cabbage homogenate is thick and can clog regular pipette tips.

2. Inoculating the phyllosphere with microbial solutions

2.1. Making glycerol stocks of inoculation strains

NOTE: Table 2.1 lists the microbial isolates that can be used in this step. Other phyllosphere isolates could also be used here.

2.1.1. Densely streak out individual colonies from a fresh streak, onto two/three new plates of the same media to get many colonies.

2.1.2. Let streaks grow for 2−5 days then scrape colonies from all plates into a 15 mL conical tube containing 15 mL of 15% glycerol, and vortex to mix thoroughly.

2.1.3. Transfer an aliquot of 1 mL of the well-mixed glycerol stock into a 1.5 mL microcentrifuge tube and store glycerol stocks at -80 °C until use. Save the remaining 14 mL of glycerol stock at -80 °C as relatively large volumes of inoculation solution are required when inoculating cabbages.

2.1.4. One week before use, thaw the 1.5 mL tube containing 1 mL of glycerol stock (from step 2.1.3) on ice, dilute, and plate at several different dilutions (e.g., 10^{-4}, 10^{-5}, and 10^{-6}) to determine the concentration (colony-forming unit [CFU] per μL) of the 14 mL of inoculation solution.

2.2. Sterilizing inoculation spray bottles

2.2.1. Disassemble the amber round Boston pump bottles (59 mL) and soak all components (pump, tube, cap and bottle) in 30% bleach solution for 30 min in a large plastic container with a tightly fitting lid.

2.2.2. After soaking, carefully pour out all bleach from the container by lifting just one corner of the lid of the container.

2.2.3. Rinse the bottles by filling the plastic container with autoclaved deionized water (~1 L depending on the container size) and carefully pour out deionized water, again by lifting the lid at one corner.

2.2.4. Sterilize a biosafety cabinet by spraying with 70% ethanol solution and turning on the UV light for 30 min.

NOTE: Continue this work in the biosafety cabinet so that there is no risk of microbial contamination of the bottles as they air dry.

2.2.5. Remove bottles from the large plastic container and fill each bottle with autoclaved deionized water using a pipette. Reassemble the pumps and place one in each bottle. Pump the deionized water through each bottle (10 sprays per bottle) to remove bleach from the pump component of the bottle.

2.2.6. Repeat step 2.2.5 to ensure that all bleach is removed from the glass bottles.

2.2.7. Test whether bottles are sterile by placing a number on each bottle (sticking lab tape to the side of the bottle when it is fully dry) then add 10 mL of 1x PBS to each of the bottles and pump 3 sprays onto a TS agar plate. After spraying, incubate the plates for one week at room temperature. If any colonies grow on a plate it indicates that the respective bottle was not sterile and should not be used for experiments.

2.2.8. Before storing the sterile bottles, remove all remaining PBS and allow the bottles to dry thoroughly in the biosafety cabinet. Store sterile bottles in a sterile plastic container (typically the container used for bleaching the bottles) until use.

2.3. Preparing the microbial inoculum and spraying germ-free cabbages

CAUTION: All steps should be performed in a biosafety cabinet, as spraying aerosolizes the microbial solutions which could contaminate work surfaces or pose a health risk if carried out on a lab bench.

NOTE: Cabbages will form true leaves after 5 days, so it is advisable to wait one week after planting the cabbage before inoculating with any microbial solutions. As the tubes are sealed, there is no need to water the cabbages. Experiments are best performed within a month of planting, as the small tubes restrict the cabbage's growth.

2.3.1. Thaw glycerol stocks on ice and dilute in 1x PBS to the desired inoculation concentration (concentration determined by thawing and plating a 1 mL aliquot in step 2.1.4).

NOTE: A variety of different inoculation levels can be used, but phyllosphere isolates can grow from 10^4 to 10^8 CFUs/mL of cabbage slurry in 10 days.

2.3.2. Add 10 mL of diluted glycerol stock to the sterile pump bottle and pump 5 sprays into a large waste collection beaker to remove any residual PBS from the bottle pump component.

2.3.3. Remove the lid from the cabbage tube, tilt the cabbage towards the spray bottle, and spray each cabbage with 3 pumps of the inoculation solution, which provides ~600 μL of inoculum.

2.3.4. After inoculating, harvest a subset of the cabbages to assess the actual input inoculation concentration. Remove the cabbage from a tube with sterilized forceps. Cut off the roots with sterile dissection scissors and then carefully place the cabbage in a preweighed sterile 1.5 mL microcentrifuge tube. Record the weight of the cabbage for future calculations if CFUs/g of cabbage is required for calculations.

2.3.5. Add 400 μL of 1x PBS to each 1.5 mL microcentrifuge tube containing cabbage and use a sterile micropestle to homogenize the cabbage into the 1x PBS by grinding it 30x.

2.3.6. Dilute cabbage homogenate (if required) and plate out the pestled cabbage mixture. Use wide orifice tips for pipetting the cabbage slurry because it will be thick and full of plant tissue pieces.

3. Preparing sterile vegetable extract

NOTE: This method is a modified version of cabbage sterile media production (Stamer et al., 1971; Yildiz and Westhoff 1981).

3.1. Purchase a cabbage from a supermarket. In the lab, remove and discard the outermost leaves of the cabbage. Chop all remaining cabbage to fit into a blender and homogenize cabbage to a fine pulp, i.e., the cabbage will not get any finer with further blending.

NOTE: Any blender which can chop cabbage to a smooth homogeneous pulp should be suitable for this method.

3.2. Weigh the blended cabbage homogenate and add 2 mL of distilled water per gram of cabbage. Filter the blended cabbage slurry through 2 layers of basket coffee filters (unbleached paper).

3.3. Dispense the cabbage slurry into centrifuge tubes (size is dependent on the centrifuge). Centrifuge the filtered cabbage slurry at 20,000 x *g* for 20 min until large particles settle out of solution.

NOTE: It is essential to centrifuge the cabbage slurry for a long period of time as cabbage particles rapidly clog the filter sterilizer.

3.4. Using a serological pipette, remove the supernatant from the pelleted cabbage debris taking care not to disturb the pelleted cabbage. If aiming to recreate fermentation conditions where standard salt concentrations are used, add 2% w/v NaCl at this step (i.e., before filter sterilization).

3.5. Filter sterilize the vegetable extract using a 0.2 μm filter (500 mL or 1 L) attached to a vacuum. Dispense into sterile tubes (either 50 mL centrifuge tubes or 15 mL centrifuge tubes) and freeze at -80 °C until use.

4. Inoculation of sterile vegetable extract

4.1. Thaw SVE and dispense 490 µL into 1.5 mL microcentrifuge tubes. Use sufficient tubes to have at least five replicates per treatment per timepoint as each timepoint measurement is destructive.

4.2. Thaw glycerol stocks of microbial isolates on ice and dilute with 1x PBS to the desired concentration. The concentration of lactic acid bacteria can be as low as 5,000 CFU per mL of SVE. To achieve this concentration, dilute stocks to 250 CFUs/µL because 10 µL will be used for inoculation of a total volume of 500 µL.

4.3. Inoculate SVE with 10 µL of diluted microbial isolate. Pipette up and down a few times to thoroughly mix. Incubate at desired temperature (14 °C for kimchi production temperature or 24 °C for warmest sauerkraut fermentation).

4.4. Measure rate of growth of microbial isolate in the SVE by harvesting replicate tubes on day 1, day 2, day 4, day 7 and day 14.

NOTE: Fermentation proceeds rapidly at the outset and slows over time. Therefore, having more initial timepoints gives greater resolution to the dynamics of how fermentation proceeds.

4.5. At each timepoint, mix the inoculated SVE well by pipetting up and down a few times. Serially dilute the inoculated SVE in 1x PBS and plate onto agar plates. Incubate the agar plates for 4−7 days before counting colonies.

NOTE: deMan, Rogosa, and Sharpe (MRS) agar should be used to enumerate all lactic acid bacteria, yeast peptone dextrose (YPD) should be used for yeast, and TS agar for most other bacteria isolates from the phyllosphere.

4.6. Record the pH of the samples at each timepoint using a micro pH probe.

NOTE: This step should be carried out after plating because the pH probe will transfer cells between tubes/treatments.

Representative Results

Growth rates of Napa cabbages

The seed sterilization method was tested with several different Napa cabbages (*B. rapa var pekinese*; **Supplemental Figure 2.1**) from a number of different suppliers and all grew consistently with similar growth rates. However, testing the methods with different species of *Brassica* (*B. rapa:* Turnip Purple Top; *B. oleracea:* Cairo Hybrid, Tropic Giant Hybrid; *B. campestris:* Pak Choi Toy Choy Hybrid; *B. juncea:* Mustard Red Giant) gave limited success (**Supplemental Figure 2.2**). Unlike Napa cabbage that forms compact neat rosettes that fit into the glass tubes, these *Brassica* spp. either had low germination rates after sterilizing or the stem elongated rapidly to make a spindly, unhealthy plant. In addition, sterilizing older seeds (>1-year-old) is not recommended as the seed coats dry out making it harder to remove them during the sterilization process. Regularly purchase new seeds and test a subset of cabbages to determine whether they are sterile before carrying out experiments.

Growth of microbial inoculants in the Napa cabbage phyllosphere

Microbial isolates (**Table 2.1**) were inoculated either as single strain isolates or in combination with another isolate to look for pairwise interactions in the Napa cabbage phyllosphere. A total of 15 germ-free cabbages were inoculated for each treatment and five cabbages were harvested immediately after inoculation, five were harvested four days after inoculation, and the remaining were harvested 10 days after inoculation. Results show that phyllosphere isolates are capable of rapid growth in the Napa cabbage phyllosphere (**Figure 2.2**).

Growth of microbial inoculants in sterile vegetable extract

Two yeasts (*Kazachstania barnetti* and *Pichia membranifaciens*) and three bacteria (*Lactobacillus koreensis*, *Pediococcus parvulus*, and *Leuconostoc mesenteroides*) were inoculated into three different types of SVE made from red, green, and Napa cabbage. All samples were incubated at 24°C and growth of the inoculates over 14 days was recorded by spot plating 5 µL of each treatment onto either MRS or YPD agar plates (n = 5). Results are shown in **Figure 2.3A**. The pH of each sample was also recorded throughout the fermentation (**Figure 2.3B**) and shows that the lactic acid bacteria were capable of acidifying the SVE to levels below pH 4 (indicating a ferment that is safe for consumption).

Figures

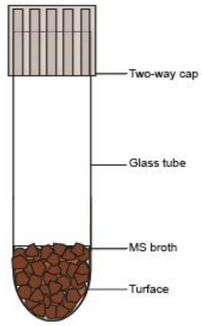

Figure 2.1 Diagram of germ-free cabbage setup.

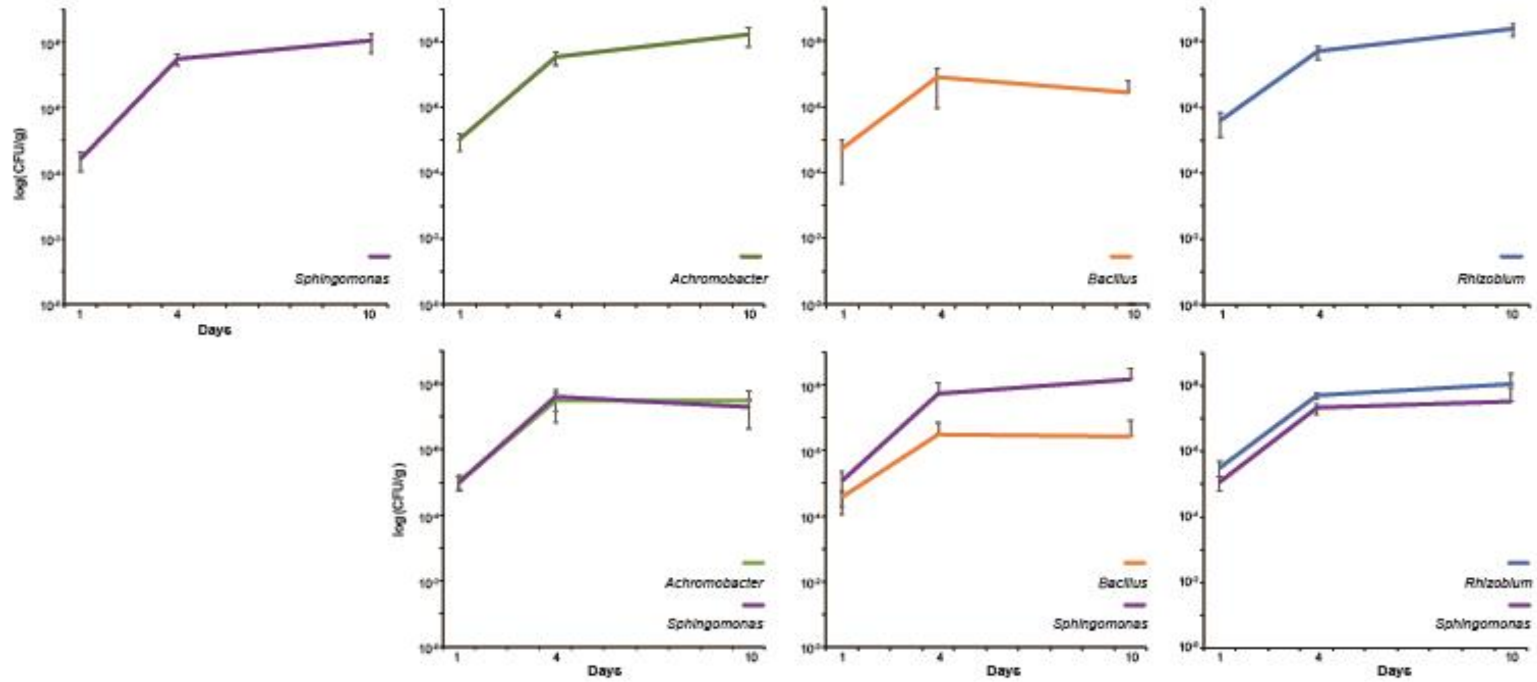

Figure 2.2 Growth rates of different bacteria on germ-free Napa cabbage.

(A) Growth of single inoculations in the phyllosphere. (B) Growth after inoculating two microbes into the phyllosphere. Growth of microbes was measured as colony forming units counted per g of cabbage homogenate plated onto either TSA or MRS media. n = 5. Error bars = standard deviation

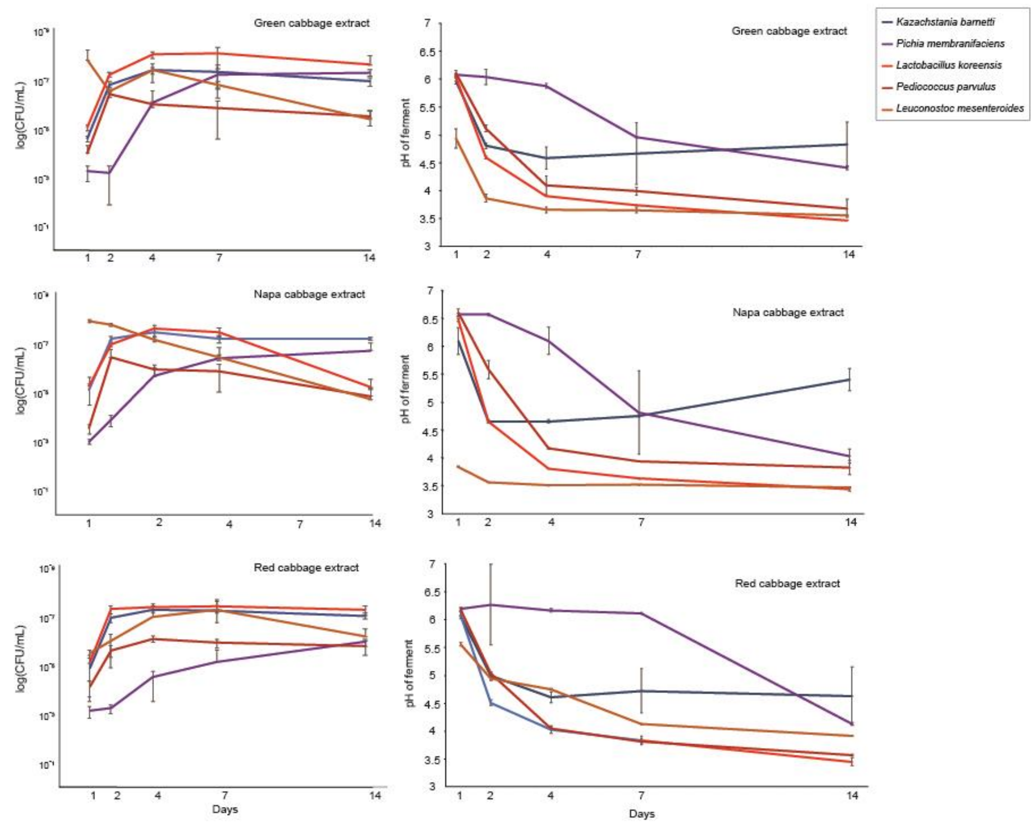

Figure 2.3 Growth of lactic acid bacteria and yeasts in sterile vegetable extract (SVE) made with red, green and Napa cabbage (A) Growth of microbial inoculants was measured by counting colony forming units per mL of SVE plated. Yeasts were plated onto YPD agar plates and bacteria onto MRS agar plates. (B) Acidification of the sterile vegetable extract as microbes grow shown as fall in pH. n = 5. Error bars = standard deviation.

Tables

Table 2.1. Microbial isolates inoculated on germ-free Napa cabbage

Phyla	Genera	Source of Microbe
Firmicutes	*Bacillus*	Phyllosphere
Firmicutes	*Lactobacillius*	Fermentation
Proteobacteria	*Achromobacter*	Phyllosphere
Proteobacteria	*Rhizobium*	Phyllosphere
Proteobacteria	*Sphingomonas*	Phyllosphere

Table 2.2. Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL microcentrifuge tubes	VWR	20170-650	
15 mL conical tubes	Falcon	352096	
7-way tray tray	Sigma Magenta™	T8654	
Amber Round Boston Glass Bottle		GPS 712OZSPPK12BR	Ordered on Amazon.com from various suppliers
Basket coffee filters	If you care		(unbleached paper) Purchased from Wholefoods

Bleach (mercury-free)	Austin's	50-010-45	
Borosilicate Glass tubes	VWR	47729-586	
Cuisinart blender	Cuisinart		Cuisinart Mini-Prep Plus Food Processor, 3-Cup
Dissection scissors	7-389-A	American Educational Products	Ordered on Amazon.com
Ethanol	VWR	89125-172	
Forceps	Aven	18434	Ordered on Amazon.com
Glycerol	Fisher Scientific	56-81-5	
KleenGuard M10	Kimberley-Clark	64240	
Light racks	Gardner's Supply	39-357	full-spectrum T5 fluorescent bulbs
Magenta tm 2-way caps	Millipore Sigma	C1934	
Man, Rogosa, and Sharpe	Fisher Scientific	DF0881-17-5	This media is for broth and 15 g of agar is added to make plates
Micro pH probe	Thermo Scientific	Orion PerpHecTTM ROSSTM 8220BNWP	
Micropestle	Carolina	215828	Also called Pellet Pestle
MS nutrient broth	Millipore Sigma	M5519	Murashige and Skoog Basal Medium
NaCl	Sigma Aldrich	S9888	

Napa cabbage seeds	Johnny's Select Seeds	2814G	B. rapa var pekinensis (Bilko)
Petri dish 100 mm * 15 mm	Fisher	FB0875712	Used to make agar plates
Phosphate buffered saline	Fisher Scientific	50-842-941	Teknova
Serologial pipettes	VWR	89130-900	
Sterile dowel	Puritan	10805-018	Autoclave before use to sterilize
Sterilizing 0.2 µm filter	Nalgene	974103	
Tryptic soy agar	Fisher Scientific	DF0370-17-3	This media is for broth and 15 g of agar is added to make plates
Tupperware container	Rubbermaid		Ordered on Amazon.com
Turface MVP	Turface	MVP	Ordered on Amazon.com from Root Naturally 6 Quart Bags
Wide orifice pipette tips	Rainin	17007102	
Yeast, peptone and dextrose	Fisher Scientific	DF0428-17-5	This media is suitable but media can also be made using yeast, peptone and dextrose, add 15 g of agar when making plates

Discussion

Germ-free Napa cabbage plants have been used to study dispersal limitation of lactic acid bacteria in the Napa cabbage phyllosphere (Miller et al. 2019). Germ-free Napa cabbages can also be used to test individual or pair-wise growth in the phyllosphere **(Figure 2.2**). Methods for making sterile vegetable extract has been tested for three different varieties of cabbage: red, green and Napa. Each of these SVEs act as a reliable growth media; inoculated microbes grow consistently across the different media. Single strain growth rates in SVE (**Figure 2.3**) show that LAB grow rapidly and acidify the media in the same way that would be anticipated in a ferment (Miller et al. 2019)

Germ-free plants and sterile vegetable extract can be used in combination to address a number of different ecological questions such as priority effects and succession in the phyllosphere or within a ferment. A synthetic community of microbes is simple to construct through plating out homogenized cabbages to obtain phyllosphere isolates, or sauerkraut to obtain lactic acid bacteria (Yu, et al. 2019). Pairwise-interactions or leave-one-out experiments with more community members can be carried out in the phyllosphere or in the SVE to assess the importance or function of community members. Environmental selection studies can be carried out in the SVE where the impact of the vegetable fermentation can be assessed. There is also potential to use both the germ-free cabbages and SVE to quantify diversification of microbial species and communities using experimental evolution.

A limitation of this germ-free cabbage system is the short timescale of the experiments. Because of the small glass tubes used, the cabbages are not able to grow for periods longer than a month as their leaves are confined by the edge of the tubes. Larger growing containers, such as plant tissue culture boxes (**Table 2.2**) could be used, but these will still not produce a full-sized cabbage plant. We have also tried growing cabbages in 0.75% agar containing MS broth, but found that this produced inconsistent growth of the cabbage seedlings. Using calcined clay as a growing substrate with enough MS broth to saturate but not flood the clay grains is the optimum method for growing healthy cabbages.

There are a few critical steps to ensure successful growth of germ-free cabbages. Ensuring that the calcined clay is fully dry when adding MS broth allows the clay to fully absorb the MS broth during the autoclave cycle. However, if there is any MS broth over the level of the clay, it must be removed before adding the seeds; seeds will not germinate if they are sitting in MS broth. Another important step to monitor is seed sterilization. Older seeds (>1 year old) will not germinate as quickly or as reliably as young seeds. Changing the size of the tube used for sterilization or overfilling the tubes can also impact sterilization. The sterilization step also helps soften and remove the seed coat so that the seeds rapidly germinate. Note here that reusing the pump spray bottles after using with microbial cultures is not recommended, as it is difficult to remove biofilms from the pump component. Of particular note, caution should be taken with *Bacillus* species as they are particularly resilient to autoclaving. Any pump bottles that have come into contact with *Bacillus* spp are not reused.

While sterile vegetable extract does not have the spatial structuring that is present in a fermentation vessel, growth dynamics of LAB suggest that it mimics fermentation progress with a rapid fall in pH and an increase in growth of lactic acid bacteria over the 14 days of fermentation. *Leuconostoc mesenteroides* is important at the outset of fermentation and it increased in abundance more rapidly than the *Lactobacillus* and *Pediococcus* spp, a trend seen in other sauerkraut succession surveys (Zabat,, et al. 2018; Lee, et al. 2015). Work in the lab has also explored using spectrophotometer to obtain optical density (OD) readings for measuring the growth of LAB in SVE dispensed into 96 well plates. Initial results with Napa cabbage extract looked promising, but SVE made with red cabbage extract changed color as the pH dropped resulting in confounded OD readings. Furthermore, using OD readings to enumerate growth limits the use of this system to single strain inoculations. Together, these limitations led us to abandon using OD readings to measure microbial growth.

Testing ecological interactions in the phyllosphere is topical as there is evidence that the phyllosphere affects crop plant health and productivity (Trivedi et al. 2017). Our model system has only been developed to work with Napa cabbage, but bacteria from the phyla Proteobacteria, Firmicutes, and Actinobacteria are common in the phyllosphere of many plant species (Knief et al. 2012; Williams and Marco 2014). While only three different varieties of cabbage have been tested, SVE can be made with other important agricultural plants. For example, studies investigating microbial community assembly during carrot juice fermentation (Wuyts et al. 2018) or microbial colonization of maize root (Niu et al. 2017) can be replicated using the protocols outlined in this paper.

Coupling the germ-free cabbage with the SVE to study community assembly in fermentation can show how changes in the phyllosphere microbiome can influence the success of fermentation. Spoilage of ferments or a failure to reach a sufficiently low pH can result if there is not a rapid initial acidification (Steinkraus 1983). These spoiled ferments might be due to manufacturing processes, but variation in phyllosphere microbiomes may also have an important influence on the success of vegetable ferments (Miller et al. 2019). The described system is a useful model for determining what microbiome assembly processes may impact the success of vegetable fermentation.

Acknowledgments

This work was supported by the USDA-NIFA grant: 2017-67013-26520. Tracy Debenport and Claire Fogan provided technical support and Ruby Ye and Casey Cosetta provide helpful comments on early versions of this manuscript.

Disclosures

The authors have nothing to disclose.

Supplementary Figures

Supplementary Figure 2.1. Growth of *Brassica rapa var pekinensis*: Bilko in germ-free conditions

Supplementary Figure 2.2. Different cabbage varieties growing in germ-free conditions.

(A) *B. rapa:* Turnip Purple Top, (B) *B. oleracea*: Cairo Hybrid, (C) *B. oleracea:* Tropic Giant Hybrid, (D) *B. campestris:* Pak Choi Toy Choy Hybrid, (E) *B. juncea:* Mustard Red Giant.

Chapter Three

Establishment limitation constrains the abundance of lactic acid bacteria in the Napa cabbage phyllosphere

Applied and Environmental Microbiology 2019

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Running title: Establishment limitation in the Napa cabbage phyllosphere

Abstract

Patterns of phyllosphere diversity have become increasingly clear with high-throughput sequencing surveys, but the processes that control phyllosphere diversity are still emerging. Through a combination of lab and field experiments using Napa cabbage and lactic acid bacteria (LAB), we examined how dispersal and establishment processes shape the ecological distributions of phyllosphere bacteria. We first determined the abundance and diversity of LAB on Napa cabbage grown at three sites using both culture-based approaches and 16S rRNA gene amplicon sequencing. Across all sites, LAB made up less than 0.9% of the total bacterial community abundance. To assess if LAB were low in abundance in the Napa cabbage phyllosphere (NCP) due to a limited abundance in local species pools (source limitation), we quantified LAB in leaf and soil samples across 51 vegetable farms and gardens throughout Northeastern US. Across all sites, LAB comprised less than 3.2% of the soil bacterial communities and less than 1.6% of phyllosphere bacterial communities. To assess whether LAB are unable to grow in the phyllosphere even if they dispersed at high rates (establishment limitation), we used a gnotobiotic Napa cabbage system in the lab with experimental communities mimicking varying dispersal rates of LAB. Even at high dispersal rates, LAB became rare or completely undetectable in experimental communities suggesting that they are also establishment-limited. Collectively, our data demonstrate that the low abundance of LAB in phyllosphere communities may be explained by establishment limitation.

Importance

The quality and safety of vegetable fermentations is dependent on the activities of LAB naturally present in the phyllosphere. Despite their critical role in determining the success of fermentation, the processes that determine the abundance and diversity of LAB in vegetables used for fermentation are poorly characterized. Our work demonstrates that the limited ability of LAB to grow in the cabbage phyllosphere environment may constrain their abundance on cabbage leaves. These results suggest that commercial fermentation of Napa cabbage proceeds despite low and variable abundances of LAB across different growing regions. Propagule limitation may also explain ecological distributions of other rare members of phyllosphere microbes.

Introduction

The phyllosphere - the aboveground portion of plants that can be colonized by microbes - plays important roles in the productivity of agricultural systems as well as the safety and quality of food (Brandl 2006; Heaton and Jones 2008; Rastogi, et al. 2013). Various bacteria, fungi, and other microbes colonize the phyllosphere, where they are antagonists, commensals, or mutualists of their hosts (Vorholt 2012; Müller et al. 2016; Schoelz and Stewart 2018; Leach et al. 2017). Many recent studies have described patterns of microbial diversity of the phyllosphere using high-throughput sequencing (Vorholt 2012; Agler et al. 2016; Peñuelas and Terradas 2014; Knief et al. 2010; Bodenhausen et al. 2014; Maignien et al. 2014; Lebeis et al. 2015; Bodenhausen, Horton, and Bergelson 2013; Beattie

2015). An extensive body of experimental work has characterized the dynamics of phyllosphere species and populations including many plant pathogens and biocontrol agents (Kinkel 1997; Andrews and Harris 2000). A mechanistic understanding of the specific ecological processes that explain patterns of phyllosphere community composition is still emerging.

Dispersal may be one ecological process that shapes phyllosphere microbiome diversity and function. When a microbe is present in a local species pool, its abundance in the phyllosphere may be constrained by how many cells successfully reach and colonize available plant leaf habitat. In plant and animal ecology, species are considered propagule-limited when propagules (seeds, spores, larvae, etc.) fail to reach all suitable habitat patches at saturating densities (Primack and Miao 1992; Cain, et al. 2000; Turnbull, et al. 2000; Clark et al. 2007). While the term propagule is not widely used in microbiology, a microbial propagule is a cell or other biological unit (e.g. spore, group of cells in a biofilm) that can generate a new microbial population when disseminated (Martiny et al. 2006). Propagule limitation can be divided into three different components: 1) source limitation, where not enough propagules are locally produced to colonize all potential habitats, 2) dispersal limitation, where enough propagules are produced, but they cannot reach all available habitats, and 3) establishment limitation, where local population sizes are constrained by availability of viable niche spaces, not by propagule abundance ((Clark et al. 2007), **Fig. 3.1A**). Several studies in other microbiomes, including the built environment (Adams et al. 2013), tree roots (Peay, et al. 2010), and aquatic communities (Bell 2010) have provided observational evidence for propagule limitation. Many studies in the phyllosphere have tracked the dispersal and movement of microbial species and populations (Kinkel 1997), but we are unaware of studies that have experimentally tested the significance of propagule limitation as a driver of phyllosphere microbiome community composition.

Lactic acid bacteria (LAB) are one commercially important group of bacteria that can be found in the phyllosphere and are widely known for their beneficial impacts in food systems (Leroy and De Vuyst 2004). In addition to fermenting vegetables to make products such as sauerkraut, kimchi, and natural pickles (Rhee, et al. 2011; Kyung et al. 2015; Plengvidhya et al. 2007), plant-associated LAB have the potential to impact human health through increasing the nutritional quality of raw food materials (Montaño et al. 2004). Despite their widespread use in food fermentation and their potential to impact human health, surprisingly little is known about the ecology of LAB in the phyllosphere (Minervini et al. 2015; Golomb and Marco 2015). Most studies have focused on the dynamics of LAB during fermentation (Wuyts et al. 2018; Di Cagno et al. 2013; Rhee, et al. 2011) and the processes that determine LAB distributions in the phyllosphere are largely unknown.

Lactic acid bacteria are essential for successful vegetable fermentations and variation in the initial abundance and composition of LAB may impact the outcome of cabbage fermentation (Gangopadhyay and Mukherjee 1971; Trail et al. 1996; Mcfeeters 2004; Harris 1998). In both large- and small-scale production, vegetable fermentations are not usually inoculated with defined starter cultures and instead rely on LAB present in the phyllosphere (Yoon et al. 2002). Microbes from local species pools in the farms where cabbages are grown colonize cabbage leaves during phyllosphere microbiome assembly. During the fermentation process, the phyllosphere microbiome undergoes a second stage of community assembly leading to a new fermented vegetable microbiome (**Fig. 3.1B**). Some studies have detected LAB in soil and water (Chen et al. 2005; Lauzon and Ringø 2012), but the sources of LAB cells in agricultural systems and their dispersal dynamics are poorly characterized.

Here, we introduce the use of the cabbage leaves as a model system for determining the ecological processes shaping phyllosphere bacterial community composition. We used Napa cabbage (*Brassica rapa* subsp. *pekinensis,* also known as Chinese cabbage), a type of cabbage grown around the world to be consumed fresh as well as for fermentation into kimchi and other fermented vegetable products (Lee, et al. 2015; Jeong, et al. 2013; Patra et al. 2016). It is a fast-growing species that can be easily grown in plant tissue culture and seeds can be sterilized to create gnotobiotic plants. Using field and lab grown plants, we first determined that LAB are low in abundance in the Napa cabbage phyllosphere (NCP). We then used field sampling and an experimental approach with gnotobiotic cabbages to determine that establishment limitation explains the low abundance of LAB in the NCP. Our work demonstrates the utility of the Napa cabbage system as a model to link patterns with processes in phyllosphere microbiomes and illustrates the importance of dispersal processes in explaining species distributions in microbial communities.

Results

LAB are in low abundance in the Napa cabbage phyllosphere. To estimate the abundance and diversity of LAB in the NCP, we grew Napa cabbages (*Brassica rapa* subsp. *pekinensis*) at three different field sites in the Boston, Massachusetts area (**Fig. S3.2**). After two months in the field (August through October), cabbages were returned to the lab where we determined phyllosphere bacterial community composition using 16S rRNA gene amplicon sequencing. We also plated cabbage leaf homogenates on de Man, Rogosa, and Sharpe (MRS) agar which is selective for many LAB taxa (De Man, et al. 1960) and tryptic soy (TS) agar which enables the growth of many phyllosphere community members (Williams et al. 2013). We paired these culture-based approaches with amplicon sequencing of the 16S rRNA gene (V4 variable region) to determine the relative abundance (RA) of LAB as all reads assigned to the order Lactobacillales compared to the total number of bacterial reads in each sample. Plating demonstrated that LAB were low in abundance in the NCP, with an average total cultural abundance of 0.76% at Site 1, 0.51% at Site 2, and 1.09% at Site 3 (**Fig. 3.2A**). Amplicon sequencing also demonstrated the low abundance of LAB with a RA of 0.30% at Site 1, 0.89% at Site 2, and 1.68% at Site 3 (**Fig. 3.2B**).

Amplicon sequencing enabled us to determine not just the relative abundance of LAB, but also the diversity of LAB genera present in the NCP. Members of the Lactobacillaceae, Leuconostocaceae, and Streptococcaceae were detected in low numbers on the cabbages from all three sites, including the genera *Lactobacillus*, *Fructobacillus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus*

(**Table S3.1**). These genera contain species that are important for vegetable fermentation including *Leuconostoc mesenteroides*, a common heterofermentative LAB detected in the early stages of many fermented vegetables (Zabat, et al. 2018) and *Lactobacillus plantarum*, a homofermentative LAB that is typically found in the later stages of fermentation (Zabat, et al. 2018). Although the NCP reads assigned to LAB were low, all of the main LAB families essential for fermenting vegetables were detected at all three sites.

Sequence-based data on cabbage phyllosphere bacterial diversity is very limited (Lee, et al. 2015; Einson et al. 2018) and our amplicon sequence data helps fill this gap. Across the three sites, Proteobacteria dominated the amplicon sequence datasets, with Bacteroidetes, Firmicutes (non-LAB), and Actinobacteria making up smaller fractions of the NCP at each site (**Fig. 3.2B, Table S3.2**). This phylum-level composition is similar to the phyllosphere microbiomes of other leafy vegetables (Williams et al. 2013; Williams and Marco 2014; Leff and Fierer 2013; Rastogi et al. 2012) and a range of other plant species where Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes dominate (Williams and Marco 2014; Bulgarelli et al. 2013; Knief et al. 2012; Reisberg et al. 2013; Rastogi, Coaker, and Leveau 2013). The most abundant bacterial genera detected across the three sites were *Sphingomonas*, *Pseudomonas*, and *Methylobacterium*, which are also abundant taxa in many phyllosphere microbiomes (Vorholt 2012; Vacher et al. 2016). While the same broad taxonomic groups of bacteria were found across the three sites, there were significant differences in phyllosphere community composition across all three sites (non-parametric PERMANOVA; F=34.02,

$p<0.001$), suggesting differences in local species pools can drive variation in NCP assembly across sites.

To determine how LAB abundances in the NCP change from phyllosphere to fermentation, we used small-scale fermentations to measure fermentation potential of NCP material from each of the three sites. Leaves were chopped into small pieces, combined with salt (2% w/w), and fermented at 24 °C for 14 days. At Site One and Site Three, the RA of LAB in the community increased as the cabbage was fermented (**Fig. 3.2B**) and we observed characteristic declines in pH in our small-scale fermentations (**Fig. S3.1**). The increase in LAB was particularly striking at site three where the LAB went from ~1% RA in phyllosphere samples to a RA of > 50% in three out of the four fermented samples (**Fig. 3.2B**). The fermentation of Site 2 phyllosphere samples diverged from Site 1 and Site 3 in that Site 2 communities were dominated by Proteobacteria and had very low amounts of LAB at the end of fermentation. As with the phyllosphere communities, the composition of fermented bacterial communities was significantly different across sites (non-parametric PERMANOVA; $F=21.43$, $p<0.01$) suggesting that initial phyllosphere composition can translate into differences in final fermentation community composition.

To confirm that LAB are consistently low in abundance in the NCP and our findings were not an artifact of our experimental manipulations, field sites, or cabbage variety, we used the same culture-based and amplicon sequencing approaches to measure LAB in Napa cabbages purchased from five supermarkets in Boston, Massachusetts. Using culture-based methods, LAB had a low abundance in all of the sampled cabbages with a total cultural abundance between 0.004% to 0.78% (**Fig. 3.2C**). Using amplicon sequencing on cabbages from one of the supermarkets, the RA of LAB in the NCP was found to be 0.16%, supporting the culture-based results (**Fig. 3.2D, Table S3.3**). Together these results indicate that LAB are not abundant in the NCP both in experimental cabbages grown in our field sites and in commercially available cabbages. Building on this observation, we next sought to determine ecological explanations for the rarity of LAB.

LAB are not abundant in local species pools across the Northeastern US. One explanation for why LAB have a low abundance in the Napa cabbage microbiome is that they are rare in local species pools in farms and gardens, or source-limited (**Fig. 3.1A**). A limited abundance of LAB in species pools would provide few opportunities for dispersal of LAB cells to the NCP. While there are many potential species pools that could be sources of LAB, such as water (Yanagida, et al. 2007), insects (Lilley et al. 1997; Vásquez et al. 2012), soil (Bai et al. 2015), and leaves (Lindemann and Upper 1985), we selected and tested the two species pools which we predicted could be the main reservoir for the phyllosphere microbial community: soil and leaves.

To determine whether LAB are source-limited, we sampled soil and leaves from 51 sites throughout the Northeastern United States (**Fig. S3.2**) and used selective plating and amplicon sequencing to determine LAB abundance in the species pools. Sites 1, 2, and 3 from above were included in this species pool survey. Sampling locations were small farms or community gardens where cruciferous vegetables including cabbage were being grown or had been grown in the recent past. At each site, soil and leaves were collected from five randomly selected locations and were then pooled and homogenized to give a site-specific sample. Soil samples were taken from the top 5 cm of soil after dead plant litter was removed. Leaf samples consisted of a mix of vegetation including crop and weed species (*Amaranthus retroflexus*, *Portulaca oleracea*, *Cerastium arvense*, *Chenopodium album*, and *Ambrosia artemisiifolia*). We did not directly sample cabbage leaves because each site had a range of cabbages of different species, varieties, and ages and because some sites were not growing cabbage while we were sampling. Weed species growing within each site provided a common potential species pool that could be consistently sampled. We used the same paired culture-based plating and 16S rRNA gene amplicon sequencing as described above to determine the RA of LAB in the NCP to estimate the RA of LAB in soil and leaf species pools.

Across all 51 sites, LAB had a low abundance in both the leaf or soil species pools. With culture-based methods, the mean RA of LAB was 1.55% in the leaves and 3.20% in the soil (**Fig. 3.3A**). Using 16S rRNA gene amplicon sequencing across a subset (n=14) of the soil and leaf samples from across the geographic range sampled, we found a much lower RA of LAB, where leaves had a RA of 0.32% and soil had a RA of 0.14% (**Fig. 3.3B, Table S3.4**). The lower RA of LAB detected using amplicon sequencing is likely due to an inability to culture many leaf and soil bacteria on TS agar and incomplete selectivity of the MRS agar leading to undercounting of non-LAB community members relative to LAB. This survey across the Northeastern US indicated that LAB are rare members in the sampled species pools of soil and leaves. These species pools could be the main reservoir for the Napa cabbage microbiome and that the scarcity of LAB in these species pools may contribute to an overall low abundance of LAB in environments where Napa cabbages grow.

To determine whether LAB that colonize NCP can be found in the soil and leaf species pools, we compared LAB detected in the NCP phyllosphere data collected from Sites 1-3 with our survey of soil and leaf samples across the 51 sites. All of the LAB taxa identified in the NCP were also detected in the soil and leaves species pools (**Table S3.1**). However, there were LAB identified in these species pools which were not detected in the NCP. For instance, *Vagococcus* and *Enterococcus* (Enterococcaceae) were present in one of the soil samples, but not present in any of the sampled NCPs. *Facklamia* and *Aerococcus* (Aerococcaceae) as well as *Alloiococcus*, *Desemzia*, *Marinilactibacillus*, *Dolosigranulum*, *Granulicatella*, *Atopoistipes* (Carnobacteriaceae) were present in a few of the sampled species pools (both soil and leaves) but were rarely detected in the NCP. These data indicate that the sampled species pools could be reservoirs of LAB that can eventually colonize cabbage leaves.

LAB are in low abundance across a global sample of soil and leaf species pools. To further examine the distribution and abundance of LAB in the environment we determined the relative abundance of taxa from the order Lactobacillales in a recently published global survey of bacterial communities (Thompson et al. 2017). At a global scale, LAB were in low abundance with a mean RA of ~2.0% (SD: +/- 9.2%). In the majority of soils and plant-associated systems, LAB were particularly rare with average RAs less than 0.001% except in steppe (mean RA= 3.7%) and dry soils (mean RA= 1.6%) (**Fig. 3.3C**). Outside of soil and plant-associated systems, LAB were present in low relative abundance across multiple ecosystems (mean RA <0.01%; **Fig. S3.2**). Several hotspots for LAB abundance were observed in insect-associated habitats (mean RA= 22.5%), human-associated habitats (mean RA= 15.1%), bird nests (mean RA=11.1%), coral reefs (mean RA= 10.9%), animal-associated habitats (mean RA=9.3%), and city environments (mean RA= 7.8%). These global data suggest that the abundance of LAB is constrained across many plant and soil environments and the rarity of LAB is not unique to the Napa cabbage phyllosphere environment.

Gnotobiotic and field-grown Napa cabbages demonstrate that LAB are establishment-limited. In addition to being source-limited, establishment limitation could also constrain the abundance of LAB in the NCP (**Fig. 3.1A**). Despite being in low abundance in the field environment, it is possible that LAB could be capable of rapid growth once they arrive in the NCP if they can easily establish. However, if LAB cannot colonize the leaf due to poor growth, weak competitive abilities, or an inability to tolerate the phyllosphere environment, they will remain at a low abundance in the cabbage phyllosphere.

To test whether LAB are establishment-limited, we developed gnotobiotic Napa cabbages. These cabbages were prepared by surface-sterilizing Napa cabbage seeds and planting them into sterilized glass tubes containing Murashige and Skoog (MS) basal salt broth and calcined clay (**Fig. 3.4A**). After the cabbages had grown for a week, we inoculated them with pure cultures of LAB along with varying levels of LAB mixed with a synthetic phyllosphere community (SPC) consisting of 14 bacterial taxa which represent the common phyla of bacteria detected in the NCP (**Table 3.1**). Four different lactic acid bacteria were tested: *Leuconostoc mesenteroides* strain BN10, *Lactobacillus plantarum* strain MKR2, *Lactococcus lactis* strain D119, and *Pediococcus pentosaceus* strain B6N. These bacteria represent some of the most common genera and species of LAB that are consistently found in fermented vegetable products (Xiong et al. 2012; Tamminen et al. 2004; Rhee, et al. 2011). The ability of LAB to establish in the NCP was measured by comparing their abundance at the start of the experiment (input) to their abundance after 10 days of growth in the NCP (output). Three different ratios of LAB relative to the SPC were inoculated onto the Napa cabbages to determine how initial LAB abundance impacts establishment: a 1:1, a 1:10, and a 1:100 initial starting ratio of LAB to SPC (**Fig. 3.4B**). The 1:100 dilution results in LAB abundances that approximate levels detected in our field experiments above. The treatment with high levels of LAB relative to the SPC (1:1) were designed to mimic high rates of LAB dispersal to the NCP (**Fig. 3.4B**).

All four of the lactic acid bacteria tested decreased in abundance when sprayed onto Napa cabbages as pure cultures, suggesting that LAB cannot grow alone on Napa cabbage leaves and instead die over time (**Fig. 3.4C**). Decreases in viable LAB cells ranged from ~3 log in *L. lactis* to ~1 log in *P. pentosaceus* (**Fig. 3.4C**). In the presence of the SPC, the decline of LAB was even greater, with all LAB decreasing when inoculated at a 1:1 ratio with the SPC. For example, in the presence of the SPC, there was a ~2 log fold decrease in viable *P. pentosaceus* cells.

Dilution of the LAB (the 1:10 and 1:100 LAB:SPC treatments) resulted in a greater decrease in LAB abundances (**Fig. 3.4C**). The most dramatic decrease was seen in *Lactobacillus plantarum*, which when co-inoculated with the SPC was not detected at any of the three LAB dilutions. In two of the tested LAB, *L. mesenteroides* and *P. pentosaceus*, no bacteria were detected after being applied at the lowest (1:100) dilution. In contrast to the decrease in abundance seen in all the LAB sprayed onto the NCP, the SPC increased in abundance in all treatments demonstrating that the experimental conditions were conducive to bacterial growth. The SPC had an average abundance of 1.6 x 108 CFU/g across all community treatments, which is comparable to levels detected in field grown cabbages (**Fig. 3.2A**).

To confirm that LAB are also establishment-limited in a field setting with larger cabbages and background levels of bacterial dispersal, three-week old Napa cabbage seedlings were inoculated with a mixed community of LAB (*Leuconostoc mesenteroides* strain BN10, *Lactobacillus plantarum* strain MKR2, and *Pediococcus pentosaceus* strain B6N; hereafter "+LAB") or phosphate buffered saline (PBS; hereafter "Control") and then planted into three treatment and three control beds in an experimental garden at Tufts University in Medford, Massachusetts in mid-May (**Fig. 3.5A**). We selected these three LAB because they are naturally resistant to vancomycin as are many members of the Lactobacillales (Campedelli et al. 2019; Swenson et al. 1990) and can therefore be easily tracked after being applied to plants in field settings. After a month of growth, a leaf was removed from each cabbage and the RA of LAB was estimated using culture-based approaches. Ten days later, the cabbages were sprayed again with the LAB mix or PBS and the abundance of LAB was determined after 17 days.

As with our lab grown gnotobiotic cabbages, LAB did not grow well in the phyllosphere of field-grown plants. After the initial inoculation, +LAB cabbages had an average LAB density of 1.0 x 106 CFU/g and only one out of 45 Control cabbages had detectable LAB (6 CFU/g, **Fig. 3.5B**). After one month, no LAB were detected in both the Control and +LAB cabbages demonstrating the same rapid die-off of LAB in the phyllosphere observed in laboratory conditions (Fig. 3.5B). At the time of the second inoculation, Control cabbages had background densities of LAB similar to what were observed in our field planted cabbages described above. This background LAB growth is likely due to colonization from local species pools or movement of LAB between treatment and control beds. The second inoculation raised the LAB density to an average density of 2.5 x 105 log CFU/g in the +LAB treatment, but LAB levels decreased to an average of 5.5 x 103 log CFU/g about two weeks later (**Fig. 3.5C**).

To determine whether supplementing field cabbages with LAB changed fermentation outcomes, we harvested Control and +LAB cabbages and fermented them in small (118 mL) sterile glass jars. Both Control and +LAB cabbages had similar fermentation dynamics, with no significant differences in total LAB abundance (**Fig. 3.5D**) or acidification (**Fig. 3.5E**) over the two weeks of fermentation (LAB abundance repeated measures ANOVA F1,4=0.01, p=0.95; acidification repeated measures ANOVA F1,4=0.18, p=0.69). These results demonstrate that low levels of LAB are sufficient for a successful fermentation to proceed and that supplementation of LAB in the field is unlikely to impact fermentation outcomes.

Discussion

Lactic acid bacteria are critical for successful vegetable fermentation and fermented vegetable producers rely on naturally occurring LAB during the production of these foods. While a number of common LAB species are detected in fermented products, there are few studies which investigate the ecology of these LAB in either the vegetable phyllosphere or the field environment (Lamont et al. 2017; Daeschel, et al. 1987). We used culture-based and sequencing methods to show that LAB are in low abundance in the phyllosphere of experimental and commercially available Napa cabbages. Using lab and field experiments, we investigated whether two components of propagule limitation - source and establishment limitation - could explain the low abundance of LAB in the NCP. Source limitation was tested by conducting a survey of two different species pools across 51 sites throughout the Northeastern US. This survey found that LAB were rare in both soil and leaf species pools and this result held true at a global scale. By inoculating LAB into the NCP it was also shown that they are unable to establish or grow, providing evidence that establishment limitation could also restrict their abundance. Surprisingly, instead of finding evidence for growth of LAB in the phyllosphere, we found that they die over time both alone and when growing with other phyllosphere bacteria. Our work demonstrates that while LAB are consistently found on Napa cabbage plants, they are always at low abundance and have limited capabilities to grow in the phyllosphere.

The culture-based and amplicon sequencing results indicate that LAB are consistently in low abundance in the Napa cabbage phyllosphere. Our study only used varieties of Napa cabbage (*Brassica rapa subsp. pekinensis*) and there are many other Brassica species and cultivars that are fermented including green, red, and savoy cabbage (all cultivars of *Brassica oleracea*). The different phylloplane structure and chemistry of these species and cultivars could influence the ability of LAB to grow and establish and LAB may not be establishment-limited for all cabbage varieties or all plant species. However, previous studies do support our observation of limited colonization by LAB in other phyllosphere systems. Metagenomic studies that were not specifically designed to study the ecology of LAB also found that Lactobacillales are generally in low abundance in the phyllosphere (Dees et al. 2015; Leff and Fierer 2013; Rastogi et al. 2012). Culture-based studies from the 1960s noted that LAB are in low abundance on the surfaces of cucumbers, beets, carrots, and other vegetables (Daeschel, et al. 1987). A recent survey of a fermentation production facility found that LAB are in low levels in the green cabbage phyllosphere (Einson et al. 2018). Collectively, these studies demonstrate a consistently low abundance of LAB across other species and varieties of cabbage as well as other vegetables.

Our survey of soil and leaf species pools from 51 sites demonstrates that LAB are rare in agroecosystems of the Northeast US. We focused on two species pools, soil and leaves, which we predicted would be the main inoculation source for the phyllosphere microbiome. Soil was thought to be a potential species pool as previous research has shown the phyllosphere is typically a subset of the bacteria detected in the rhizosphere (Bai et al. 2015). Leaves were also considered a potential species pool for the phyllosphere microbiome as neighboring plants growing in proximity to Napa cabbages could facilitate dispersal of microbes as plant leaves brush against one another or as other vectors (farm tools, humans, insects) move bacteria from one plant to another. LAB were in low abundance in both of these tested species pools which demonstrates the potential for source limitation of LAB in agroecosystems.

We acknowledge that there are other potential reservoirs of LAB that might colonize cabbage leaves that were not sampled in this study. For example water (Yanagida, et al. 2007), air (Lindemann and Upper 1985), and herbivores that feed on the cabbage leaf (Vásquez et al. 2012; Welte et al. 2016) could all contain LAB. Insects could be an especially important species pool to consider as certain insects, including *Scaptomyza* spp. and *Mamestra brassicae*, have been reported to vector bacteria between plants as they feed on the leaf (Lilley et al. 1997; Groen et al. 2016). Honey bee species have been shown to have LAB in their intestinal tracts (Vásquez et al. 2012) and although bees do not usually feed on leaves they could have other interactions with plant leaves which might transfer bacteria into the phyllosphere. Our analysis of the Earth Microbiome Project data revealed that members of the Lactobacillales were highest in abundance in insect-associated systems as well as in association with birds, and were low in abundance in water, air, and soil. This suggests that herbivory or excretion from birds may contribute

LAB to phyllosphere microbiomes. Future experiments that experimentally manipulate insect herbivory in the Napa cabbage phyllosphere could help assess the contribution of this microbial species pool to dispersal dynamics of LAB.

LAB were not able to grow well in the phyllosphere and levels decreased despite inoculating the cabbages with high amounts of LAB. Initial LAB inoculum lost viability over time and fell to levels comparable to those detected in the Napa cabbages planted out at the three field sites. These data suggest that there may be a low carrying capacity for LAB on the leaf or that there is a constant influx of LAB which do not survive once they arrive in the phyllosphere. It is not surprising that LAB do not colonize the phyllosphere in high levels as they lack many of the microbial traits that are found in phyllosphere-adapted bacteria. For example, many phyllosphere bacteria are pigmented and use these pigments to protect against high levels of ultraviolet light on leaf surfaces (Bringel and Couée 2015; Lindow and Brandl 2003; Jacobs, et al. 2005). LAB generally lack photoprotective pigments (Carr, et al. 2002) and are likely poorly equipped to deal with damage caused by UV.

Studies using next-generation sequencing have shown a common pattern in microbial community composition where a few common species make up the majority of the community along with a ‘long tail’ of rare species (Lynch and Neufeld 2015; Sogin et al. 2006). These rare species might play a disproportionate role in ecosystem functioning or could function only when the environment changes (Jousset et al. 2017). Conditional rarity describes bacterial taxa that remain in low abundance until environmental conditions enable them to rapidly increase in abundance (Shade et al. 2014) and may be aptly applied to LAB in a farm-to-ferment framework (**Fig. 3.1B**). While the agroecological function of LAB was not tested in this study, it is possible that they remain rare in the environment but shift to an increased abundance following plant decomposition at the end of the growing season when plants are harvested. LAB are also important in creating silage where plants are fermented in anaerobic conditions on a farm (Ni et al. 2015; Driehuis, et al. 2001). Release of plant sugars and a shift to an anaerobic environment are two of the main environmental changes which are also present in fermented vegetable production. LAB are not good at persisting in the Napa cabbage phyllosphere, but the anaerobic, salty, and sugar-rich conditions of the fermentation environment allow these bacteria to become dominant members of the fermented food microbiome.

This research focused exclusively on the bacteria that make up the NCP microbiome. Most plants are colonized by fungi, protists, and other microbes that may contribute to the ecological distributions of LAB in the phyllosphere. For example, many fungi can live in the phyllosphere of cabbages as pathogens (El-Mohamedy and El-Mougy 2009; O'Hara, et al. 2016) and the phyllosphere yeast *Rhodotorula* is known to cause defects in fermented vegetable products (Fred and Peterson 1922; Barth et al. 2009). Future work should determine the contributions of these other microbes in the phyllosphere and fermentation microbiome assembly process.

We developed gnotobiotic Napa cabbages to dissect microbial community dynamics in the phyllosphere. Our model system integrating germ-free plants with a culture collection spanning the diversity of cabbage phyllosphere bacteria adds to the growing number of gnotobiotic systems being used to study how microbial communities assemble in the phyllosphere and rhizosphere (Lebeis et al. 2015; Bai et al. 2015; Niu et al. 2017). We acknowledge that young cabbages in test tubes with artificial soil medium do not fully recapitulate the dynamics of full-sized field-grown cabbages, but our results in the lab were supported by similar field experiments. The Napa cabbage phyllosphere model provides future opportunities to explore how other ecological processes, including microbe-microbe interactions, explain patterns of phyllosphere diversity and impact the quality of fermented vegetables.

Materials and Methods

Determining the abundance of LAB in the Napa cabbage phyllosphere. 'Kaboko' F1 hybrid organic Napa cabbage seeds (High Mowing Seeds) were surface sterilized by soaking in 10% bleach for 40 min, then rinsed eight times with sterile deionized water. Seeds were sown in plant pots (5.5 x 5.5 x 5 cm) and watered as necessary with autoclaved (121ºC for 20 minutes) deionized water. Pots were filled with Sunshine Mix 1 (Sun Gro Horticulture) that was autoclaved (121ºC for 20 minutes) to reduce its microbial load. Pots were then placed in sterile Sun bags (pore size 0.02 µm, Sigma-Aldrich) to minimize contamination with microbes from the lab environment. Pots were kept under light racks (full-spectrum T5 fluorescent bulbs) with a 16-h light cycle at 24°C.

After cabbages had grown at least three true leaves (about four weeks of growth), they were planted out at three sites in mid-August. Site 1 was an urban community garden with a mix of vegetables and flowers in Boston, Massachusetts, Site 2 was a raised garden bed used regularly for plant research projects at Tufts University (Medford, Massachusetts), and Site 3 was a small, rural farm used for vegetable production about 25 miles outside of Boston (Lincoln, Massachusetts, **Fig. S3.1**). To help reduce variation in soil conditions between sites, peat pots (Jiffy pots 10.16 cm wide by 10.16 cm deep) containing Sunshine Mix soil were first placed into the site in a grid formation (~25 cm spacing between pots) and the cabbages were planted into the peat pots. All cabbages were watered as needed throughout the growing season. Each plant was fertilized with 50 mL of fertilizer (3:4:4 N:P:K, 5% calcium, 1% magnesium, 2% sulfur) prepared in a concentration of 15 g/L and filter sterilized before application.

After two months (mid-October), cabbages were cut at the base of the head and placed in a Ziploc bag for transporting back to the lab. In the lab, leaf fragments were taken from each cabbage for amplicon sequencing and for plating. For amplicon sequencing, leaf fragments (~ 4 cm^2) were taken from three randomly selected leaves from across the plant (both inner and outer leaves) and frozen at -80°C for processing at a later date. Additionally, three leaf fragments per plant were taken for culture-dependent analyses. The leaf fragments were homogenized with 500 µL PBS in a 1.5 mL microcentrifuge tube using a sterile micropestle. The cabbage homogenate was then plated on tryptic soy (TS) agar plates containing cycloheximide (100 mg/L) to estimate total cultural phyllosphere bacteria and de Man, Rogosa, and Sharpe (MRS) agar plates containing cycloheximide (100 mg/L) to estimate total LAB abundance. After a week of aerobic growth at 24°C, colonies were counted from TS and MRS plates to determine CFU per gram of cabbage.

After samples of Napa cabbage were taken for plating and sequencing, the remaining cabbage was shredded using a Cuisinart DLC-2ABC Mini Prep Plus food processor for ~2 minutes and 2% (w/w) salt was added. Cabbage homogenate was then compacted into sterile 5 mL screwtop vials (Axygen Scientific) and maintained at 24ºC for 14 days. On days 1, 2, 4, 7, 10 and 14, four replicate ferments were harvested. Ferments were homogenized in a Whirl-pak bag. A 100µL sample of the liquid ferment was then frozen at -20^{o}C for DNA extraction and amplicon sequencing (see below). We acknowledge that these small-scale fermentations do not completely reflect the conditions in home or industrial fermentations where the production environment or other ingredients may introduce LAB (Lee, et al. 2015; Zabat, et al. 2018). However, these assays do provide an estimate of the fermentation potential of vegetable materials.

To determine the LAB abundance and diversity in commercial Napa cabbages, we purchased cabbages from five different supermarkets in the Boston, Massachusetts area. For each supermarket sampled, three Napa cabbages were purchased on the same day and returned to the lab where 20 g of cabbage from each of four outer leaves was placed into 118 mL Whirl-Pak bags. Additionally, 20 g of cabbage was taken from four inner leaves (leaves which were not exposed to environment) and also placed into 118 mL Whirl-Pak bags. Napa cabbage leaves were then homogenized in 50 mL of 1 x PBS for 1 min per bag. The cabbage homogenate was then plated on TS agar plates containing 21.6 mg/L of natamycin to estimate total cultural phyllosphere bacteria and MRS agar plates also containing 21.6 mg/L of natamycin to estimate total LAB abundance.

Amplicon sequencing of the 16S rRNA gene. DNA was extracted from leaf fragments or ferment liquid using the MoBio Powersoil kit (Qiagen) according to the manufacturer's instructions. To generate amplicon libraries, a portion of the 16S rRNA gene was amplified using the primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) (Caporaso et al. 2011). Golay barcodes (12 bp) were incorporated in reverse primer constructs to allow for multiplexing. Promega PCR Master Mix was used for duplicate PCRs in 25 µL reactions with 0.2 µM of each primer and 1µL of DNA template using the following thermocycler conditions: initial denaturation at 95°C for 5 minutes; 35 cycles of 45 seconds at 95°C, 60 seconds at 50°C, and 90 seconds at 72 °C; a final elongation at 72 °C for 10 minutes. PCR amplicons were pooled, cleaned and normalized using SequalPrep Normalization Plates (Life Technologies). Equimolar concentrations were pooled for sequencing on an Illumina MiSeq in the CU Boulder BioFrontiers Sequencing Center using the v2 300-cycle kit (Illumina Inc.).

QIIME (Gregory Caporaso et al. 2010) and UPARSE (Edgar 2013) were used to process amplicon sequencing data as described by Andrei et al. (Andrei et al. 2015) with some modifications. Sequences were demultiplexed using the Golay barcodes via QIIME v1.9.1 (Gregory Caporaso et al. 2010). The following options were used to obtain raw forward and reverse fastq read files: split_libraries_fastq.py -q 0 --max_bad_run_length 250 --min_per_read_length_fraction 0.0001 --sequence_max_n 250 --store_demultiplexed_fastq. Paired-ends were merged in usearch v8 (Edgar 2010). Data from two independent runs on an Illumina MiSeq were then combined and then reads were quality filtered and OTU tables were constructed using the UPARSE pipeline (Edgar 2013). OTUs were clustered at 97% sequence similarity with de novo chimera detection enabled. The following parameters were used to modify the UPARSE pipeline: the –minh option of -uchime_ref was set to 1.5 for reference based chimera removal to reduce the false positive detection of chimeras; the OTU table was generated by mapping quality filtered reads back to the OTU seeds by setting the following –usearch_global parameters: -maxaccepts 0 -maxrejects 0, in order to avoid over-inflation of specific OTU counts and ensure that individual reads are correctly mapped to their respective OTUs. Consensus taxonomy was assigned using QIIME v1.9.1 (Caporaso et al. 2010) on a custom database sourced from SILVA v128 (Pruesse et al. 2007). Because LAB are rare in the Napa cabbage phyllosphere, we did not rarefy the amplicon sequencing data as this could lead to the loss of rare LAB reads.

Identifying LAB in local species pools. To determine whether soil or neighboring plants are major reservoirs of LAB, we quantified the abundance of LAB at 51 farms or community gardens throughout the Northeastern United States using culture-based and amplicon sequencing approaches. Farmers were recruited using local extension agencies and regional farm networks. From each farm, five randomly selected sites were sampled and a total of 20 g of leaf material and 20 g of soil from the top 5 cm of soil were collected. Samples were kept at 4°C for one or two days before being processed at the lab. Slurries of either leaf or soil were made by placing the collected material into a Whirl-Pak bag together with 50 mL of PBS and then macerated the sample for 5 mins. Equal volumes (10 mL) of each of the five soil and five leaf samples from each site were pooled to create a pooled leaf mix and a pooled soil mix.

A culture-based approach was used to determine the abundance of LAB in all leaf and soil samples homogenates. Soil and leaf samples were plated onto both MRS and TS plates containing 21.6 mg/L natamycin. Colonies were counted after aerobic incubation at 24°C for one week and the ratios of CFU growing on MRS plates to TS plates were used to provide an estimate of the RA of LAB in the phyllosphere community. LAB colonies were distinguished from Enterobacteriaceae colonies which can grow on MRS plates as LAB are small white, creamy, and/or translucent colonies whereas Enterobacteriaceae colonies are large, vicious, and cream colored. In addition, amplicon sequencing was performed on a subsample (14 out of 51 sites) of leaf and soil slurries to confirm the plating data. DNA was extracted using a MoBio Powersoil DNA extraction kit and amplicon libraries were prepared and sequenced as previously described.

Gnotobiotic Napa cabbages. Calcined clay (Turface) was washed repeatedly with tap water to remove fine particles and dust. The clay was then autoclaved (121ºC for 20 minutes) to reduce bacterial load. Cabbage growth chambers were prepared by adding 10 g of calcined clay and 10 mL of Murashige and Skoog (MS) basal salt broth (4.4 g/L of basal salt to water) to 15 cm x 2.5 cm glass test tubes. Tubes were covered with 22 mm Magenta two-way test tube caps (Sigma-Aldrich) and autoclaved at 121ºC for 60 mins. 'Kakabo' Napa cabbage seeds were sterilized by placing 100 seeds in a 1.5mL microcentrifuge tube, vortexing the seeds for 5 mins in 1 mL of 70% ethanol, and then vortexing again for 5 mins in 1 mL of 50% bleach. Ethanol and bleach were rinsed away using four washes in sterile deionized water with vortexing for 5 mins. The seeds were left soaking in the sterile deionized water for at least one hour to help soften any remaining seed coat. Using sterilized tweezers, one sterile seed was added to each prepared tube and the lid was firmly replaced. Tubes were placed under light racks (full-spectrum T5 fluorescent bulbs) with a 16-h light cycle at 24°C. The relative humidity inside of the test tubes was 55%.

Creating a synthetic phyllosphere community for Napa cabbage. A synthetic phyllosphere community (SPC) was created by sampling a range of fresh Napa cabbage leaf samples collected from the cabbage field experiment previously described (Sites 1, 2, and 3). We isolated a range of phyllosphere bacteria by plating leaf homogenates onto the following media: yeast extract peptone dextrose, tryptic soy, Pseudomonas isolation, M17, and de Man, Rogosa, & Sharpe. Single colonies of different morphotypes were streaked out and identified using 16S rRNA gene sequencing with primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT) (Weisburg et al. 1991). Identities and NCBI accession numbers of strains are given in Table 1. Strains were made into experimental glycerol stocks by growing each strain as a overnight liquid culture (TS broth) and pelleting the overnight at 3000 x g for 5 mins at 4ºC. The supernatant was discarded and the pellet was then washed with 1 x PBS before it was resuspended in 15% glycerol in 1 x PBS.

Inoculating gnotobiotic cabbages to test establishment limitation. Inocula of all 14 members of the SPC and four LAB (*Leuconostoc mesenteroides* strain BN10, *Lactobacillus plantarum* strain MKR2, *Lactococcus lactis* strain D119, and *Pediococcus pentosaceus* strain B6N) were prepared from frozen glycerol stocks. These glycerol stocks were prepared by growing each strain on solid media (TS agar for SPC strains and MRS agar for LAB) and then scraping cells of each of the strains into sterile 15% glycerol before storage at -80°C. Multiple tubes were prepared for each strain and one tube was thawed and plated before experiment setup to determine viable cell densities (CFU/µL).

All of the SPC members were diluted in 1 x PBS to 10,000 CFU/ µL, and 8 mL of each stock was combined to create a solution containing equal concentrations of each of the 14 members. Each of the four LAB were also diluted in PBS to 10,000 CFU/ µL. Multiple treatments with different mixing ratios of LAB:SPC were prepared: 1:1, 1:10, and 1:100. These ratios were prepared by mixing equal volumes of the full strength LAB and SPC inocula (1:1) or by mixing 10-fold dilutions of the LAB inoculum with the full strength SPC inoculum for 1:10 and 1:100 concentrations. Inocula of each of the LAB alone was also prepared for a total of 16 treatments: each LAB alone and each LAB in a mix with the SPC in ratios of 1:1, 1:10, and 1:100. A negative control bottle containing just 1 x PBS was also prepared to test for contamination of sterile plants, bottles, or reagents.

All 16 treatments were sprayed onto plants using amber Boston round glass bottles (59.15 mL) with an atomizer. Bottles and atomizers were sterilized by soaking them in a 30% bleach solution for at least 30 minutes and then rinsing them with sterile deionized water. Three pumps of inoculum (approximately 600 µL) were applied to each cabbage using the atomizer with 16 replicate cabbages per treatment. After spraying, half the cabbages (n=8) were harvested to enumerate the input bacteria inoculated. To harvest, cabbages were removed from the tubes with sterilized tweezers and roots were cut off using sterilized dissection scissors. The entire mass of the cabbage was weighed in a 1.5 mL microcentrifuge tube and 400 µL of 1 x PBS was added to each tube. Leaves were then homogenized with a sterile micropestle and the cabbage slurry was then diluted and plated onto TS agar and MRS agar plates to quantify total phyllosphere bacteria and LAB abundance, respectively. Cabbages were left to grow at 24°C under light racks as described earlier for 10 days. All remaining cabbages in each treatment were harvested in the same manner as the inputs to get output counts on TS agar and MRS agar.

Testing establishment limitation in the field. To test whether LAB are establishment-limited in a field setting, seeds of the Napa cabbage variety ‘Bilko F1 OG’ were surface sterilized as described above and planted in pots containing Sunshine Mix soil. We switched seed varieties in this experiment (from ‘Kakabo’ used above) due to a limited availability of ‘Kakabo’ seeds. Pilot experiments comparing the growth of LAB and SPC isolates found no differences between the ‘Bilko F1 OG’ and ‘Kakabo’ varieties. For our field experiments, we did not use the sterile tissue culture approach described above because preliminary experiments suggested cabbages grown in ambient lab conditions had no detectable lactic acid bacteria. We did not attempt to control the composition of other phyllosphere bacteria in this experiment. After the cabbages had grown under light racks (same conditions described above) for three weeks, they were inoculated with a mixed community of LAB including *Leuconostoc mesenteroides* BN10, *Lactobacillus plantarum* MKR2, and *Pediococcus pentosaceus* B6N (+ LAB) or sprayed them with PBS as a control (Control). Inoculum was added using the same approach described above for gnotobiotic lab plants where three pumps of inoculum (approximately 600 µL) was sprayed using an atomizer attached to a sterile glass bottle. This is “First Inoculation” in Figure 3.5. One leaf was removed from each replicate cabbage post-inoculation (n=45 per treatment) to determine the density of LAB inoculated per gram of cabbage (on MRS agar) and the density of background phyllosphere bacteria (on TS agar). The cabbage leaf was weighted in a 1.5 mL microcentrifuge tube then 400 µL of PBS was added. The cabbage leaf was homogenized with a sterile micropestle and the cabbage slurry was then diluted and plated on MRS agar plates with 21.6 mg/L of natamycin to inhibit fungi and 100 mg/L of vancomycin to reduce the growth of Enterobacteriaceae. The cabbages were then planted into raised beds (183x 122 x 38 cm) filled with a mix of field soil and composted cow manure in an experimental garden at Tufts University in mid-August. Three beds were assigned as controls and three were assigned as treatments. After a month of growth (mid-September), a leaf was removed from each replicate cabbage (n=40), placed into a 29.57 mL Whirl-Pak filter bag (Nasco, Modesto, CA) and taken back to the lab for processing. This is "First Output" in Figure 3.5. Approximately 0.4 g of the cabbage leaf was placed into a 1.5 mL microcentrifuge tube with 400 µL of PBS and homogenized with a sterile micropestle. The homogenized cabbage slurry was then diluted and plated on MRS plates with 21.6 mg/L of natamycin to inhibit fungi and 100 mg/L of vancomycin to reduce the growth of Enterobacteriaceae (Mathur and Singh 2005). Ten days after the first harvest, the cabbages were sprayed again with LAB inoculum or PBS ("Second Inoculation" in Figure 3.5) and the abundance of LAB was tracked after 17 days (early October, "Second Output" in Figure 3.5) via plating as was just described.

Fermentation of field-grown cabbages. Field-grown cabbages from the Control and +LAB treatments were fermented in 4 ounce (~118 mL) sterile glass canning jars with metal screwtop lids. Cabbages from each Control or +LAB plot were pooled (Control Bed 1 = 13 cabbages, Control Bed 2 = 15 cabbages, Control Bed 3 = 15 cabbages, +LAB Bed 1 = 15 cabbages, +LAB Bed 2 = 12 cabbages, +LAB Bed 3 =14 cabbages) and finely sliced using a knife sterilized with 70% ethanol. This chopped cabbage was then mixed for 5 mins with salt to obtain 2% (w/w) salt concentration. Equal amounts of the cabbage mix was distributed into the sterile jars, tightly compacted, and the lids were then sealed. Jars were incubated in the dark at 24^{o}C. LAB abundance and pH was measured at 0, 1, 2, 4, 7, and 14 days. At each timepoint the jar of sauerkraut was emptied into a sterile 118.29 mL Whirl-Pak bag and fully mixed to homogenize the sample. LAB abundance was determined by plating cabbage juice from the ferment onto MRS agar. pH was determined by inserting a pH probe into the Whirl-Pak bag with approximately 20 mL of liquid.

Analysis of the global distribution of LAB. To further understand the ecology of LAB at a global scale, we investigated their relative abundance in the recently published Earth Microbiome Project (Thompson et al. 2017). A rarefied OTU table (10,000 sequences per sample) that was generated from closed reference OTU clustering was retrieved from the EMP website (ftp://ftp.microbio.me/emp/release1). The table was filtered to identify members of the order Lactobacillales in QIIME and analyzed in R.

Statistical analysis. Statistical analyses were performed using R version 3.5.1. All CFU data were log transformed prior to analysis. PERMANOVAs were conducted using the vegan package. Repeated measures analysis was carried out using the lme4 package and the post hoc test Anova was carried out with the car package. T-tests were corrected for repeat sampling using a Hochberg correction.

Data availability. Partial 16S rRNA gene sequences of bacterial isolates have been deposited in NCBI (see Table 3.1 for accession #s). Amplicon sequence data have been deposited in NCBI as Bioproject PRJNA510140 with Sequence Read Archive accession numbers SAMN10612113-SAMN10612199.

Acknowledgements

We are very grateful to the numerous farmers and community gardeners that provided access to their land for various aspects of this work. We are especially grateful to Erik Jacobs for providing farm access and support for pilot experiments. All amplicon library preparation and sequencing was performed by Jonah Ventures. This work was supported by USDA NIFA grant 2017-67013-26520. Megan Biango-Daniels, Casey Cosetta, and Freddy Lee provided helpful feedback on previous versions of this manuscript. Elizabeth Landis provided support with plant care. Kinsey Drake, Claire Forgan, and Emily Van Doren provided research support in the lab.

Tables

Table 3.1. Bacterial isolates used to make the synthetic phyllosphere community in gnotobiotic cabbages.

Strain ID	Phylum	Family	Genus/Species	NCBI Accession # of 16S rRNA gene sequence	Isolation source
BSC5	Alphaproteobacteria	Rhizobiaceae	Rhizobiaceae sp.	MK308543	Napa cabbage phyllosphere
BSC6	Gammaproteobacteria	Enterobacteriaceae	*Pantoea* sp.	MK308544	Napa cabbage phyllosphere
BSC12	Gammaproteobacteria	Enterobacteriaceae	*Pantoea* sp.	MK308545	Napa cabbage phyllosphere
BSC13	Actinobacteria	Microbacteriaceae	*Microbacterium* sp.	MK308546	Napa cabbage phyllosphere
BSC14	Bacteroidetes	Sphingobacteriaceae	*Pedobacter* sp.	MK308547	Napa cabbage phyllosphere
BSC44	Alphaproteobacteria	Caulobacteraceae	*Brevundimonas* sp.	MK308548	Napa cabbage phyllosphere
BSC45	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas* sp.	MK308549	Napa cabbage phyllosphere
BSC46	Actinobacteria	Micrococcaceae	Micrococcaceae sp.	MK308550	Napa cabbage phyllosphere
BSC51	Gammaproteobacteria	Xanthomonadaceae	*Xanthomonas* sp.	MK308551	Napa cabbage phyllosphere
BSC57	Betaproteobacteria	Alcaligenaceae	*Achromobacter* sp.	MK308552	Napa cabbage phyllosphere
BSC58	Alphaproteobacteria	Sphingomonadaceae	*Sphingomonas* sp.	MK308553	Napa cabbage phyllosphere
GR2	Actinobacteria	Microbacteriaceae	*Curtobacterium* sp.	MK308554	Napa cabbage phyllosphere
GR11	Actinobacteria	Microbacteriaceae	Microbacteriaceae sp.	MK308555	Napa cabbage phyllosphere
GR29	Bacteroidetes	Sphingobacteriaceae	*Sphingobacterium* sp.	MK308556	Napa cabbage phyllosphere
BN10	Firmicutes	Leuconostocaceae	*Leuconostoc mesenteroides*	MK329278	Napa cabbage phyllosphere
MKR2	Firmicutes	Lactobacillaceae	*Lactobacillus brevis*	MK329281	Fermented vegetable product
B6N	Firmicutes	Lactobacillaceae	*Pediococcus pentosaceus*	MK329280	Napa cabbage phyllosphere
D119	Firmicutes	Streptococcaceae	*Lactococcus lactis*	MK329277	Farm soil

Figures

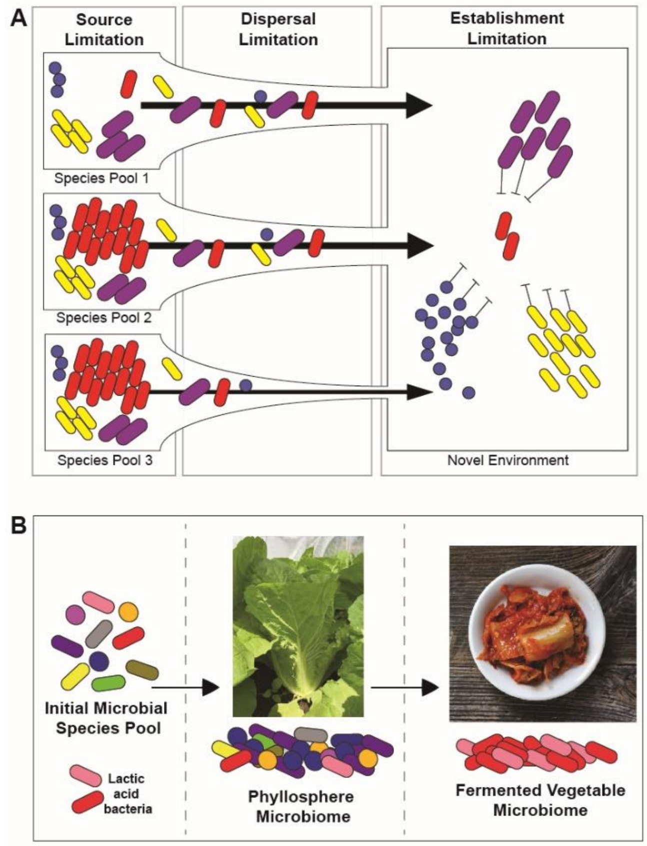


Figure 3.1. Conceptual overview of propagule limitation and the farm-to-ferment assembly of fermented vegetable microbiomes. (A) The three types of propagule limitation are source, dispersal, and establishment. Source limitation is where not enough propagules are present in local species pools to disperse to the phyllosphere. Dispersal limitation is where enough propagules are produced in local species pools, but they cannot reach all available habitats due to lack of dispersal opportunities. Establishment limitation is where local population sizes are constrained by the availability of habitats that can be colonized, for example by competition with other phyllosphere bacteria. (B) Local species pools consist of bacteria that could disperse to cabbage leaves to form the phyllosphere microbiome. When chopped up and fermented, the phyllosphere microbiome is reassembled to become the fermented vegetable microbiome.

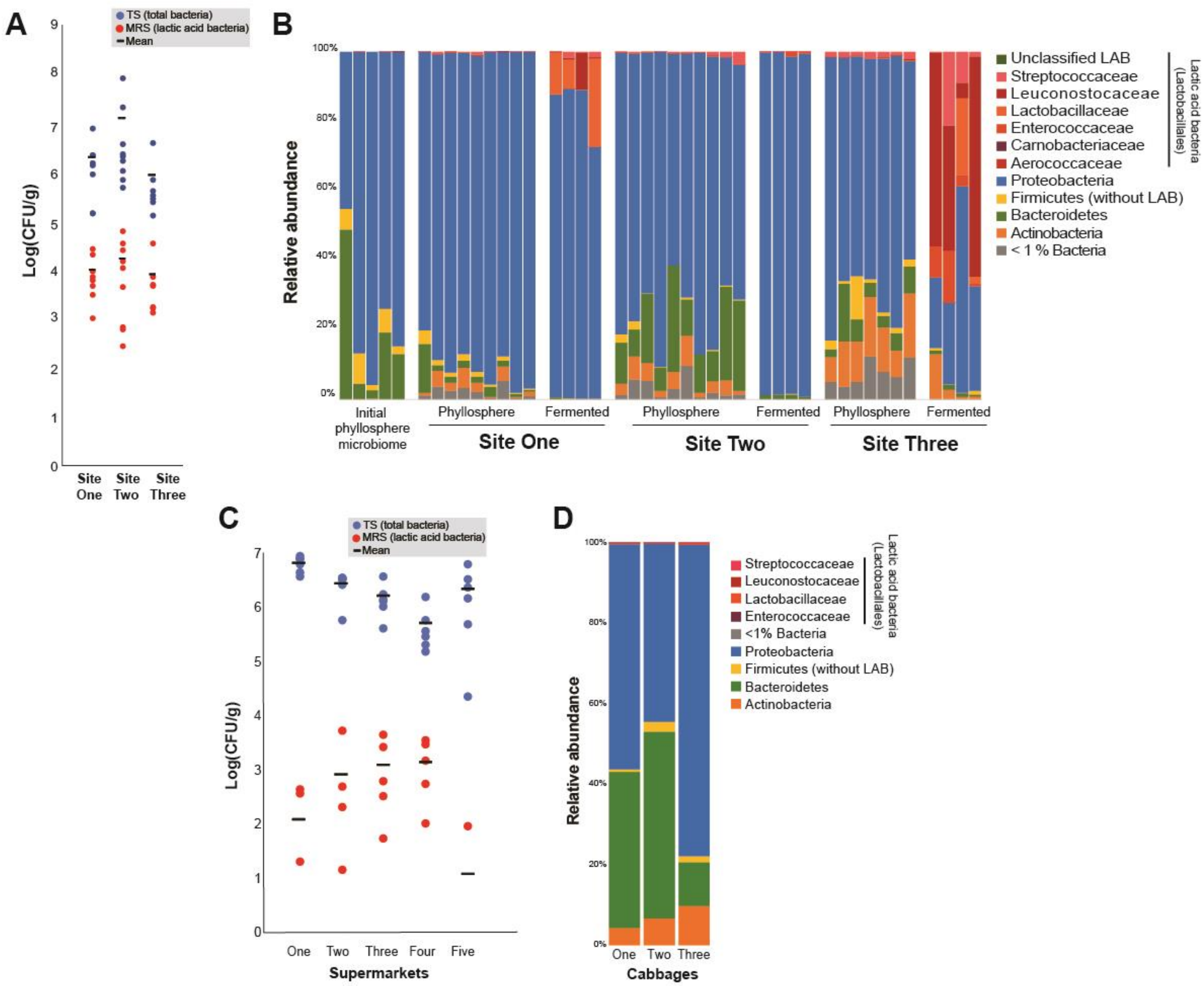


Figure 3.2. Microbial diversity and LAB abundance in the Napa cabbage phyllosphere

(A) Abundance of total culturable bacteria (TS agar in blue) and lactic acid bacteria (MRS agar in red) in the Napa cabbage phyllosphere at three sites in the Boston, area, MA. (B) Relative abundance of bacterial phyla and Lactobacillales families identified in the Napa cabbage phyllosphere using amplicon sequencing of the 16S rRNA gene. Each column represents an individual cabbage showing the initial phyllosphere microbiome of the lab-grown cabbage (n=5), Site 1 phyllosphere (n=9) and fermented (n=4), Site 2 phyllosphere (n=10) and fermented (n=4), and Site 3 (n=7) and fermented (n=4). Members of the Lactobacillales are shown at the family level in order to indicate the prevalence of major LAB groups. (C) Abundance of total culturable bacteria (TS agar in blue) and lactic acid bacteria (MRS agar in red) in the Napa cabbage phyllosphere from cabbages purchased at five supermarkets. For each supermarket n=6. (D) Relative abundance of phyla identified in the Napa cabbage phyllosphere purchased from one of the five supermarkets. Each column represents an individual cabbage. As with (B), members of the Lactobacillales are shown at the family level in order to indicate the prevalence of major LAB groups.

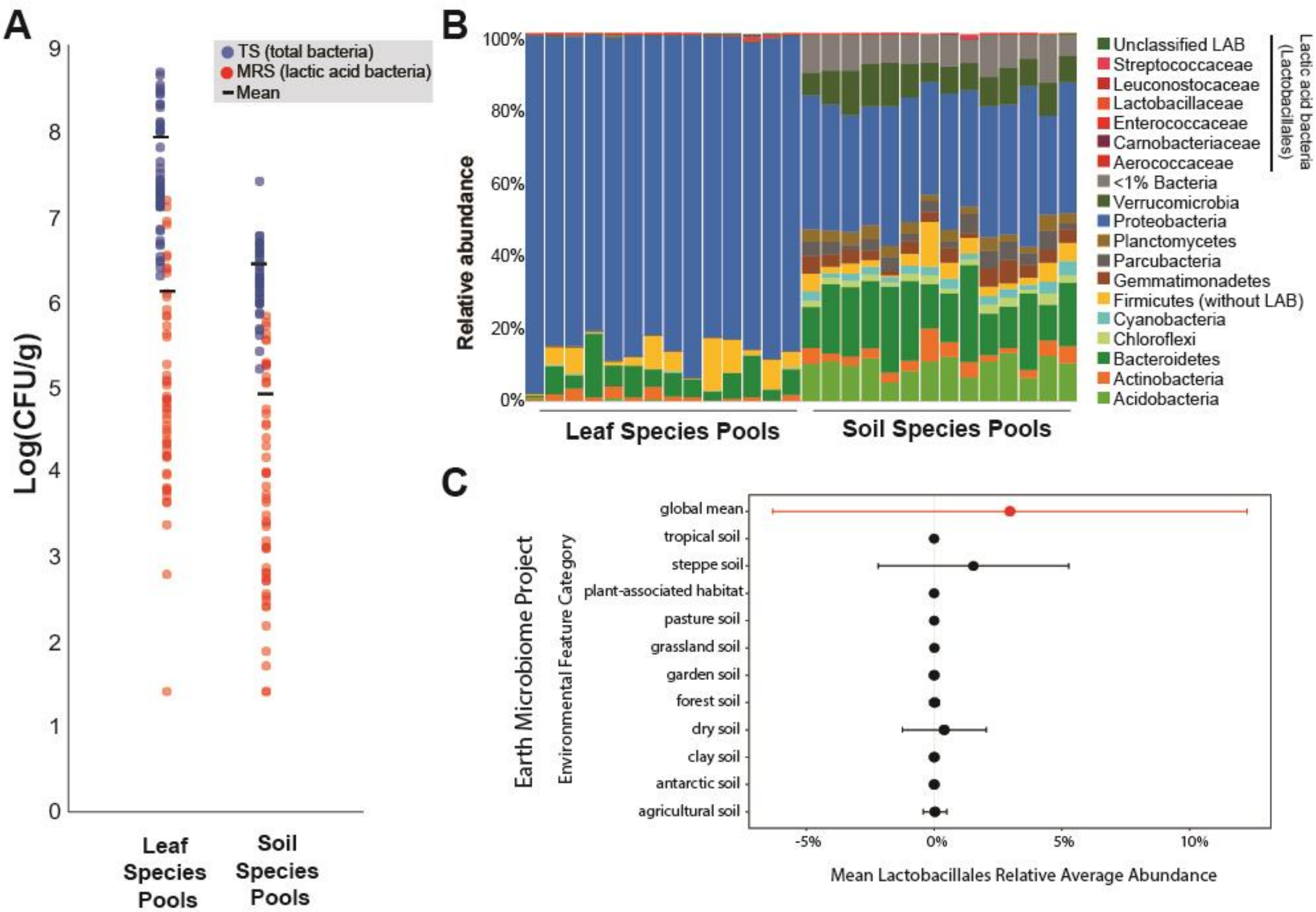


Figure 3.3. LAB are rare in species pools.

(A) Abundance of total culturable bacteria (TS agar in blue) and lactic acid bacteria (MRS agar in red) in leaf and soil species pools from 51 farms through the Northeastern United States. Each point represents CFU data collected from a pool of five leaf or soil samples from each site. (B) Relative abundance of bacteria found in leaf samples and soil samples at a subset of farms from (A) as determined with amplicon sequencing of the 16S rRNA gene (V4 region). As with Figure 3.2, members of the Lactobacillales are shown at the family level in order to indicate the prevalence of major LAB groups. Each column represents sequence data collected from a pool of five leaf or soil samples from each site. Data from 14 sites is presented. (C) Relative abundance of Lactobacillales in amplicon sequence data collected as part of the Earth Microbiome Project. Points indicate means and error bars indicate one standard deviation.

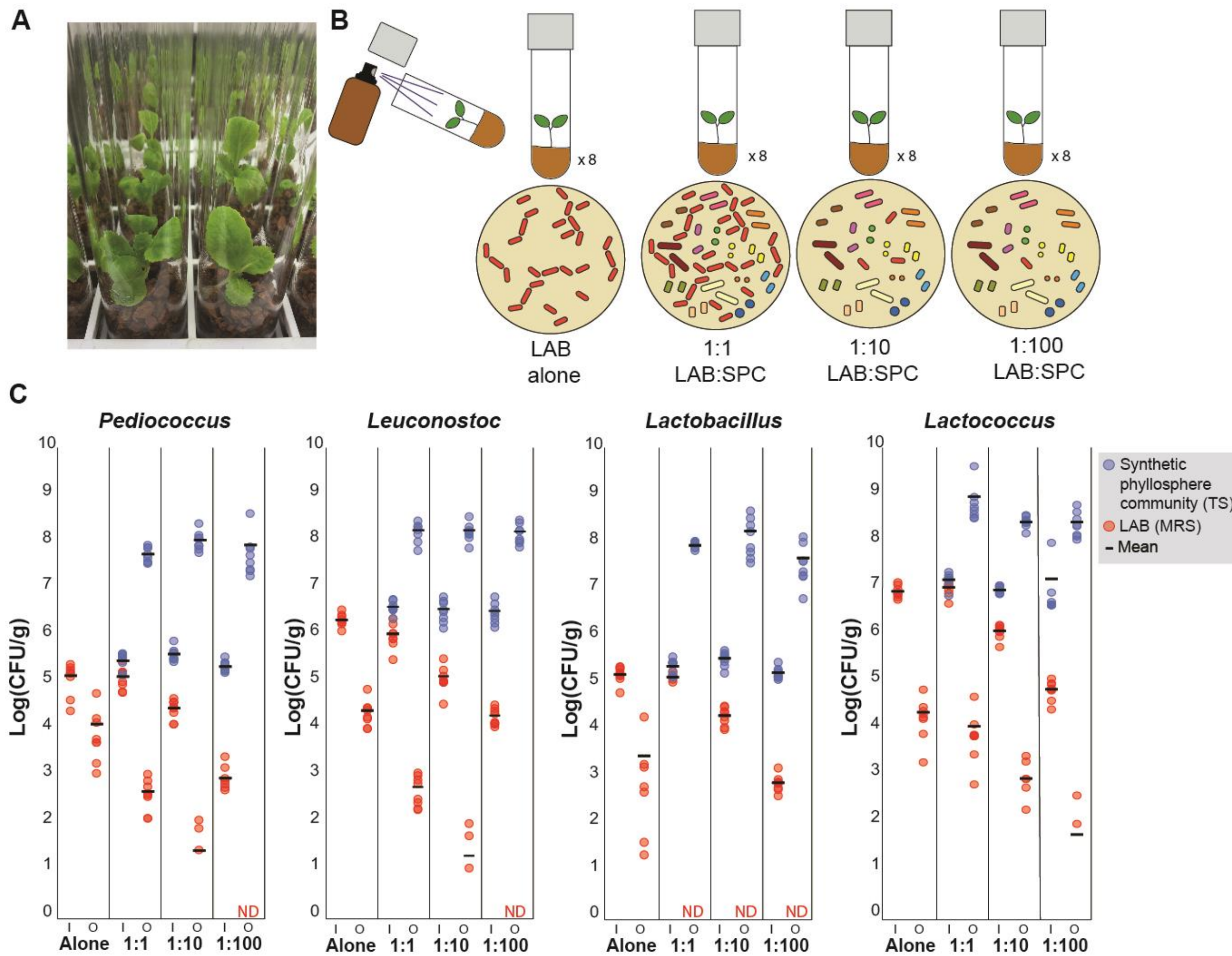


Figure 3.4. Gnotobiotic cabbages demonstrate that LAB are establishment-limited in the Napa cabbage phyllosphere.

(A) Photograph of gnotobiotic Napa cabbages used in experiments. (B) Overview of experimental design showing four treatments (LAB grown alone, LAB grown 1:1 with synthetic phyllosphere community or SPC, LAB grown 1:10 with SPC, LAB grown 1:100 with SPC). Treatments were applied using sterile brown amber spray bottles. Four lactic acid bacteria were used in these experiments: *Pediococcus pentosaceus* strain B6N, *Leuconostoc mesenteroides* strain BN10, *Lactobacillus plantarum* strain MKR2, and *Lactococcus lactis* strain D119. (C) Abundance of the SPC (TS agar in blue) and LAB (MRS agar in red) in the input (I) inoculum and output (O) after 10 days of growth in the NCP are shown. Input and output were significantly different from one another in all tested LAB species (t-test, $p < 0.5$, corrected for repeat sampling using a Hochberg correction). n= 8. ND = not detected.

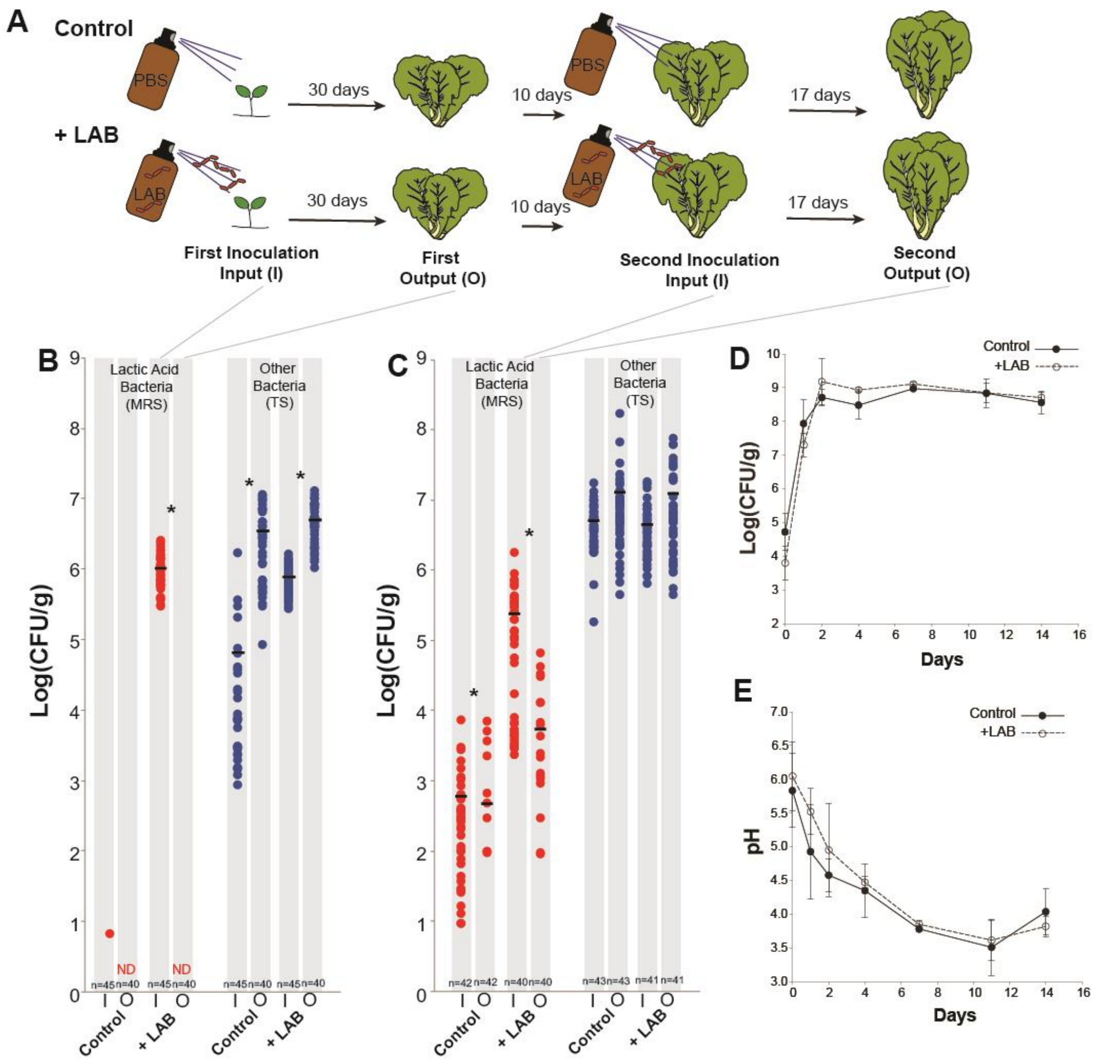
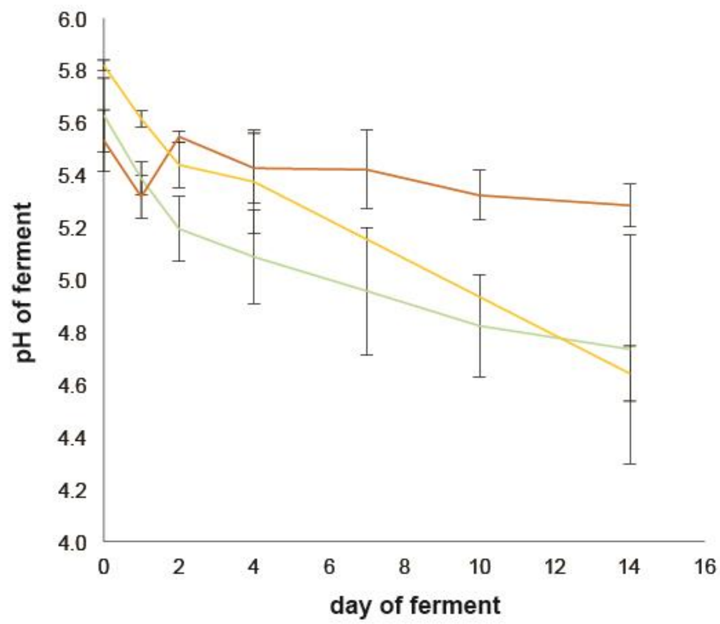


Figure 3.5. LAB are establishment-limited in the phyllosphere of field-grown Napa cabbages.

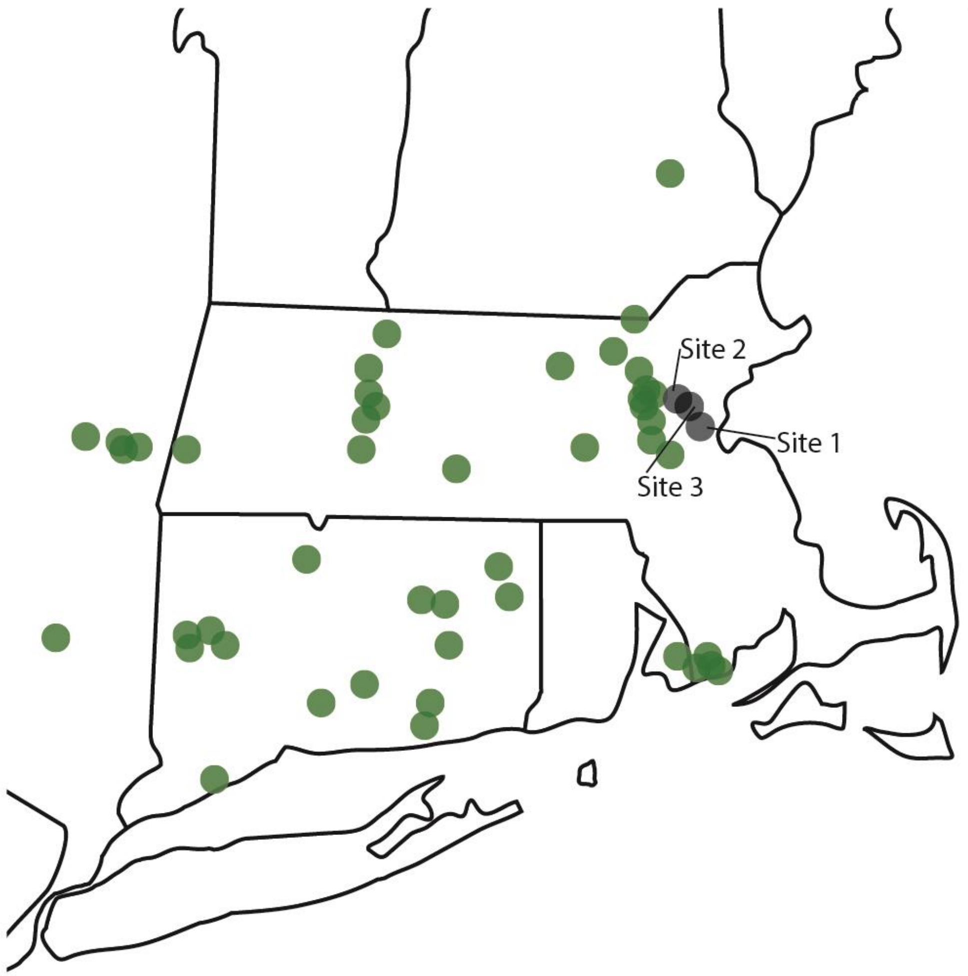
Figure caption 3.5 continued

(A) Overview of the experimental design. Napa cabbages were grown in the lab and were sprayed with either phosphate buffered saline (PBS) as a Control or an equal mix of three LAB (*Pediococcus pentosaceus* strain B6N, *Leuconostoc mesenteroides* strain BN10, *and Lactobacillus plantarum* strain MKR2) for the +LAB treatment. Input data was collected right after inoculation and output data were collected after 30 days of growth in the field. After 10 days of growth, cabbages were inoculated again with the same treatments. Input data were collected at the second inoculation and output data were collected after 17 more days of growth. (B) Abundance of LAB (MRS agar in red) and other phyllosphere bacteria (TS agar in blue) in the input (I) inoculum and output (O) leaf harvests for the first period of the experiment. Asterisk indicates significant difference between input (I) and output (O) bacterial densities (t-test, $p < 0.5$, corrected for repeat sampling using a Hochberg correction). The number of replicates is indicated at the bottom of the graph. ND = not detected. (C) Same as B, but for the second set of inputs and outputs. (D) Abundance of LAB during fermentation of cabbages from the Control and +LAB treatments in (B) and (C). Points indicate mean values (n=3). Error bars represent one standard deviation. (E) pH during fermentation of cabbages from the Control and +LAB treatments in (B) and (C). Points indicate mean values (n=3). Error bars represent one standard deviation. Control and +LAB treatments were not significantly different from one another in (D) or (E) (see text for repeated measures ANOVA statistics).

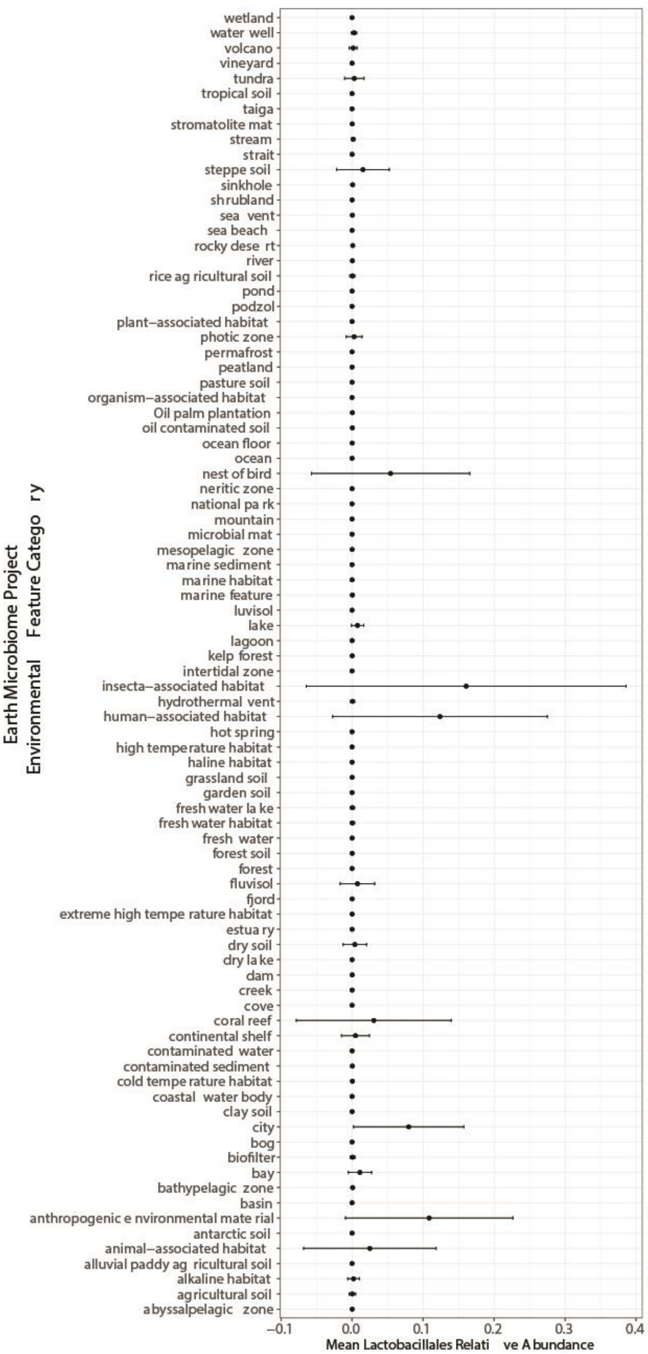
Supplementary Figures



Supplementary Figure 3.1. pH of cabbage ferments made with Napa cabbage phyllospheres from three different sites. Points represent replicate ferments (n = 4) sampled at each time point. Error bars represent one standard deviation.



Supplementary Figures 3.2. Locations of the 51 sites sampled in this study. Sites 1-3 used to generate data in Figure 3.1 are indicated. All sites were used to generate leaf and soil species pool data in Figure 3.3.



Supplementary Figures 3.3. LAB abundance in Earth Microbiome Project database. Data show the relative abundance of OTUs assigned to the Lactobacillales from various environments defined by the Earth Microbiome Project. These data include more categories

Supplementary Tables

Supplementary Tables 3.1. Genera of lactic acid bacteria detected in amplicon sequencing of the Napa cabbage phyllosphere from three sites, Napa cabbages purchased at five supermarkets, and soil and leaf species pools

					Napa Cabbage Phyllosphere - Site 1	Napa Cabbage Phyllosphere - Site 2	Napa Cabbage Phyllosphere - Site 3	Supermarket Napa Cabbage	Leaf Species Pool	Soil Species Pool
Phylum	**Class**	**Order**	**Family**	***Genus***	**Detected at Site?**	**Detected at Site?**	**Detected at Site?**	**# Supermarkets detected (out of 5)**	**# sites detected (out of 14)**	**# sites detected (out of 14)**
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Aerococcaceae	*D_5__Facklamia*	No	Yes	Yes	0	0	3
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Aerococcaceae	*D_5__Aerococcus*	No	No	No	0	1	2
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Carnobacteriaceae	*D_5__Alloiococcus*	No	No	No	0	0	0

D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Carnobacteriaceae	*D_5__Desemzia*	No	No	No	0	0	1
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Carnobacteriaceae	*D_5__Marinilactibacillus*	No	Yes	Yes	0	1	1
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Carnobacteriaceae	*D_5__Dolosigranulum*	No	No	No	0	0	1
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Carnobacteriaceae	*D_5__Granulicatella*	Yes	No	No	0	2	0
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Carnobacteriaceae	*D_5__Atopostipes*	No	No	No	0	0	1
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Enterococcaceae	*D_5__Vagococcus*	No	No	No	0	0	1
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Enterococcaceae	*D_5__Enterococcus*	No	No	No	0	1	1
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Lactobacillaceae	*D_5__Lactobacillus*	Yes	Yes	Yes	3	13	10

D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Lactobacillaceae	*D_5__Pediococcus*	No	No	No	0	2	0
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Leuconostocaceae	*D_5__Fructobacillus*	Yes	Yes	Yes	4	7	5
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Leuconostocaceae	*D_5__Leuconostoc*	Yes	Yes	Yes	1	3	2
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Leuconostocaceae	*D_5__Weissella*	No	No	No	0	1	0
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Streptococcaceae	*D_5__Lactococcus*	Yes	Yes	Yes	1	7	5
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Streptococcaceae	*D_5__Streptococcus*	Yes	Yes	Yes	3	11	7
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	D_4__Streptococcaceae	*Other*	Yes	Yes	Yes	4	12	10
D_1__Firmicutes	D_2__Bacilli	D_3__Lactobacillales	Other	*Other*	Yes	Yes	Yes	1	3	7

Four additional supplementary tables (see below) are available online at:

https://aem.asm.org/content/85/13/e00269-19/figures-only

Table S2. Summary of all OTUs identified in the Napa cabbage (*Brassica rapa* subsp. *pekinensis)* microbiome grown in the lab (before transplanted to field), after growing at three sites, and after fermentation

Table S3. Summary of all OTUs identified in the Napa cabbage (*Brassica rapa* subsp. *pekinensis*) purchased from a supermarket in the Boston, Massachusetts (USA) area

Table S4. Average abundance of OTUs identified in the species pools of soils and leaves

Chapter Four

Diversity of yeast and lactic acid bacteria in fermented vegetable products sold in the United States

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Abstract

Fermented vegetables are increasing in popularity in North America, but little is known about the diversity of their microbial communities. Shotgun metagenomic sequencing was used to determine the bacterial and fungal diversity of 17 different fermented cabbage products (sauerkraut and kimchi) made in the United States and Canada. We found an average of 10.8 species of lactic acid bacteria (LAB) per product using metagenomic sequencing along with low levels (<1% relative abundance of *Kazachstania*) of yeast in some of the products. The most common LAB species was *Enterococcus faecium,* which was found in high abundance in all products. Other LAB detected in products were *Leuconostoc mesenteroides*, *Lactobacillus (Lb.) plantarum*, *Lb. sakei*, and *Lb. brevis* which are commonly isolated from most fermented vegetable products. We were surprised to find yeast in over half of the sampled products, in some instances at a greater cell count than LAB. Yeast is known as a spoilage organism in fermented vegetable products and can reduce the quality of flavor and texture profiles. Further experiments using *in vitro* fermentations and a six-member synthetic LAB and yeast community show that the presence of yeast is predominantly explained by the temperature of the fermentation, although the salt concentration and type of cabbage used in the fermentation can also influence yeast abundance. These results provide the first comprehensive survey of North American fermented vegetable products and demonstrate how temperature, salt, and ingredients can drive the community structure of bacterial and yeast communities in these products.

Introduction

The consumption of fermented vegetable products has increased in the United States, in part due to the purported health benefits of fermented foods, but also as consumers seek to explore new flavors (Martins et al. 2013; James and Wang 2019; Rezac et al. 2018; Tamang et al. 2016; Park et al. 2014). Sauerkraut, a ferment made using salted cabbage, originates from eastern Europe, while kimchi, a fermented cabbage product containing chili, garlic, ginger and fish sauce, originates from Korea (Park et al. 2014; Jung et al. 2011; Zabat, et al. 2018). Fusions of sauerkraut and kimchi recipes are popular with many North American fermenters and result in a variety of spiced vegetable products (such as vegan kimchi) (Zabat, et al. 2018). Generally these vegetable products are spontaneously fermented by lactic acid bacteria (LAB) that naturally occur on the surface of vegetables (Park et al. 2014). While LAB can have a varied or low abundance in the phyllosphere at the outset of fermentation (Yu, et al. 2019; Miller et al. 2019), the high salt and anaerobic conditions of the ferment select for their rapid growth (Di Cagno et al. 2013). Fermentation proceeds as a succession of LAB with heterofermenters predominating at the outset of fermentation followed by homofermenters (Lee, et al. 2015).

While production and consumption of fermented vegetables has grown throughout North America, very little is known about the microbial diversity of the products made in this geographic region compared to other parts of the world. If the microbes that ferment vegetables are globally distributed then North American fermented vegetables could have very similar microbial compositions to the fermented vegetable microbiomes described in surveys of sauerkraut and kimchi in Europe and Asia (Johanningsmeier et al. 2007; Jung et al. 2011; Zabat, et al. 2018; Chang et al. 2008; Nam et al. 2009; Jeong, et al. 2013; Jeong, Lee, et al. 2013). Alternatively, they could have unique microbial compositions as their microbiomes are formed by dispersal from local communities into the ferment (Jung et al.

2018). In addition, the fermentation practices and novel recipes developed for some North American ferments could result in changes in the community composition (Jeong, et al. 2013). A few studies have characterized the diversity of sauerkraut and kimchi in single production facilities (Zabat, et al. 2018; Einson et al. 2018), but a comprehensive survey of store-bought ferments from a range of producers in the United States has not been conducted.

Based on previous studies, kimchi has a more diverse LAB community than sauerkraut (Tamang, et al. 2016). Lee et al (2015) used sequencing methods to show how the microbiome of raw ingredients can contribute to the overall microbiome of kimchi (Lee, et al. 2015). Only one heterofermenter, *Leuconostoc mesenteroides,* is commonly isolated from sauerkraut (Johanningsmeier et al. 2007) although many heterofermentative LAB have been isolated from kimchi including *Leuconostoc* spp (*L*), *L. mesenteroides, L. kimchii, L. citreum, L. inhae, L. gelidum, L. carnosum, L. gasicomitatum* and *Weissella (W)* spp. *W. koreensis, W. kimchii, W. cibaria* (Fusco et al. 2015). Likewise, only a few homofermenters are typically isolated from sauerkraut which include *Lactobacillus (Lb) plantarum*, *Lb. brevis*, *Lb sakei*, and *Pediococcus (P) pentosaceus* and *P. cerevisiae* (Wouters et al. 2013; Johanningsmeier et al. 2007). In kimchi, researchers have isolated *Lb. delbrueckii*, *Lb. buchneri*, *Lb. brevis*, *Lb. fermentum*, *Lb. curvatus*, *Ped. acidilactici*, and *Lactococcus lactis* (Jung et al. 2013; Park et al. 2010; Nam et al. 2009; Lee, et al. 2015).

While the diversity of LAB in fermented vegetable products has been thoroughly characterized, the abundance and diversity of yeasts in fermented products has received less attention and the factors that contribute to their presence are not well understood. Yeasts are capable of growing in acidic environments and are important components of other fermented foods such as sourdough and cheese where they grow alongside LAB (De Vuyst and Neysens 2005; Beresford et al. 2001). Despite their association with other fermented foods, yeasts are undesirable in vegetable ferments due to their production of yeasty off flavors, role in vegetable softening, and gaseous spoilage (Fleming et al. 1983; Moon et al. 2014; Viander, et al. 2003). There are several genera of yeasts which have been isolated from kimchi products, including *Candida*, *Halococcus*, *Haloterrigena*, *Kluyveromyces*, *Lodderomyces*, *Natrialba*, *Natronococcus*, *Pichia*, *Saccharomyces*, *Sporisorium,* and *Trichosporon* (Tamang, et al. 2016). A study of kimchi found five abundant yeast species in Korean kimchi, these were *Pichia kluyveri, Yarrowia lipolytica, Candida sake, Hanseniaspora uvarum,* and *Kazachstania servazzii.* (Kim et al. 2019). *Kazachstania* spp. (*K. exigua* and *K. pseudohumilis*) were also identified as spoilage colonies in Japanese kimchi fermented at a warmer temperature (Suzuki et al. 2018).

We conducted a metagenomic survey to provide a detailed understanding of the microbial communities associated with a range of fermented vegetable products from North America. Metagenomic sequencing enables all members of the microbial community to be identified without primer bias and provides information about the viral, bacterial and fungal components of the community (Jung et al. 2011). We also paired metagenomic sequencing with culture-based counts to relate the actual abundance of viable yeast and bacterial cells to the relative abundance seen in the sequencing data. Our survey found that while most samples did contain several LAB species, the abundance of yeast within the fermented products was highly variable with some samples containing more colony forming units of yeast than bacteria and others no yeast at all. Using *in vitro* vegetable ferments in the lab we determined that the structure of the microbial community could be influenced by the temperature, salt concentration and type of vegetable being fermented. Our metagenomic survey is the first analysis of microbial diversity in North American fermented vegetable products and showed that high levels of yeast are present in some products. Our *in vitro* experiments determined that the presence of yeast in a community can be varied depending on fermentation parameters.

Materials and Methods

Summary of purchased fermented vegetable products. Seventeen varieties of fermented vegetable products (all cabbage based) were purchased from supermarkets and health food stores in the Boston (MA) area. Three jars of each variety were purchased and details of each product are summarized in **Table S4.1.** Where possible all three jars came from the same batch or date of production. Products labelled as sauerkraut have between two and five ingredients with a minimum of cabbage and salt. Two of these sauerkraut products also have caraway seeds and one product also contains cucumbers. Kimchi and kimchi-style products have between seven and twelve ingredients; all contain cabbage, salt, garlic, hot pepper (that could be one or more types of hot pepper, such as red pepper flakes, sweet pepper and hot pepper), onions or scallions or both. Most of the kimchi products also contain ginger, some also contain other vegetables such as carrots, radishes, Korean radishes, leeks, and one product contains apples. Most of the products are made in the northeastern United States, including Rhode Island, Massachusetts, Maine, New Hampshire, New York and Vermont; two products were made in California and one in Canada. One does not report where the product was made, and another only states the United States.

DNA extraction. From each product, 50g of fermented vegetable product was placed into a 710 mL Whirl Pak bag (Nasco). Samples were mixed thoroughly for 1 minute to homogenize the product. From these samples 200 μL of liquid was removed for DNA extraction using a DNeasy PowerSoil Kit (Qiagen). DNA extraction was carried out according to the manufacturer's protocol (only 50 μL of sample was eluted in the final step to increase the concentration of DNA in the elution). Glycerol stocks of each of the samples were taken by placing 750 μL of the fermented vegetable liquid (remaining in the Whirl pak bags) into cryovials containing 750 μL 30% glycerol (made with 1x phosphate buffered saline). All glycerol stocks were stored at -80ºC.

Library preparation and metagenomic sequencing of samples. Libraries were prepared using a similar approach previously described for whole-genome sequencing of bacterial and fungal genomes (Bodinaku et al. 2019). We used the NEBNext® Ultra II FS DNA Library Prep Kit (New England Biolabs) and followed the manufacturer's instructions for low input (<100 ng) libraries. For each product sampled, we fragmented ~ 100 ng of DNA (in 26 μL volume) for 20 minutes using the NEBNext Ultra II FS Enzyme Mix. This fragmented DNA was then used for Illumina adapter ligation and PCR enrichment using 8-based index primers (NEBNext® Multiplex Oligos for Illumina®). Libraries were sequenced at the Tufts University Core Facility on a NextSeq 500 using paired-end high-output with 150 cycles. Only reads from clusters that passed the default quality filter on the Illumina NextSeq 500 were used in downstream analyses.

Assigning taxa to metagenomic sequences. Fastq reads were uploaded to KBASE and analyzed using Kaiju. This program takes forward and reverse reads and assigns reads to taxa by comparison to the nr_euk database (containing archaeal, bacterial, fungal and virus protein sequences) provided by NCBI (Menzel, et al. 2016). Default settings were used in Kaiju which allows for three mismatches, a 65% match score and a minimum required match length of 11 for classifying reads. All fastq were subsampled at 10% in Kaiju and taxa assigned at < 0.5 % abundance in each sample were pooled. Output files were collated in order to plot relative abundance. This data was then used to determine dissimilarity values between products.

Enumerating bacteria and yeast. Samples from each type of fermented vegetable product were diluted with 1x PBS to a range of dilutions before plating onto yeast peptone dextrose (YPD) agar plates supplemented with 50 mg/L chloramphenicol to enumerate all yeast species and deMan, Rogosa and Sharpe (MRS) agar plates supplement with

21.6mg/L natamycin to enumerate all bacteria species. Plates were incubated aerobically for seven days by which time colonies could be counted to obtain colony forming units (CFUs)/ mL.

Each morphologically distinct colony from these plates was streaked onto fresh MRS or YPD plates and incubated to obtain pure cultures that were stored as glycerol stocks. All bacterial colonies were also identified using Sanger sequencing of the rRNA gene that was amplified using the primers, 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT) (Weisburg et al. 1991). All yeast colonies were identified by amplifying part of the internal transcribed spacer (ITS) region using the primers, ITS1f (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC). Species identification of yeast isolates have been summarized in **Table S4.2.**

Creating a synthetic fermentation community. From this culture collection, six morphologically distinct species were selected to be part of a sauerkraut synthetic community. These included three yeasts and three lactic acid bacteria. The three yeasts were: *Kazachstania barnetti*, *Pichia membranifaciens* and *Rhodoturula* sp. The *Rhodoturula* sp. was isolated from the phyllosphere and added to the synthetic community as it is common in the phyllosphere and has been reported as a spoilage yeast (Whipps et al. 2008). The three bacteria were two homofermenting bacteria: *Lactobacillus korenesis* and *Pediococcus paravalus* and one heterofermenting bacteria *Leuconostoc mesenteroides*. (Johanningsmeier et al. 2007). As the *L. mesenteroides* isolate identified from the fermented vegetable products was not viable when plated from glycerol stocks, a phyllosphere isolate previously identified was used in this community (Miller et al. 2019). Glycerol stocks of each of the six members of the sauerkraut synthetic community were made by streaking all LAB onto MRS agar plates and all yeast species onto YPD agar plates. Plates were incubated (~ 3 days) then colonies were scraped from each plate and placed into 15% glycerol diluted in 1x PBS. Glycerol stocks were stored at -80°C. A glycerol stock of each isolate was thawed on ice and diluted in 1x PBS. LAB were plated onto MRS agar plates and yeast species were plated onto YPD agar plates; all species were plated at a range of dilutions in order to count colony forming units per mL to estimate accurate stock concentrations one week before in vitro experiments.

***In vitro* experiment to investigate microbial community structure.** In order to determine the factors that influence the structure of the microbial community, a sterile vegetable extract (SVE) was prepared following the methods outlined in Chapter 2 from three different cabbages, Red cabbage (*Brassica oleracea*), green cabbage (*Brassica oleracea*) and Napa cabbage (*Brassica rapa*). These three types of SVE (red, green and Napa) were prepared at three different salt (NaCl) concentrations (0% w/v, 2% w/v and 4% w/v). To set up the community composition experiment; 1.5 mL microcentrifuge tubes were each filled with 490 μL of SVE from one of the three cabbage types and the three salt concentrations. This setup was prepared at two different incubation temperatures (14°C and 24°C). To make the synthetic sauerkraut community a glycerol stock of each species was thawed on ice and diluted to a concentration of 5000 CFUs /mL in 1 x PBS. All six diluted stocks were mixed together thoroughly and 10 μL of this mixed community was added to eight replicate 1.5 mL microcentrifuge tubes for each of the 18 treatments. Eight additional tubes for each treatment were set-up for immediate analyses as input values. Input tubes were all plated at a ten-fold dilution on MRS + 21.6 mg/ L natamycin to count LAB and YPD + 50 mg/L chloramphenicol to count the yeast. Plates were incubated aerobically for ~ 5 days, then scanned and counted. Colony morphology was used to distinguish between all the input species. After plating, the pH of each sample was taken using a micro pH probe (Orion PerpHecTTM ROSSTM 8220BNWP). After one week of incubation at the respective temperatures, samples were harvested as per the input samples at 10^{-3} and 10^{-4} dilutions on MRS plates for bacteria and 10^{-2} and 10^{-3} dilutions for YPD plates for yeast.

Glycerol stocks of remaining samples were stored at -80ºC and were used where necessary to obtain replate counts.

Statistics. Data was analyzed using a Vegan package (Oksanen, et al. 2019) in R studio (Version 1.2.5033). Principle Coordinate analysis was carried out using PAST statistical package (Hammer 2004).

Results

Sauerkraut and kimchi ferments are dominated by LAB taxa. Shotgun metagenomic sequencing was used to estimate the taxonomic diversity of 17 brands of fermented vegetable products. The total number of reads obtained was 44,937,133 with an average of 898,742 reads per sample. Taxa were assigned to metagenomic sequencing reads using Kaiju (Menzel, et al. 2016) and the relative abundance of genera which comprised > 0.1% relative abundance (RA) was calculated using the total number of reads assigned to a specific genera relative to all classified reads. The average RA of genera in each product was calculated from three replicate samples, except for CUB and DTL as samples 4- CUB and 43 - DTL were removed due to low sequencing reads (**Figure 4.1A)**. The proportion of classified to unclassified reads was consistent across all samples, except for sample 43 which was removed from all analysis due to low read counts (**Figure S4. 1)**. Over 80% of classified reads in each product are members of LAB, these include *Lactobacillus*, and *Leuconostoc* along with many of other genera commonly detected in fermented vegetable products. Genera detected at low levels include many Proteobacteria, including *Pseudomonas*, *Enterobacter* and *Serratia.* These taxa are known to be prevalent in the phyllosphere of vegetables so it is not unexpected to find them in these fermented vegetable products (Bai et al. 2015; Carlström et al. 2019; Lindow and Brandl 2003). Two yeast genera, *Kazachstania* and *Pichia* were identified in the fermented vegetable products.

Kazachstania was found in four products (all three samples of the products NPL and PGT, two samples of NJM and just one of the samples of UKT), *Pichia* was detected in one product (two of the PGT samples). The Kaiju database used in our analyses (nr_euk) could assign reads as viral but was not capable of further resolving the taxonomy of these reads; brands contained on average 0.4% viral reads. The most abundant LAB species identified in the fermented vegetables was *Enterococcus faecium* (**Table 4.1**). Another *Enterococcus* species, *Enterococcus faecalis*, was also found in every product sampled. *Leuconostoc mesenteroides* (detected in 15 brands) was the second most abundant species. Four *Lactobacillus* species, *Lb. plantarum, Lb. sakei, Lb. brevis* and *Lb. curvatus* were all also detected at levels >1% of the reads. Together with *Le. gelidum* and *Lactococcus lactis*, these LAB species are commonly detected in many kimchi and sauerkraut products and suggest that a core microbiome common to all fermented vegetable products exists worldwide (Zabat, et al. 2018; Johanningsmeier et al. 2007; Tamang, et al. 2016; Jung et al. 2011; Hong et al. 2014; Lee, et al. 2015). All reads which could be assigned to a species were used to calculate a dissimilarity matrix using Bray-Curtis. This analysis uses the relative abundance of all species present within each sample compared to all samples and assigns the sample a value indicating how similar it is to the other communities. Samples of the same product were not significantly different to one another; however, a slight difference in community composition was found when comparing sauerkraut (nine products) to kimchi (8 products) (PERMANOVA analysis; $F= 2.17$, $p=0.09$) (**Figure 4.1B**).

Culture-based analysis shows that yeast abundances are high in some fermented food products A limitation of culture-independent analysis is that dead cells often still contain viable DNA which can bias sequencing results (Carini et al. 2016). Therefore, we supplemented our culture-independent analysis using culture-based techniques. Average CFUs of culturable yeast and bacteria per product were calculated per mL of plated fermented liquid (**Figure 4.1C)**. One product, CUB, had no culturable yeast or bacteria suggesting that this product has been pasteurized. By plating the fermented vegetables, we found that the amount of yeast in the products was highly varied. In two products yeast CFUs were more numerous than bacteria, in seven products the yeast CFUs are similar in abundance to the bacterial CFUs. In the remaining seven products, the bacteria were more numerous than the yeast; including two products where bacteria were cultured in high numbers and there were no culturable yeast samples. Yeast appears to be more readily detected using culture-based approaches than culture-independent sequencing techniques and there appears to be a large variation in the presence of yeast between the products of fermented vegetables.

Characterizing yeast diversity in ferment vegetable products As only one species of yeast (*Kazachstania saulgeensis*) was detected in the fermented products using metagenomic sequencing; culture-based techniques were used to further characterize the yeast diversity of the samples. Yeasts which were morphologically distinct when they were plated for enumeration were also identified using Sanger sequencing of the ITS region of the genome (**Table S4.3**). Most of the species identified were *Pichia* and *Kazachstania* spp and included *P. fermentans*, *P. membranifaciens*, *K. humilis*, *K. bulderi*, *K. exigua* and *K. barnettii*. *Saturnispora mendoncae* was also isolated; this species was originally described from a jar of sauerkraut from the Netherlands (Kurtzman 2006). *Cystobasidium laryngis* a species synonmous with *Rhodotorula laryngis* was also detected, *Rhodoturula* are common phyllosphere yeast and have been reported as spoilage organisms in sauerkraut (Beuchat 1987).

Conditions during fermentation influence microbial community structure In order to investigate what causes the observed variation in yeast abundance across products, we carried out *in vitro* fermentation experiments with three LAB species (*Lactobacillus korenesis*, *Pediococcus paravalus* and *Leuconostoc mesenteroides*) and three yeast species

(*Kazachstania barnetti*, *Pichia membranifaciens* and a *Rhodotorula* sp). Three treatment conditions that are predicted to play a role in microbiome assembly in fermented foods were tested: temperature, salt concentration and cabbage variety. The temperature of incubation when making fermented cabbage can vary from cool temperatures seen in many kimchi ferments (10ºC - 18ºC) to warmer temperatures that I observed while visiting sauerkraut fermentation facilities (Wood 2014). Temperature has been shown to influence the abundance of yeast in kimchi products, with higher temperatures favoring yeast growth (Suzuki et al. 2018; Fred and Peterson 1922). Likewise, salt concentration can also be important in structuring fermented food microbiomes. *Rhodotorula* sp for example, can persist in higher salt conditions (> 3%) that would be detrimental to many bacteria (Fred and Peterson 1922). Reports on the presence of yeast in fermented vegetable products are more common in kimchi (typically made with *Brassica rapa* varieties) than in sauerkraut (usually produced with *Brassica oleracea*) which might suggest that the cabbage forming the base of the ferment might be a factor that can alter the structure of the microbiome (Kim et al. 2019; Suzuki et al. 2018; Moon et al. 2014; Chang et al. 2008; Fred and Peterson 1922).

Shifts in the microbial community structure across the treatments, determined via relative abundance of each of the six microbial species, were observed after seven days of *in vitro* fermentation (**Figure 4.2A**). All tested conditions had a significant impact on the community structure (PERMANOVA 'adonis', incubation temperature R^2=0.2, $p= <0.001$; cabbage R^2=0.09, $p=<0.001$; and salt R^2=0.06, $p=< 0.001$),

Impact of temperature on community structure. Yeast is more prevalent at 24ºC with an average of abundance of 36% (total average sum of yeast for all 24ºC treatments) compared to 27% abundance at 14ºC (total average sum of yeast for all 14ºC treatments). As *Rhodotorula* isn't significantly different between the two temperatures this difference is driven by levels of *Kazachstania* and *Pichia*.

Rhodotorula, Pediococcus and *Leuconostoc* were not significantly impacted by the incubation temperature (ANOVA, *p=>0.05*, with 'Bonferroni correction'). *Lactobacillus* was present in all communities incubated at 24ºC although in Napa SVE and green SVE at 4% salt, levels were very low (<1%). All other 24ºC treatments had levels of *Lactobacillus* between 14 - 66% of the total treatment community. *Pediococcus* is very abundant in red cabbage SVE at 0% salt incubated at 14ºC but is not prevalent in any other communities and remains below 7% in all other treatments.

Impact of salt on community structure *Kazachstania* and *Rhodotorula* were not significantly impacted by levels of salt (ANOVA, *p=>0.05*, with 'Bonferroni correction'); however, all other species in the community were. The most striking impact of salt of the community was that the presence of salt influenced community function. A fall in pH to 4.2 is required for a successful fermentation (IFT/FDA 2001), but not all communities reached a pH of 4.2. Communities incubated at 0% salt and 14ºC did not consistently reach the low pH required for fermentation. Likewise, at 24ºC the pH of all treatments incubated at 0% salt was higher and more varied than communities containing 2% and 4% salt (**Figure 4.2B**). The variation in community assembly between individual replicates was markedly larger at 0% salt compared to 2% and 4% (**Figure S4.2**).

Impact of cabbage type on community structure Cabbage type had a slight influence on the community structure which is driven predominantly by differences in *Pichia and Rhodoturula* (ANOVA,p = < 0.05 and p=<0.001 respectively, with 'Bonferroni correction'). *Kazachstania Leuconostoc* and *Lactobacillus* were not influenced by cabbage type and *Pediococcus was* only weakly impacted by cabbage type. Overall, the relative abundance of bacteria in red cabbage communities is greater than other cabbage varieties. There is a RA of 86% for all bacteria in red cabbage treatments, compared to 61% in green cabbage and 57% in Napa cabbage. All types of cabbage have different chemical compositions which could influence how they are fermented (Heaney et al. 1987; Kim et al. 2013). While all three treatment conditions were significant using PERMANOVA analysis and can be seen to cluster when plotted as an ordination (Bray-Curtis) (**Figure 4.2C**), incubation temperature explains a greater proportion of the variation in community structure than cabbage and salt.

Total CFU counts of yeast relative to bacteria in the *in vitro* ferments compared to the fermented vegetable products In our *in vitro* fermentation experiment only four out of the 18 treatments resulted in communities where the yeast was more prevalent than the bacteria, three of these were in Napa cabbage and one was in green cabbage. In comparison two of the tested fermented vegetable products contained yeast in greater abundance than the bacteria. This could be as a result of the relative prevalence of yeast and bacteria in the environment, while yeasts, particularly *Rhodotorula* spp, are known to colonize the phyllosphere. Perhaps *Kazachstania* and *Pichia* are less common in these environments.

Discussion

Fermented vegetable products are regularly produced and consumed across North America, but little is known about the diversity of their microbial communities. Many studies have used culture-dependent and independent methods to show that Asian kimchi products and European sauerkrauts are fermented by a range of lactic acid bacteria (Nguyen et al. 2013; Johanningsmeier et al. 2007; Lee, et al 2015; Jung et al. 2011). Using metagenomic sequencing we measured the taxonomic diversity of North American fermented foods and found that *Enterococcus*, *Lactobacillus* and *Leuconostoc* species make up over 80% of all classified reads. In a study looking at succession in North American sauerkraut, Lactobacillales was seen to dominate the microbial community after two days of fermentation (Zabat, et al. 2018). In another study, vegetable fermentation facilities were shown to be dominated by *Lactobacillales* and *Leuconostoc* during fermentation (Einson et al. 2018). Identifying similar LAB species across a range of North American ferments to those found in production facilities as well as worldwide indicates a core global fermented vegetable microbiome.

At the species level, the metagenomic sequencing identified many taxa which are common to fermented vegetable products including sauerkraut and kimchi. All the products contained a number of LAB species with an average of 10.8 LAB species per brand. The most abundant species identified in all products was *Enterococcus faecium*. *Enterococci* spp. are common in many fermented foods and beverages including meats such as salami, dairy and fermented vegetables (Tamang et al. 2020; Tamang et al. 2016; Rhee, et al. 2011; Rho et al. 2017; Jung et al. 2011) and have been detected at the outset of fermenting vegan kimchi (Zabat, et al. 2018). *Enterococcus* spp. are prevalent in many foods and supplements either as starter cultures or probiotics (Hanchi et al. 2018; Rho et al. 2017). Despite their prevalence in the human diet they have been identified as the cause of nosocomial infections (Bello Gonzalez et al. 2017), in part as they are naturally resistant to a number of antibiotics (Kürekci et al. 2016; Bello Gonzalez et al. 2017; Hanchi et al. 2018; Ben Braïek and Smaoui 2019). Comparing the taxa obtained using sequencing with those found using culturing methods show conflicting results. Our plating methods did not isolate any *Enterococcus* spp. and only one *Leuconostoc* spp. was obtained from one jar of fermented vegetable product. This result, together with the detection of high levels of yeast using culture dependent techniques indicate that complementing sequencing surveys with plating data can help to fully understand diversity of a system.

Leuconostoc and *Weissella* spp. are important at the outset of vegetable fermentation (Lee, et al. 2015). We found that 15/17 products contained *Le. mesenteroides* and this was the second most abundant species detected. Of the two products that didn't contain *Le. mesenteroides*, one contained *Weissella cibaria*, and the other *Le. gelidum*. We also identified four species of *Lactobacillus* at greater than 1% abundance in the fermented vegetable products. These were *Lb. plantarum*, *Lb. sakei*, *Lb. brevis*, and *Lb. curvatus.* These four species have been frequently detected in the later stages of vegetable fermentation (Zabat, et al. 2018; Tamang, et al. 2016; Vogelxy et al. 1993). *L. lactis* was also detected in the fermented products; this species is common in many dairy ferments (Beresford et al. 2001; Quigley et al. 2013) but is also found in vegetable ferments and has even been tested as a potential starter culture (Kristek et al., 2004.; Cheigh et al. 2002).

In addition to LAB, low levels of non-fermenting bacteria were identified, including common phyllosphere species (Lindow and Brandl 2003; Bai et al. 2015). Non-LAB genera identified in this study included *Bordetella*, *Pseudomonas*, *Salmonella*, *Krytobacter*, *Mycobacterium*, *Pantoea* and *Serratia*. *Enterobacter* was also isolated in three of the seventeen brands of fermented vegetables. This species is commonly detected in fermented pickles where it can cause spoilage (Pérez-Díaz et al. 2019). Other studies have detected many Proteobacteria in the ingredients, (Lee, et al. 2015; Einson et al. 2018) at the outset of fermentation (Zabat, et al. 2018) and in fermented foods (Jung et al. 2011). The low levels of Proteobacteria seen in North American products is not unexpected when compared to other surveys of fermented vegetable products. *Salmonella enterica* was detected in nine fermented vegetable products; however, this might be as other non-pathogenic Enterobacteriaceae spp are less well described in the reference database used to assign taxa than the pathogenic *Salmonella enterica* species.

Using metagenomic sequencing and plating techniques, yeast were identified in four out of the 17 products sampled. As only one species of yeast was identified with metagenomic sequencing, we used plating and Sanger sequencing of morphologically distinct yeast colonies to further explore yeast diversity. The two most commonly detected genera using Sanger sequencing were *Kazachstania* and *Pichia*; these two yeasts are common in other acidic fermented foods suggesting that they can withstand the low pH generated by LAB (De Vuyst and Neysens 2005). Yeast is reported as undesirable in kimchi as they produce flavors that can lower the perceived quality of the product and can soften the vegetables making the texture unappealing (Savard et al. 2002; Moon et al. 2014; Savard, et al. 2002). Some reports suggest that yeasts are able to persist in fermentation conditions when there is not a rapid fall in pH at the outset of fermentation or when there are unfermented sugars in the vegetable products (Le Dinh and Kyung 2006). It is likely that it is not the low pH of the ferment but the type of organic acids present that prevents yeast growth. A range of organic acids such as lactic acid, acetic acid and propionic acid are produced by early colonizing heterofermentative lactic acid bacteria and it could be that this mix of acids is what represses the yeast (Savard et al. 2002). Incomplete fermentation, where sugars remain in high levels in the vegetable ferment, could also enable yeast to increase in abundance at later stages of fermentation or potentially after a product has been sold to a consumer. Incomplete fermentation has been detected in fermented cucumbers, however, the spoilage yeasts identified were *Saccharomyces bayanus* and *Saccharomyces unisporus, neither of* which were identified in our survey (Savard et al. 2002).

There were some surprising anomalies when comparing yeast metagenomic and culture-based data in terms of the relative abundance of yeast in each sample. In our plating of the fermented vegetable products we detected yeast in 14 of the 17 products. In some of these samples the yeast counts were more abundant than the colony counts for bacteria (**Figure 4.1C**). This failure to detect a high relative abundance of yeast in the metagenomic sequencing could be for several reasons including the quality of the nr_euk database used to assign taxonomies to the reads. For instance, some bacteria are very well described with genomes of multiple strains representing each species. Disproportionate representation in the protein database nr_euk will bias the output towards these well described species. In addition, bacteria have smaller genomes than yeast so the read depth required to obtain full coverage of bacterial genomes is less than that of yeasts. It is also likely that some of the bacteria detected in our samples were not living, this would further bias results. While our samples contained adequate read coverage for detecting bacteria in the samples a greater read depth would have helped to elucidate the taxonomy of the yeast. By complementing our sequencing data with culture-based approaches we can obtain a holistic understanding of the microbial community of fermented vegetable products.

To understand what drives the variability of yeast abundance in fermented vegetable products, we developed a synthetic *in vitro* fermentation community. We tested the impact of several abiotic factors, temperature, salt and cabbage type on the structure of the fermented microbiome. We found that the temperature of the fermentation had an influence on the structure of the microbial community. At 24°C incubation *Lactobacillus* was more prevalent in the community while incubation at 14°C resulted in a community dominated by *Leuconostoc*. These differences could be as a result of the timing of the harvest; community succession might advance at a faster rate at 24°C, therefore continuing the 14°C fermentation for a few more days might result in a community with fewer *Le. mesenteroides* and more *Lb. koreensis* as in the 24°C fermentation. It could also be that *Le. mesenteroides* is psychrophilic and grows well in cooler temperatures (Eom, et al. 2007) enabling it to dominate the community at low temperatures.

We found that salt had a clear influence on the community structure; at 0% the community structure was more varied than the community at 2% and 4% salt. Research into low salt production of kimchi, sauerkraut and fermented pickle products has shown that a dramatic reduction in salt can still result in a successful and safe ferment; (Viander, et al. 2003; Choi et al. 1994; Johanningsmeier et al. 2007; McMurtrie and Johanningsmeier 2018; Song et al. 2019) however, we show that even in our experiment containing just LAB and yeast, salt is required for a successful ferment at 14°C. Another study found that a high salt concentration of 7% was detrimental to yeast species (Mheen and Kwon 1984). It could be that 4% concentration of salt was not sufficiently different to 2% salt concentration to see a marked repression of yeast growth in the ferments. It does however look as if *Pichia* growth is improved in low salt conditions at warmer temperatures (approximately x10 more *Picha* at 0% salt and 24ºC than other conditions) which suggests that salt and temperature could be limiting this yeast species.

Cabbage type influenced the structure of the fermented vegetable microbiome. Red, green cabbage (*Brassica oleae)* and Napa cabbage (*Brassica rapa*) have different chemical compositions. These chemical differences are likely to be what drives the changes in the microbiome structure. Not only are the ingredients likely to have an impact on the dominant species isolated in the fermented vegetable product but the source of the ingredients can also influence the community composition (Hong et al. 2014; Nguyen et al. 2013). A study looking at kimchi products made across different regions of China found that the region as well as the ingredients influenced the microbial community (Peng et al. 2018). Likewise, Lee et al. (2015) investigated kimchi made from ingredients made from different markets and found that ingredients are an important source of microbial diversity.

Next steps for this research would be to look at biotic factors which influence the presence of yeast in the fermented food community. A first experiment would be to test the impact that each of the LAB have on shaping the fermented food microbiome. For example, *Le. mesenteroides* is a heterofermenter and typically first in the fermented food community succession however, without this species would the homofermenters be capable of lowering the pH sufficiently and outgrowing the yeast species? A leave-one-out experiment which removes one LAB from the community at a time will show their importance in shaping community structure. After carrying out this experiment, it would be important to test how the sauerkraut community is structured in the sterile vegetable extract in the presence of other phyllosphere community members. Many LAB are known to produce bacteriocins in addition to organic acids. Bacteriocins are small antimicrobial peptides that are effective against related bacteria even at low concentrations (Sidooski et al. 2019) and have been isolated from fermented foods (Cleveland et al. 2001). Like bacteriocins, organic acids (including acetic acid and lactic acid) are produced by LAB as they ferment vegetables (Zalán et al. 2009). These antimicrobial compounds together with the change in abiotic conditions throughout fermentation are thought to shape the microbial community by preventing the growth of undesirable microorganisms.

Another improvement to our survey of fermented vegetable products would be to further explore the role of viruses in shaping the community. Bacteriophages can shape the communities of lactic acid bacteria of fermented foods, particularly in the dairy industry (Marcó, et al. 2012), but also in fermented vegetables (Lu et al. 2003). All the fermented vegetable products contained viruses and it is likely that some of these viruses are bacteriophages.

The structuring of fermented food microbiomes can have important implications for the quality, shelf life and safety of these products. We show that the structure of fermented food microbiomes can be driven in part by the abiotic conditions in which they are fermented. Fermenting at cooler temperatures can improve the growth of heterofermentative LAB, as shown with *Leuconostoc mesenteroides*. *Leuconostoc* spp. cause a rapid drop in pH while growing as it produces a broad range of organic acids including acetic acid and lactic acid. It is likely that a more complex mix of organic acids coupled with a rapid fall in pH could result in less yeast present in the fermented vegetable product.

Overall from our survey of 17 fermented vegetable products we did not see any major differences in the community structure across the products. This shows that the ingredients and area in which the ferment was produced did not influence the structure, but rather that there is a robust core fermented food microbiome which forms during fermentation. We also found that yeast are present in many North American fermented vegetable products and their abundance could be influenced by fermentation temperature and to a lesser extent the level of salt and ingredients used. The same core species of LAB are found in fermented vegetables all over the world, showing that there is a global distribution of these species in vegetable environments.

Acknowledgements

We appreciate the help of undergraduate students in the Spring 2019 semester of the Tufts University Bio 106 course (Microbiology w/ Lab) for helping to collect culture-based data from the products. This project was funded in part by a grant from the USDA-NIFA (2017-67013-26520) and from a grant from the Tufts University Graduate School of Arts and Science. We appreciate feedback on previous versions of the manuscript from Emily Putnam, Megan Biango-Daniels, Elizabeth Landis and Ruby Ye. Assistance with statistical methods and metagenomic sequencing analysis came from Yue Yu.

Tables

Table 4.1 Summary of most abundant microbial species detected in fermented vegetable products using Kaiju

Phylum	Family	Species	% of total microbial reads	# brands this product was detected in
Firmicutes	Enterococcaceae	*Enterococcus faecium*	24.07	17
Firmicutes	Leuconostocaceae	*Leuconostoc mesenteroides*	8.91	15
Firmicutes	Enterococcaceae	*Enterococcus faecalis*	4.81	17
Firmicutes	Lactobacillaceae	*Lactobacillus plantarum*	4.29	15
Firmicutes	Lactobacillaceae	*Lactobacillus sakei*	3.76	12

Firmicutes	Lactobacillaceae	*Lactobacillus brevis*	1.96	13
Firmicutes	Leuconostocaceae	*Leuconostoc gelidum*	1.45	5
Firmicutes	Streptococcaceae	*Lactococcus lactis*	1.42	10
Firmicutes	Lactobacillaceae	*Lactobacillus curvatus*	1.1	9

Figures

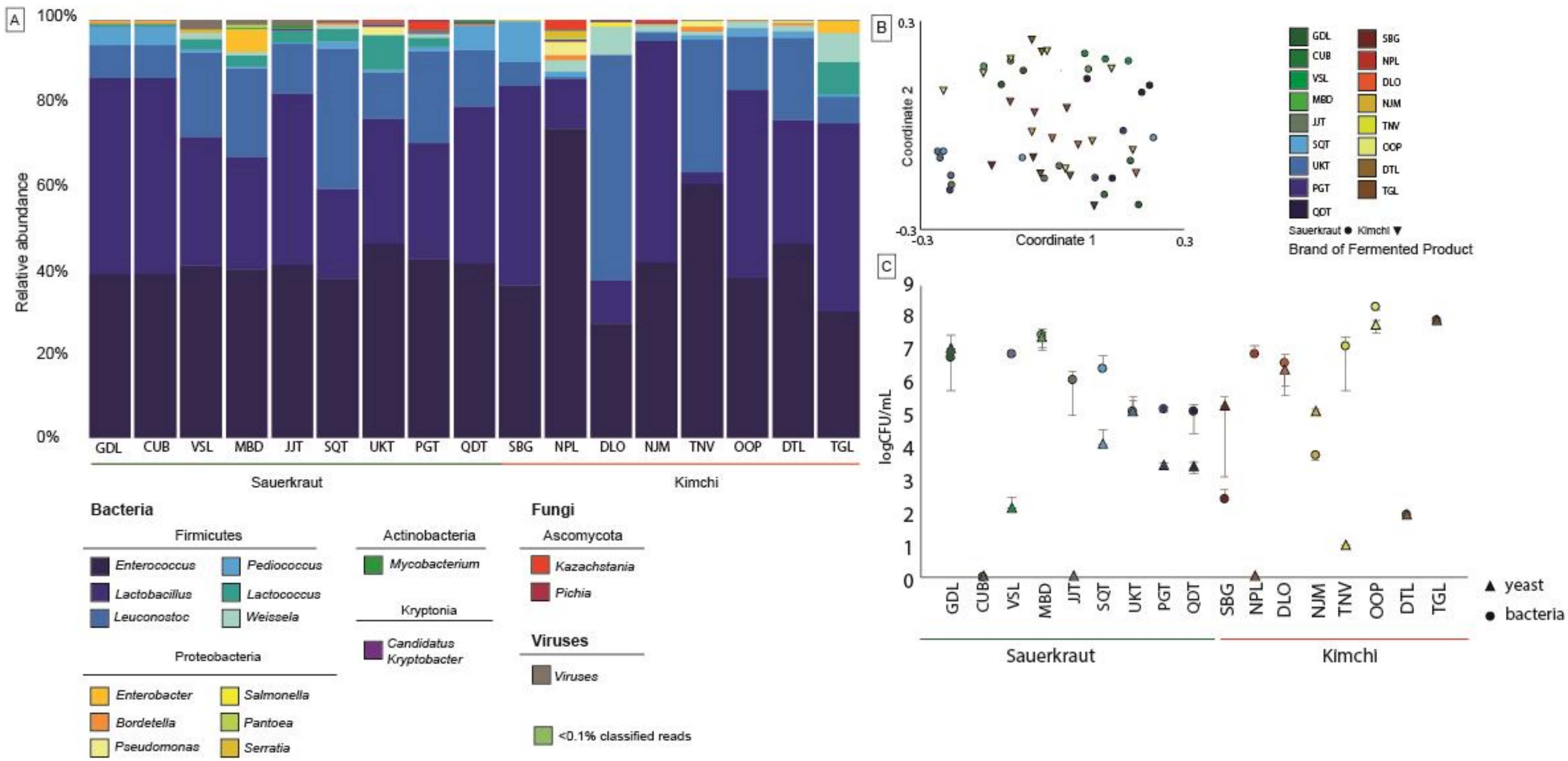


Figure 4.1. Microbial diversity and abundance in North American fermented vegetable products

Figure 4.1 caption continued

(A) Average relative abundance of genera detected in fermented vegetable products. Relative abundance was calculated as the percentage of reads assigned to a particular generus compared to all classified reads. All brands contain three replicates except for CUB and DTL as samples 4 and 43 were removed due to low read counts. Sauerkraut samples are ordered to the left of the chart (denoted by the green line below the x-axis) and all kimchi samples are ordered to the right of the chart (denoted by the red line). All genera are ordered according to their phyla in the legend below the chart (B) Principal coordinate analysis of all fermented vegetable products. Principal coordinate analysis was carried out using a Bray-Curtis dissimilarity matrix. Samples 4 and 43 were not included in this analysis. All sauerkraut samples are shown as circles and all kimchi samples as inverted triangles. Colors relate to the legend to the right of the chart. (C) Abundance of bacteria and yeast cultured from fermented vegetable products. For products GDL, CUB, SBG, NPL, VSL, DLO, MBD, JJT, NJM, SQT and TNV an average abundance is plotted (n=3), for products UKT, PGT, OOT and DTL an average abundance is plotted (n=2) and products QDT and TGL one value is plotted are points show logCFU/ mL of bacteria plotted as circles and logCFU/ mL yeasts are plotted as triangles. Error bars denote standard deviation.

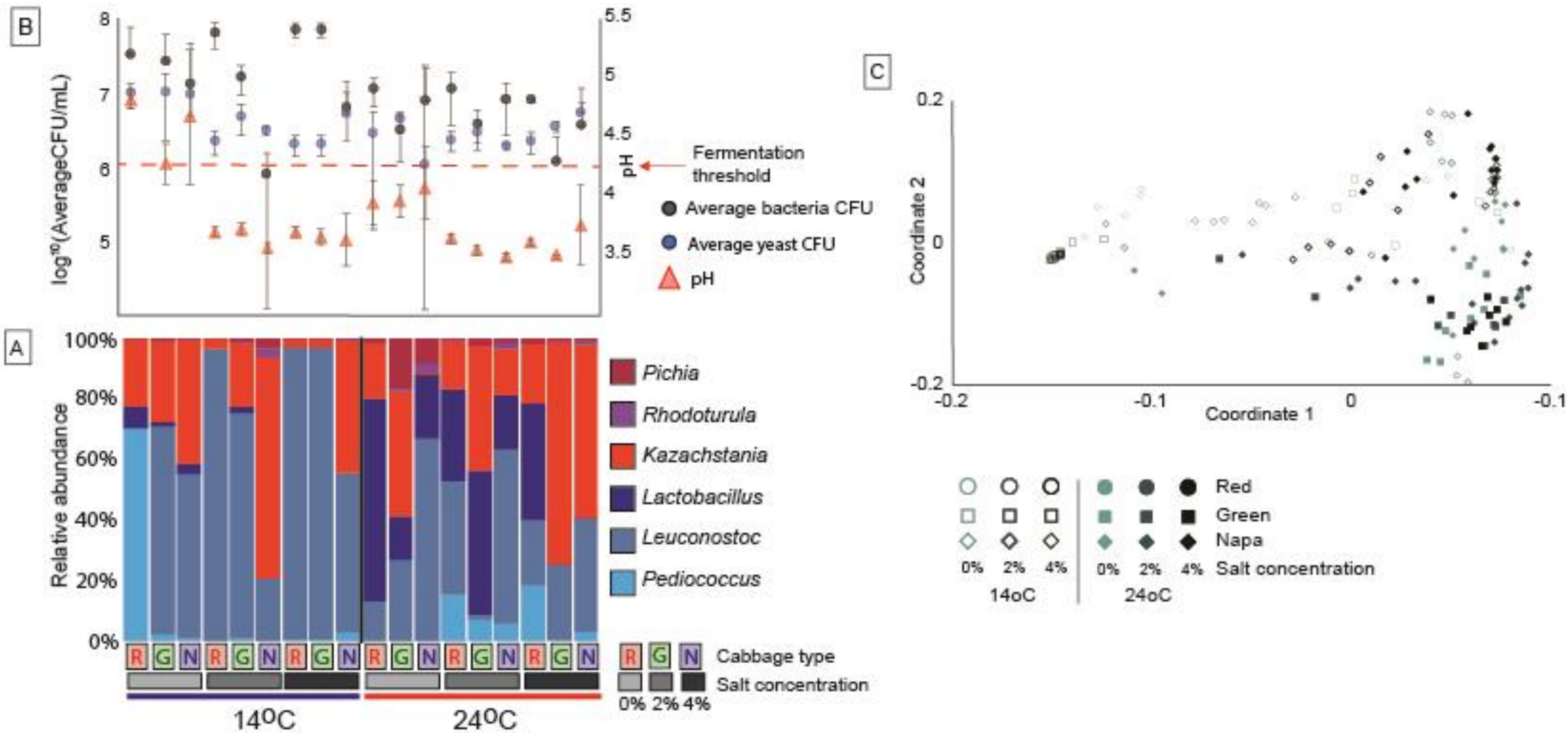


Figure 4.2. Microbial community structure and fermentation dynamics across 18 *in vitro* treatment environments.

Figure 4.2 caption continued

(A) Average relative abundance of each of the six community members across all treatments. The average relative abundance of the six-members of the synthetic community across all treatments. All treatments at 14◦C are shown to the left of the figure and all 24◦C treatments to the right. Salt concentrations for each treatment is shown below the chart in grey, and all cabbage types are also labelled below the figure with R for red cabbage, G for green cabbage and N for Napa cabbage. For all treatments ($n=8$) except for Red, 14◦C 0% salt where ($n=6$). **(B) Average abundance of bacteria and yeast in each community and ferment pH.** LogCFU/ mL of bacteria are plotted as black circles while logCFU/ mL of yeast are plotted as blue circles and x-axis is to the left of the plot. Average pH of ferments is shown as red triangles, x-axis to the right of the figure. In all treatments ($n=8$) except for Red, 14◦C 0% salt where ($n=6$). Error bars for all plots =standard deviation. (C) **Principal coordinate analysis of *in vitro* ferment communities.** Principal coordinate analysis of all synthetic ferment communities. Principal coordinate analysis was carried out using a Bray-Curtis dissimilarity matrix. Colors relate to the legend below the chart

Supplementary Tables

Supplementary Table 4.1 Metadata of fermented vegetable products.

Sample	Type of ferment	pH	Ingredients	Location Made
1 - GDL	Sauerkraut	3.59	green cabbage, brine (water, sea salt), caraway	Watsonville, CA
2 - GDL	Sauerkraut	N/A	green cabbage, brine (water, sea salt), caraway	Watsonville, CA
3 - GDL	Sauerkraut	3.48	green cabbage, brine (water, sea salt), caraway	Watsonville, CA
4 - CUB	Sauerkraut	3.67	cabbage, artesian well water, salt	Stockton, Canada

5 - CUB	Sauerkraut	3.68	cabbage, artesian well water, salt	Stockton, Canada
6 - CUB	Sauerkraut	3.73	cabbage, artesian well water, salt	Stockton, Canada
7 - SBG	Kimchi	3.56	green cabbage, napa cabbage, radish, onion, carrots, korean hot peppers, unrefined sea salt, ginger, garlic	Alna, Maine
8 - SBG	Kimchi	3.48	green cabbage, napa cabbage, radish, onion, carrots, korean hot peppers, unrefined sea salt, ginger, garlic	Alna, Maine
9 - SBG	Kimchi	3.52	green cabbage, napa cabbage, radish, onion, carrots, korean hot peppers, unrefined sea salt, ginger, garlic	Alna, Maine
10 - NPL	Kimchi	3.77	napa cabbage, red pepper flakes, garlic, ginger, scallions, cilantro, lime juice, salt, sugar, bhut jolokia powder	Brooklyn, NY
11 - NPL	Kimchi	3.99	napa cabbage, red pepper flakes, garlic, ginger, scallions, cilantro, lime juice, salt, sugar, bhut jolokia powder	Brooklyn, NY
12 - NPL	Kimchi	3.86	napa cabbage, red pepper flakes, garlic, ginger, scallions, cilantro, lime juice, salt, sugar, bhut jolokia powder	Brooklyn, NY
13 - VSL	Sauerkraut	3.61	green cabbage, salt salt, garlic	Santa Rosa CA
14 - VSL	Sauerkraut	3.59	green cabbage, salt salt, garlic	Santa Rosa CA

15 - VSL	Sauerkraut	3.6	green cabbage, salt salt, garlic	Santa Rosa CA
16 - DLO	Kimchi	4	napa cabbage, korean radish, garlic, salt, red pepper powder, first press fish sauce (anchovy, sea salt), sugar, scallions, onion, ginger	Pawtucket, RI
17 - DLO	Kimchi	4.04	napa cabbage, korean radish, garlic, salt, red pepper powder, first press fish sauce (anchovy, sea salt), sugar, scallions, onion, ginger	Pawtucket, RI
18 - DLO	Kimchi	4.06	napa cabbage, korean radish, garlic, salt, red pepper powder, first press fish sauce (anchovy, sea salt), sugar, scallions, onion, ginger	Pawtucket, RI
19 - MBD	Sauerkraut	3.51	cabbage caraway seeds, sea salt	Cranston RI
20 - MBD	Sauerkraut	3.5	cabbage caraway seeds, sea salt	Cranston RI
21 - MBD	Sauerkraut	3.55	cabbage caraway seeds, sea salt	Cranston RI
22- JJT	Sauerkraut	3.56	green cabbage, sea salt	Pittsfield, MA
23 - JJT	Sauerkraut	3.56	green cabbage, sea salt	Pittsfield, MA
24 - JJT	Sauerkraut	3.37	green cabbage, sea salt	Pittsfield, MA
25 - NJM	Kimchi	3.51	napa cabbage, yellow onion, green onion, red chile pepper flakes, salt, fresh garlic, fresh ginger, sugar	Long Island City, NY

26 - NJM	Kimchi	3.5	napa cabbage, yellow onion, green onion, red chile pepper flakes, salt, fresh garlic, fresh ginger, sugar	Long Island City, NY
27 - NJM	Kimchi	3.51	napa cabbage, yellow onion, green onion, red chile pepper flakes, salt, fresh garlic, fresh ginger, sugar	Long Island City, NY
28 - SQT	Sauerkraut	3.42	Northeast-grown cabbage, filtered water, unrefined sea salt, caraway seed, dill seed	Greenfield, MA
29 - SQT	Sauerkraut	3.46	Northeast-grown cabbage, filtered water, unrefined sea salt, caraway seed, dill seed	Greenfield, MA
30 - SQT	Sauerkraut	3.46	Northeast-grown cabbage, filtered water, unrefined sea salt, caraway seed, dill seed	Greenfield, MA
31 - TNV	Kimchi	4.09	All natural cabbage, carrots, green onion, sea salt, sweet red peppers, garlic, leeks, ginger.	Waterbury, VT
32- TNV	Kimchi	4.04	All natural cabbage, carrots, green onion, sea salt, sweet red peppers, garlic, leeks, ginger.	Waterbury, VT
33 - TNV	Kimchi	3.95	All natural cabbage, carrots, green onion, sea salt, sweet red peppers, garlic, leeks, ginger.	Waterbury, VT
34 - UKT	Sauerkraut	3.67	cabbage, persian cucumbers, sea salt, garlic	No details
35 - UKT	Sauerkraut	3.56	cabbage, persian cucumbers, sea salt, garlic	No details

36 - UKT	Sauerkraut	3.54	cabbage, persian cucumbers, sea salt, garlic	No details
37 - PGT	Sauerkraut	3.42	cabbage, water, salt	product of USA
38 - PGT	Sauerkraut	3.46	cabbage, water, salt	product of USA
39 - PGT	Sauerkraut	3.33	cabbage, water, salt	product of USA
40 - OOT	Kimchi	3.43	cabbage, carrot, scallion, garlic, ginger, chilies, himalayan pink salt	Weare, NH
41 - OOT	Kimchi	3.45	cabbage, carrot, scallion, garlic, ginger, chilies, himalayan pink salt	Weare, NH
42 - OOT	Kimchi	3.47	cabbage, carrot, scallion, garlic, ginger, chilies, himalayan pink salt	Weare, NH
43 - DTL	Kimchi	4.47	Napa cabbage, garlic, sugar, salt, shrimp, anchovy sauce, anchovy, salt, sugar, (fructose), red pepper, apple, radish, ginger, hot pepper powder, onion	Maspeth, NY
44 - DTL	Kimchi	3.93	Napa cabbage, garlic, sugar, salt, shrimp, anchovy sauce, anchovy, salt, sugar, (fructose), red pepper, apple, radish, ginger, hot pepper powder, onion	Maspeth, NY
45 - DTL	Kimchi	3.91	Napa cabbage, garlic, sugar, salt, shrimp, anchovy sauce, anchovy, salt, sugar, (fructose), red pepper, apple, radish, ginger, hot pepper powder, onion	Maspeth, NY
46 - QDT	Sauerkraut	3.34	Cabbage, Sea salt, Dill	Gloucester, MA
47 - QDT	Sauerkraut	3.47	Cabbage, Sea salt, Dill	Gloucester, MA

48 - QDT	Sauerkraut	3.38	Cabbage, Sea salt, Dill	Gloucester, MA
49 - TGL	Kimchi	3.65	Napa cabbage, Korean Hot pepper flakes, Anchovy Fish Sauce (Anchovy, Salt, Water), Salt, Sugar, Carrots, Garlic, Chives, Daikon radish, Onion.	Kittery, Maine
50 - TGL	Kimchi	3.6	Napa cabbage, Korean Hot pepper flakes, Anchovy Fish Sauce (Anchovy, Salt, Water), Salt, Sugar, Carrots, Garlic, Chives, Daikon radish, Onion.	Kittery, Maine
51 - TGL	Kimchi	3.74	Napa cabbage, Korean Hot pepper flakes, Anchovy Fish Sauce (Anchovy, Salt, Water), Salt, Sugar, Carrots, Garlic, Chives, Daikon radish, Onion.	Kittery, Maine

Supplementary Table 4.2. All taxa identified at the species level with metagenomic sequencing. This includes taxa identified > 1% and < 1% relative abundance compared to all classified reads.

Phylum	Family	Species	Relative abudance of species compared to all classified reads	Number of brands containing this species
Species making up > 1 % RA of all classified reads				
Firmicutes	Enterococcaceae	*Enterococcus faecium*	24.03	17
Firmicutes	Leuconostocaceae	*Leuconostoc mesenteroides*	8.82	15
Firmicutes	Enterococcaceae	*Enterococcus faecalis*	4.83	17
Firmicutes	Lactobacillaceae	*Lactobacillus plantarum*	4.24	12
Firmicutes	Lactobacillaceae	*Lactobacillus sakei*	4.12	15
Firmicutes	Lactobacillaceae	*Lactobacillus brevis*	2.02	13
Firmicutes	Leuconostocaceae	*Leuconostoc gelidum*	1.55	5
Firmicutes	Streptococcaceae	*Lactococcus lactis*	1.38	10
Firmicutes	Lactobacillaceae	*Lactobacillus curvatus*	1.19	9
Species making up < 1 % RA of all classified reads				
Firmicutes	Lactobacillaceae	*Lactobacillus herbarum*	0.63	3
Firmicutes	Lactobacillaceae	*Pediococcus inopinatus*	0.58	
Firmicutes	Lactobacillaceae	*Lactobacillus buchneri*	0.43	3
Firmicutes	Lactobacillaceae	*Lactobacillus rapi*	0.3	2
Firmicutes	Lactobacillaceae	*Lactobacillus fabifermentans*	0.3	1
Firmicutes	Lactobacillaceae	*Pediococcus acidilactici*	0.27	3
Firmicutes	Leuconostocaceae	*Leuconostoc citreum*	0.26	13
Firmicutes	Lactobacillaceae	*Lactobacillus koreensis*	0.24	4
Proteobacteria	Alcaligenaceae	*Bordetella bronchiseptica*	0.24	1

Firmicutes	Leuconostocaceae	*Weissella viridescens*	0.24	3
Firmicutes	Lactobacillaceae	*Lactobacillus parabrevis*	0.23	10
Firmicutes	Leuconostocaceae	*Leuconostoc carnosum*	0.21	4
Firmicutes	Leuconostocaceae	*Weissella cibaria*	0.2	3
Proteobacteria	Enterobacteriaceae	*Salmonella enterica*	0.18	9
Firmicutes	Lactobacillaceae	*Lactobacillus carnosum*	0.18	1
Krytobacter		*Candidatus Kryptobacter tengchongensis*	0.17	5
Actinobacteria	Mycobacteriaceae	*Mycobacterium gastri*	0.15	8
Firmicutes	Lactobacillaceae	*Lactobacillus kimchiensis*	0.15	4
Firmicutes	Lactobacillaceae	*Lactobacillus xiangfangensis*	0.15	1
Firmicutes	Leuconostocaceae	*Leuconostoc fallax*	0.13	2
Firmicutes	Lactobacillaceae	*Pediococcus parvulus*	0.09	1
Firmicutes	Lactobacillaceae	*Lactobacillus nantensis*	0.09	4
Firmicutes	Lactobacillaceae	*Lactobacillus heilongjiangensis*	0.08	1
Firmicutes	Lactobacillaceae	*Pediococcus pentosaceus*	0.08	1
Firmicutes	Lactobacillaceae	*Lactobacillus paraplantarum*	0.06	3
Proteobacteria	Enterobacteriaceae	*Enterobacter cloacae*	0.06	2
Proteobacteria	Enterobacteriaceae	*Leclercia adecarboxylata*	0.04	2
Firmicutes	Streptococcaceae	*Lactococcus garvieae*	0.04	1
Firmicutes	Lactobacillaceae	*Lactobacillus malefermentans*	0.04	1
Firmicutes	Leuconostocaceae	*Leuconostoc lactis*	0.03	1
Actinobacteria	Micrococcaceae	*Paenarthrobacter nicotinovorans*	0.03	1
Proteobacteria	Enterobacteriaceae	*Kluyvera intermedia*	0.03	1
Proteobacteria	Yersiniaceae	*Serratia sp. Leaf51*	0.03	3
Firmicutes	Lactobacillaceae	*Lactobacillus hammesii*	0.02	1
Firmicutes	Lactobacillaceae	*Pediococcus damnosus*	0.02	1
Proteobacteria	Enterobacteriaceae	*Enterobacter mori*	0.02	1

Firmicutes	Lactobacillaceae	*Lactobacillus coryniformis*	0.01	1
Firmicutes	Leuconostocaceae	*Leuconostoc pseudomesenteroides*	0.01333	1
Firmicutes	Lactobacillaceae	*Lactobacillus paracollinoides*	0.01212	1
Firmicutes	Lactobacillaceae	*Lactobacillus backii*	0.00925	1
Proteobacteria	Moraxellaceae	*Acinetobacter baumannii*	0.00923	1
Firmicutes	Staphylococcaceae	*Staphylococcus aureus*	0.00817	1
Firmicutes	Lactobacillaceae	*Lactobacillus hokkaidonensis*	0.00736	1
Proteobacteria	Vibrionaceae	*Vibrio sp. CECT 9027*	0.0058	1
Firmicutes	Leuconostocaceae	*Weissella confusa*	0.00302	1
Proteobacteria	Sphingomonadaceae	*Sphingomonas sp. LK11*	0.00018	1
Proteobacteria	Caulobacteraceae	*Brevundimonas sp. GW460-12-10-14-LB2*	0.00016	1
Proteobacteria	Erwiniaceae	*Pantoea agglomerans*	0.00013	1
Proteobacteria	Enterobacteriaceae	*Enterobacter sp. 638*	0.00011	1
Proteobacteria	Yersiniaceae	*Serratia proteamaculans*	0.00009	1
Firmicutes	Lactobacillaceae	*Lactobacillus crustorum*	0.00006	1
Fungal species				
Ascomycota	Saccharomycetaceae	*Kazachstania saulgeensis*	0.16	4
Virus				
Viruses			0.67	17

Supplementary Table 2.3 Taxonomy of yeasts isolated from fermented vegetable products.

Brand	BLAST ID	BLAST % Pairwise Identity
GDL	*Pichia fermentans*	99.1
	Pichia fermentans	98.8
	Pichia fermentans	98.8
	Pichia membranifaciens	99.5
NPL	*Saccharomyces barnetti/ Kazachstania barnetti*	100/ 99.9
	Saccharomyces barnetti/ Kazachstania barnetti	100/ 100
	Kazachstania sp.	99.8
NJM	*Kazachstania humilis/ Kazachstania bulderi*	100/ 100
	Kazachstania bulderi/ Kazachstania humilis	100/ 99.7
	Kazachstania bulderi/ Kazachstania humilis	100/ 99.6
	Kazachstania bulderi	100
UKT	*Kazachstania bulderi/ Kazachstania humilis*	99.3 / 99.2
	Kazachstania bulderi/ Kazachstania humilis	99.5/ 99.3
SQT	30_FUN_2_Pichia membranifaciens	FUN27_ITS1F
	30_FUN_1_Kazachstania exigua	FUN28_ITS1F
	Kazachstania exigua/ Kazachstania barnettii	87.5/ 86.4

	Kazachstania exigua	100
	Kazachstania exigua	100
PGT	*Saturnispora mendoncae*	91.4
	Pichia membranifaciens	99.8
	Kazachstania humilis/ Kazachstania bulderi	100/ 100
	Kazachstania bulderi/ Kazachstania humilis	99.9 / 99.7
DLO	*Cystobasidium laryngis*	99.9

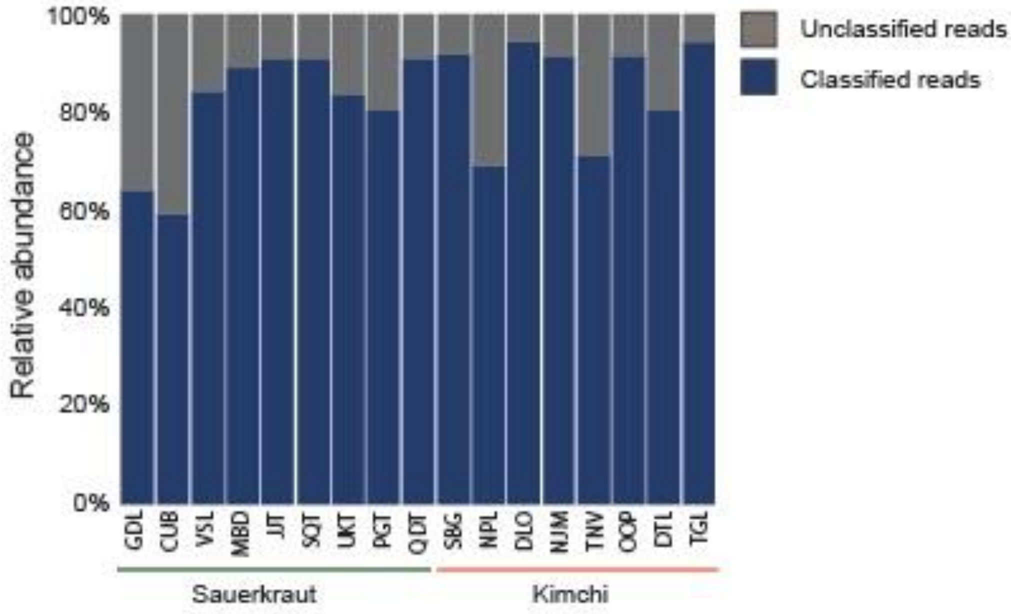
Supplementary Table 4.4 Summary of metagenomic sequencing data

Sample	Number of reads	Number of bases	%GC content
1 - GDL	1105097.75	18616061	43.9
2 - GDL	858224.25	20138882	43.6
3 - GDL	902973.5	19813984	44.4
4 - CUB	198*	*	*
5 - CUB	1345465	3591189	38.9
6 - CUB	673151.5	4812295	39.6
7 - SBG	642524.5	16119428	43
8 - SBG	738365.5	17332450	42.9
9 - SBG	689456.5	16689330	43
10 - NPL	460723	7764546	42.9
11 - NPL	661455.25	13879207	45.7
12 - NPL	965081.75	12263013	44.7
13 - VSL	863279.5	14102480	41.3
14 - VSL	1666298.75	17871669	41
15 - VSL	945131	18119452	42.8
16 - DLO	140834.75	7436584	41.3
17 - DLO	2561179.75	13309375	40.3
18 - DLO	1063354.5	9176678	40.5
19 - MBD	1967670.75	44846675	44.7
20 - MBD	2085504.25	50321687	42.7
21 - MBD	1977151.25	53243290	43.7
22- JJT	915115.5	11174101	40.5
23 - JJT	1634653.25	13078979	41.2
24 - JJT	616422	10263126	40.7
25 - NJM	546652.75	8778090	42.7
26 - NJM	393545.25	8953682	42.5
27 - NJM	360380.75	8680646	42.5
28 - SQT	1026852.5	12325164	39.7
29 - SQT	646783.75	10603788	39.7

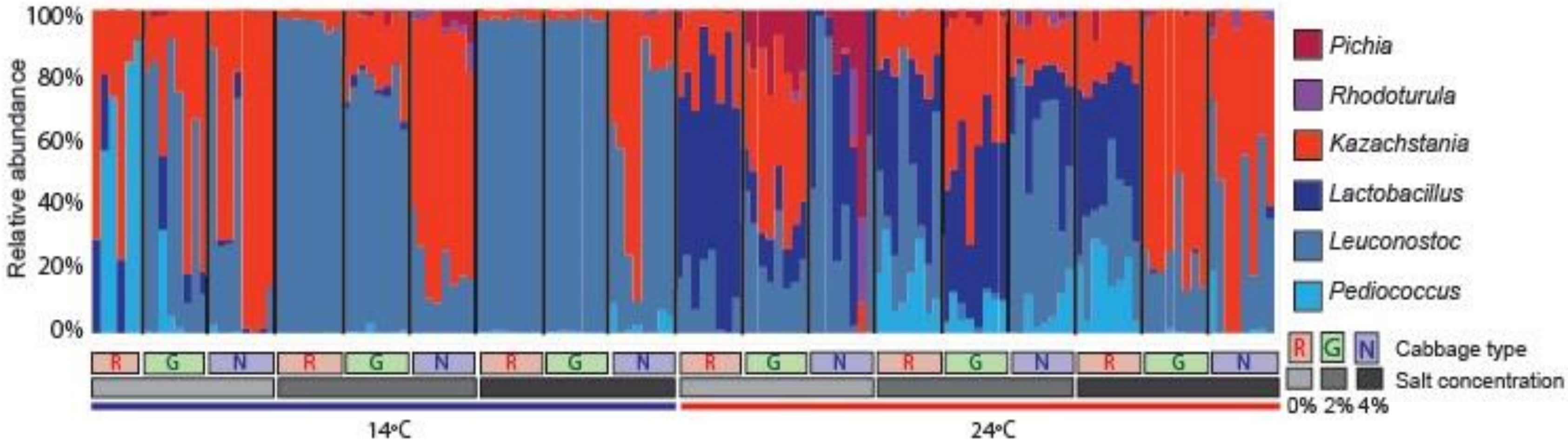
30 - SQT	609773.25	9527684	40.5
31 - TNV	752552	11962025	39.9
32- TNV	522809	9407335	38.5
33 - TNV	698624.25	10177895	38.7
34 - UKT	734614	20903746	43.1
35 - UKT	450695	12680794	40.9
36 - UKT	534206	11442335	39.9
37 - PGT	934490.75	23527293	39.4
38 - PGT	495742.75	16325382	40.3
39 - PGT	628749.75	19741489	40.6
40 - OOT	596919.75	18575419	41.5
41 - OOT	1189746	28391820	41.7
42 - OOT	765662	22201348	41.3
43 - DTL	204428.25	289819	38.7
44 - DTL	1024443.5	2099805	41.3
45 - DTL	597231	942144	40.3
46 - QDT	1342109.5	17398859	40.4
47 - QDT	710684.5	14883792	40.7
48 - QDT	1126694.75	17091685	40.4
49 - TGL	902044.75	22837942	41.8
50 - TGL	639324.25	21633210	41.8
51 - TGL	1022260	29679037	43.7

* sample removed from further analysis due low read counts

Supplementary Figures



Supplementary Figure 4.1. Relative abundance of classified and unclassified reads in all fermented vegetable products. Calculated using all reads and averaged over three replicate samples for each product except for products CUB and DTL as samples 4 and 43 were removed as these samples had particularly low reads.



Supplementary Figure 4.2. Variation in the relative abundance of various community members at 0% salt treatments.

The relative abundance of the six-members of the synthetic community across all treatments. Each bar represents a replica community. All treatments at 14ºC are shown to the left of the figure and all 24ºC treatments to the right. Salt concentrations for each treatment is shown below the chart in grey, and all cabbage types are also labelled below the figure with R for red cabbage, G for green cabbage and N for Napa cabbage. For all treatments (*n=8*) except for Red, 14ºC 0% salt where (*n=6*)

Chapter Five

The role of dispersal in controlling the diversity of fermented food microbiomes

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Abstract

Patterns in the structure of fermented food microbiomes have been described, but the underlying processes shaping the microbial diversity of these systems are poorly characterized. Like other microbiomes, fermented food microbial communities have not been considered dispersal-limited, leaving researchers to assume that selection (e.g. abiotic filtering and biotic interactions) is the main process shaping them. In this review, we highlight the importance of dispersal in structuring the diversity of fermented food microbiomes and propose using a propagule limitation framework to help anchor future microbial dispersal research in fermented foods. Using this framework, we show how microbial dispersal influences many fermented food microbiomes, highlighting cheese, sourdough, and fermented vegetable products as case studies. We also use this framework to propose areas for future research into the role of dispersal in fermented foods. Dispersal of organisms into fermented food microbiomes has the potential to improve food flavor and quality as microbes that produce novel metabolites disperse into the food. Controlling dispersal processes can also prevent the introduction of pathogens and spoilage organisms into ferments. Overall, we believe that understanding how dispersal structures microbiomes will help develop critical control points and measures to improve food production.

Introduction

Using culture-dependent and -independent techniques, common patterns in the structure of fermented food microbiomes have been observed (Cao et al. 2017). Fermented foods are dominated by a few abundant organisms and many low abundance or rare species (Tamang et al. 2020; Tamang, et al. 2016; Połka et al. 2015; Jung et al. 2011; Bokulich et al. 2012). Many fermented foods can be fermented by a range of different species or strains of organisms showing that functional redundancy exists in many fermented microbiomes

(Tamang, et al. 2016). Most fermented foods show a clear community succession throughout fermentation where the growth of early colonizers modifies the environment enabling other organisms to grow within the community (Lee, et al. 2015; Walsh et al. 2016; Ahn et al. 2014). One of the most notable features of fermented foods is that these communities are reproduced worldwide in a reliable way with a robust community structure regardless of location (Wolfe and Dutton 2015). Moving from exploring these patterns of microbiome diversity to understanding the ecological and evolutionary processes that determine the structure of microbiomes is the next step for both microbial ecologists and food fermenters (Muller 2019; Coutinho et al. 2018). Being able to shift food microbiomes into new stable states, or recover from disruption (Shade 2018) could help to innovate microbial techniques for correcting failed fermentations (Kamelamela et al. 2018), or improving quality, flavor, and safety of fermented food products (Bokulich et al. 2016).

Dispersal is one of four ecological and evolutionary processes that shape how communities assemble (Vellend 2010). These processes - dispersal, selection, diversification and drift - were shown to be applicable in microbiomes by Nemergut (2013) and Hanson (2012). Traditionally there has been less focus on dispersal's role in structuring microbiomes as Baas-Becking's rule (developed while studying the microbiome of milk) "everything is everywhere, but the environment selects", has been assumed to be true for many communities (Finlay 2002). In some spontaneously fermented foods this is particularly relevant as fermenting organisms are considered ubiquitous. For instance, some lactic acid bacteria (LAB) such as *Leuconostoc mesenteroides* are detected worldwide in fermented vegetables (Di Cagno et al. 2013). Likewise, natural wine yeasts are common on grapes all over the world (Fleet, et al. 1984; Martínez et al. 2004; Wang et al. 2012). While many microbes detected in fermented foods have a broad distribution, there could still be subspecies or strain level differences that show distinct spatial distributions indicating that everything is not necessarily everywhere (Chitlapilly Dass et al. 2010; Kvitt and Colorni 2004).

The balance between the processes of dispersal and selection is essential for structuring plant and animal communities, but how these processes work together in microbiomes is still emerging. Dispersal consists of immigration, the movement from one place or community into another or emigration, movement away from a place or community (Clobert et al. 2012). In this review we will focus on immigration where microbes disperse into the fermented food from a source community, as these are the microbes that have the potential to contribute to the fermented food microbiome (Sloan et al. 2006; Kinkel 1997). High levels of dispersal can reduce variability between populations and is considered a homogenizing process (Fodelianakis et al. 2019). As more dispersal results in the same microbes being detected in a greater number of microbiomes. Dispersal has the potential to dramatically influence the quality, reproducibility and safety of food microbiomes; either by dispersal of spoilage organisms into the fermented food system or via low levels of desirable fermenting organisms dispersing into the food. While dispersal is a homogenizing process, selection is considered a diversifying process which can drive differences between communities (Fodelianakis et al. 2019). In fermented food production, an emphasis on selection in structuring microbiomes is not misplaced, as strong environmental selection (for example, low pH, high salt, and anaerobic conditions) is often required to create a desirable microbiome (Ross, et al. 2002).

Studying dispersal in fermented foods also provides an exciting opportunity to link food producers to microbial ecologists. Many small-scale fermenters claim their facilities have a “microbial terroir”: a unique microbiome associated specifically with the geography of their ingredients or production facility (Spano and Capozzi 2011). This can be seen in many wine growing regions in France where producers claim their unique soil mineral content and micro-climate help to create a distinct regional microbiome (Gilbert, et al.

2014). Likewise, cheese producers attribute regional variation in milk as well as the conditions of the caves where the cheese is aged as imparting a unique microbial terroir to their cheeses (Turbes et al. 2016). Sharing the specialized knowledge food producers have regarding their food's microbiome will enhance ecological understanding of the processes involved in microbiome structuring.

This review will summarize the role of dispersal in structuring the microbiomes of a range of fermented foods with an emphasis on cheese, sourdough and fermented vegetables. We also suggest using a framework to help organize current research on dispersal to identify areas where more research is needed. The goal of this review is to demonstrate how studying dispersal could improve production quality by highlighting critical control points for making safe reproducible ferments.

Propagule limitation as a framework for dispersal in fermented food microbiomes.

The propagule limitation framework can be used to consider dispersal by breaking it into three distinct parts. The power of compartmentalizing the dispersal process is that it enables researchers and food fermenters to pinpoint exact areas where this process could influence microbial community structure (Primack and Miao 1992; Miller et al. 2019; Clark et al. 2007). In plant ecology, propagules are typically thought of as seeds but in this review, propagules refer to microbial cells, spores, or any microbial entity that is capable of dispersal and regrowth from a parent population into a new habitat. Propagule limitation can be broken into three component parts: **source limitation**, **dispersal limitation,** and **establishment limitation** (**Figure 1**) (adapted from Miller et al. 2019).

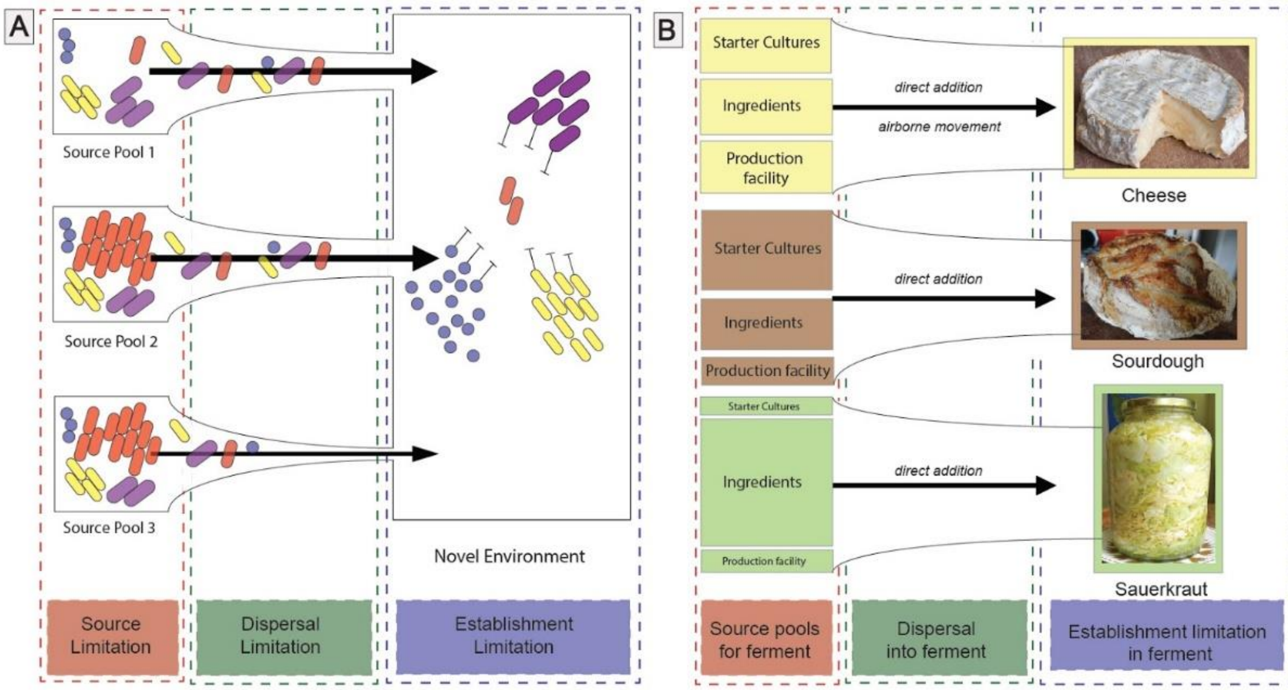

Figure 5.1. Conceptual overview of propagule limitation

(A) The three types of propagule limitation are source, dispersal, and establishment. Source limitation is where not enough propagules are present in local species pools to disperse into the fermented food microbiome. Dispersal limitation is where enough propagules are produced in local species pools, but they cannot reach all fermented food microbiomes due to lack of dispersal opportunities. Establishment limitation is where local population sizes are constrained by the availability of habitats that can be colonized, for example, by competition with other microbes. (B) Overview of propagule limitation in three fermented foods. In cheese (upper), sourdough (middle) and sauerkraut (lower) source limitation is considered on the left of the figure. Colored boxes suggest potential species pools for the food microbiome and the relative size of the box indicates the contribution of that species pool to the ferment microbiome. In the center of the figure, mechanisms for dispersal into the food are considered. On the right of the figure the novel environment is shown, establishment limitation in fermented foods is often driven by abiotic conditions

Source limitation refers to either a low abundance or a complete absence of propagules in feeder communities **(species pools**). Absence of an organism in species pools that feed into a novel community directly limits its abundance in the novel community. Source limitation has a more profound impact on foods that are spontaneously fermented, or those that rely on uncontrolled dispersal from any number of species pools (such as adventitious dispersal of fungal species from cheese caves walls) to contribute additional complexity to their microbiome. Finding the species pools that contribute to specific fermented foods can be essential to understand how source limitation would influence community structure.

Dispersal limitation describes a lack of direct movement of a propagule from the species pool to a new community. Dispersal limitation is not often considered in microbial communities as bacterial and fungal spores are small enough to be passively dispersed for long distances by air currents or moving water (Lindström and Östman 2011). Bacteria have even been shown to disperse 5000 km in air currents (Prospero et al. 2005). Active dispersal where a host or vector such as an insect or even plant material (i.e. wood) facilitates microbial dispersal can also move bacteria and fungi long distances (Martini et al. 2015; Golan and Pringle 2017). As another form of active dispersal, many human-associated microbes have been transported worldwide through the movement of people and products. Anthropogenic dispersal is particularly relevant to fermented foods (Zhu et al. 2017) as human populations have migrated with fermented foods and their associated microbes (i.e. populations bringing sourdough cultures to North America from Europe) (Gobbetti and Gänzle 2013). While this transport of microbes is clearly intentional, the spread of plants and transport of goods worldwide (i.e. oak barrels) has led to an unintentional dispersal of microbes worldwide (Martínez et al. 2004; Goddard et al. 2010). Despite the possibility of high levels of dispersal in microbial populations, dispersal limitation is still detected in communities. For instance, fungal communities across pine forests show regional endemism (Talbot et al. 2014).

Establishment limitation arises when a propagule disperses into a novel habitat but is unable to thrive (growth and reproduce) as a result of either biotic or abiotic selection (Nathan and Muller-Landau 2000). Biotic selection where residents of the novel habitat prevent establishment (He et al. 2014) or abiotic selection where the environment is unsuitable for the dispersing propagule (Zabat et al. 2018; Montel et al. 2014), can both contribute to establishment limitation. With specific reference to microbes, unsuitable establishment conditions can result in the organism entering dormancy. This can influence its apparent abundance in a community when using sequencing techniques as it will be detected in the sequencing data but it is not functioning in the community (van Vliet 2015). Establishment limitation is prevalent in fermented foods as changes in the abiotic conditions of the food i.e. high salt concentration or a low pH are common features of ferments. These abiotic conditions are encouraged by fermenters as they prevent the growth of spoilage organisms, enabling food to be preserved by microbes that tolerate these conditions. Once an established community is present in fermented foods, biotic selection by the abundant community of fermenting organisms can result in exclusion of any invading organisms.

Scales of dispersal in fermented foods microbiomes

Direct dispersal of a microbe can be considered at three scales (**Figure 2**). **Small-scale dispersal** (micrometers - centimeters) describes the movement of microbes across or within fermented foods. Microbial dispersal within food will decrease the heterogeneity of the food microbiome. Imagine for instance, a sourdough that is not fully mixed, pockets of flour will not be broken down by lactic acid bacteria. These pockets of unfermented flour could be areas where spoilage microbes could invade as there are no organic acids produced by LAB in this region of the sourdough to inhibit their growth. Another example can be seen in Stilton cheese. In this cheese the small-scale spatial distribution of microbes can influence flavor development as microbes metabolize the cheese (Ercolini, et al. 2003). Having an even distribution of all microbes across the rind is important to produce uniform flavors in cheese. Some microbes found in fermented foods are motile, for instance many Proteobacteria found in cheese have flagella (Kearns, 2010) and other species such as Bacillus (common in milk) can move across moist surfaces by swarming behavior (Kearns and Losick, 2003). Motile bacteria have also been shown to actively disperse along fungal hyphae (Zhang et al. 2018), showing that interactions in fermented foods can be important for small-scale dispersal. More studies that experimentally address the role of small-scale dispersal in microbial community assembly will help improve our understanding of this process in ferments.

Medium-scale dispersal (centimeters - meters) describes the direct dispersal of microbes either between fermented products or from the production facility to the food product. Medium-scale dispersal is particularly important in fermented foods that are left open as they age, i.e. cheese and salami. Aging provides opportunities for dispersal within the production facility, in fact, medium-scale dispersal can even be promoted by placing "good cheeses" within an aging environment. There is less opportunity for medium-scale dispersal in ferments that are produced in sterile sealed vats, for instance sauerkraut. These ferments are also often sealed (with one-way valves to allow gas leakage) to create anaerobic conditions. In some production facilities, there might still be deliberate transfer (dispersal) of the sauerkraut microbiome from one vat to another. For instance, a slow vegetable ferment can be sped up by back-slopping from a rapid successful ferment (Gibbons and Rinker 2015). This would be an example of medium-scale dispersal mediated by human food producers.

Large-scale dispersal (meters to kilometers) can be studied in foods that are produced over a broad geographic range. Many fermented foods and drinks are produced globally, for example wine fermentation is carried out in Europe, the United States, Australia, South America, and South Africa and many studies have looked at wine microbiomes (Pinto, et al. 2015; Setati et al. 2012; Bokulich, et al. 2016). While it is challenging to directly trace the movements of microbes at a global scale. Microbiologists often use the similarity between microbial communities collected across large distances, to infer large-scale dispersal (Talbot et al. 2014; Finkel et al. 2012; Bell 2010; Astorga et al. 2012). The relationship between geographic distance and community composition is referred to as distance-decay (**Box 1**) and is frequently used to address questions about dispersal (Nekola and White 1999). Returning to our wine example, if South African and European wine microbiomes had more similar communities than European and Australian wine microbiomes, this could indicate that there is more microbial dispersal between Europe and South African than Europe and Australia. It can be hard to confidently attribute community differences to dispersal when there are environmental gradients between sampling points For example as two points differ in location, differences in community structuring might be due to environmental differences not dispersal limitation (Soininen, et al. 2007; Cottenie 2005). In fermented food products, the strong environmental selection associated with fermentation could enables researchers to have greater confidence when attributing distance-decay measurements to dispersal. Take for example two types of fermented vegetables, sauerkraut and kimchi. Sauerkraut, (lacto-fermented cabbage), is common throughout North America and Europe (Zabat et al. 2018) and kimchi (lacto-fermented spiced Napa cabbage) is made in Asia (Swain et al. 2014). Vegetable fermentation requires strong environmental selection in order to reach a standard product (addition of salt and exclusion of oxygen) (Di Cagno et al. 2013). To some degree this strong selection enables differences between communities seen in distance-decay relationships to be attributed to dispersal-limitation, not to environmental gradients.

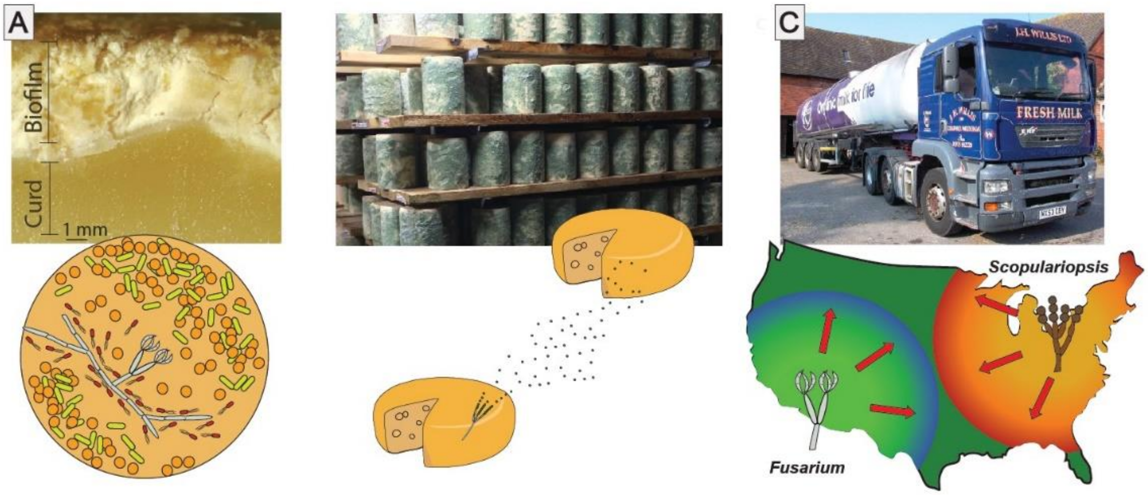

Figure 4. Scales of dispersal within a maturing cheese wheel.

Cheese rind microbiomes illustrate the multiple scales of dispersal that can occur within and between fermented food microbiomes**. (A) Small-scale dispersal.** (lower) Dispersal of motile bacteria (shown in red) facilitated by filamentous fungi (grey) alters the cheese rind microbiome (shown as yellow and orange bacteria and yeast colonies) (NOTE: Not to scale). (upper) Cross-section of a cheese rind, showing a biofilm at the cheese - air interface. **(B) Medium-scale dispersal.** (lower) Dispersal of microbes within a production facility can influence the microbial community. Fungal spores from one cheese can drift onto a neighboring cheese wheel. (upper) Cheeses aging in a cheese cave, showing proximity to potential source microbial communities such as the cave walls and other cheeses. **(C) Large-scale dispersal** (lower) Fungal spores are capable of dispersing long distances across broad geographic areas. In a fictional example, *Fusarium* spores radiate from a cheese facility in the Southern US while a *Scopulariopsis* disperses spores from the

North Eastern US. (upper) Trucks travelling across the United States carrying milk could be one mechanism for fungal dispersal.

Temporal shifts in microbial dispersal

In many spontaneously fermented foods, there is known to be a seasonality to the ferment microbiomes. This seasonality is likely to be driven by climatic conditions and can impact the success of fermentations. One seasonal fermented beverage are lambics, or wild fermented beers, these are highly seasonal and only produced between October and March when the weather is cooler (Spitaels et al. 2014). Rates of kimchi fermentation have been reported to change with seasons, with faster fermentation occurring in the spring compared to the winter. This seasonality has been linked to changes in salt content and quality of the Napa cabbage (*Brassica rapa*) used during this fermentation (Lee et al. 2018). Likewise, there have been reports of seasonal shifts in the lettuce microbiome from higher levels of Firmicutes in the spring (June) to Proteobacteria in later months (August and October) (Williams et al. 2013). Although not reported, perhaps this shift in the phyllosphere microbiome composition could also occur in vegetables. Many Proteobacteria are spoilage organisms and this seasonality could influence the prevalence of spoilage organisms fermented vegetables. Another spoilage organism, *Botrytis cinerea* increases during wet hot conditions adversely impacting wine production (Steel et al. 2011). Considering the high seasonal variation in microbiomes is important for food producers and should also be taken into account by ecologists using fermented foods to study dispersal.

Dispersal dynamics in three fermented foods microbiomes

Cheese, sourdough, and fermented vegetables are three fermented foods that have been well-studied. In these fermented foods systems, there is experimental evidence for source limitation, dispersal at different scales, and establishment limitation. Dispersal dynamics in other fermented foods has been summarized in **Table 5.1** showing wherever possible the research carried out in these foods and how it fits within the propagule limitation framework. Also suggested within the table are areas where more research is required to fully understand the role of dispersal in shaping the fermented food community.

Cheese Rind Microbiome

Microbes are involved in two steps in the production of surface-ripened cheese. Cheese is initially fermented by LAB that are added to the milk, and then aged with surface microbes to ripen the cheese. Ripening involves the growth of bacteria, yeasts, molds, and other filamentous fungi (Macori and Cotter 2018). Culture-dependent and -independent surveys of have been used to determine the structure of the cheese microbiome (Irlinger et al. 2015). Up to 44 different genera of bacteria from three prokaryotic phyla (Actinobacteria, Firmicutes and Proteobacteria) dominate the cheese rind, as well as yeasts from 10 genera and several different genera of filamentous fungi. (Wolfe et al. 2014; Irlinger et al. 2015). Some bacteria are common and abundant in many cheese rinds such as *Brevibacterium* spp. which are present in 78% of cheeses and makes up 20% of the relative abundance (Wolfe et al. 2014). But many species detected in the rinds are only found in low abundance. For instance, several species of Microbacteria have been shown to make up < 1% of all species detected in a cheese rind survey but were found in many samples (at least 23% of all sampled cheeses). Many of isolates detected in the cheese rinds are not added as starter and adjunct cultures but colonize the cheese from other species pools such as ingredients and production facilities. This uncontrolled dispersal would be a particularly interesting area for food producers to consider medium-scale dispersal processes.

Cheese starter cultures that shape the initial microbiome contain a mix of LAB including various *Lactococcus lactis* subsp., *Streptococcus thermophilus, Lactobacillus delbrueckii,* and *Lactobacillus helveticus* (Buyong, et al. 1998; Bachmann et al. 2009; Cotter and Beresford 2017). These starter cultures acidify the environment as they make lactic acid and produce bacteriocins such as pediocin that inhibit the growth of pathogenic organisms such as *Listeria monocytogenes* (Buyong, et al. 1998; Bachmann et al. 2009) Non-starter LAB spp. (*Pediococcus, Enterococcus,* and *Leuconostoc*) and non-LAB species are often included in cheese manufacturing as secondary microbiota. Non-LAB cultures can include a wide range of species such various *Propionibacteria* (*Propionibacterium freudenreichii, Propionibacterium acidipropionici, Propionibacterium thoenii,* and *Propionibacterium jensenii*) (Fröhlich-Wyder et al. 2017), and fungal species such as *Penicillium camemberti, Geotrichum candidum,* and various yeasts including *Kluyveromyces lactis* and *Debaryomyces hansenii* (Spinnler 2017). The milk microbiome includes many LAB genera such as *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Enterococcus* (Irlinger and Mounier 2009; Macori and Cotter 2018) non-LAB species such as *Pseudomonas* and *Acinetobacter* can also be present in milk. Relative to the density of the starter cultures added to cheese, the milk does not contribute a significant microbial load to the cheese microbiome, particularly after milk is pasteurized (Cotter and Beresford 2017). However, microbes that originate from milk can become a significant component of the microbiome of raw milk cheeses. In addition to milk, other cheese species pools can be the terrestrial environment such as cows (teats are known as sources of yeast) (Garnier, et al. 2017) and production facilities i.e. the walls and vats used to produce cheese. Even some Proteobacteria found in cheese have been traced to the ocean environment, dispersing into the cheese facility via sea salt (Yeluri Jonnala et al. 2018; Bokulich and Mills 2013; Wolfe et al. 2014). Studies that investigate cheese spoilage highlight potential routes of contamination, or species pools. For example the source of blue contaminating *Pseudomonas fluorescens* was found to be the water used for cheese production (Martin et al. 2011). Identifying species pools for fermented foods will help producers to set up controls for these microbial inputs to ultimately create consistent food products.

The specific aging techniques used in cheese making can result in different opportunities for dispersal, and the aging conditions can change establishment limitations in the rind. In washed-rind cheeses the rind of an aged cheese is washed in a briny solution. This solution is then used to inoculate fresh young cheeses (Goerges et al. 2008). *Brevibacterium linens* is a dominant species in washed-rind cheeses, and other Actinobacteria are common in these cheese rinds including, *Arthrobacter* spp., *Corynebacterium* spp, *Microbacterium* spp, and *Rhodococcus* spp (Brennan et al. 2002; Percival and Percival 2017). Washing disperses microbes between cheeses and changes the overall rind conditions. Washed-rind cheeses are moister environments and microbes on the rind must be able to withstand the physical disturbance of washing. In natural-rind cheeses, species such as *Staphylococcus equorum*, which can tolerate dry conditions dominate (Yeluri Jonnala et al. 2018; Wolfe et al. 2014). Bloomy-rind soft-cheeses such as St. Marcellin are colonized by a yeast spp. *Geotrichum candidum.* This yeast is usually added to the cheese as a starter culture, but after a cheese maker uses this yeast it can be detected in their cheese making facilities (Jacques et al. 2017; Marcellino et al. 2001). Wolfe et al, (2014) also found the yeast *Candida* to be common in many cheese rinds, as well as filamentous fungi, *Penicillium* and *Scopulariopsis*.

Dispersal in cheese has been studied at all three spatial scales (**Figure 2**). At small spatial scales, microbes will move across the cheese rind. While LAB and *Propionibacteria* are not motile, Proteobacteria are motile, most species have flagella and those which don't can move through bacterial gliding (Kearns 2010). This motility is likely to be affected by the moisture content of the cheese as wetter environments promote the swimming and swarming motility of bacteria (Mounier et al. 2009; McSweeney et al. 2017). *Bacillus* spp. are also detected in cheese rinds and are able to move via swarming (Kearns and Losick 2003). Microbial interactions in cheese rinds can also facilitate small-scale dispersal. This is seen in washed and natural-rind cheeses where fungal hyphae growing on the rind (such as *Mucor lanceolatus*) can be used as a dispersal route by motile bacteria (such as *Serratia proteamaculans*) (Zhang et al. 2018) (**Figure 5.2A**). Dispersal has important consequences for rind development as more dispersal will homogenize the microbiome creating an even rind. For example, consider a bloomy-rind cheese without an even coverage of *Penicillium camemberti*. Gaps in the rind could be problematic for several reasons, the uninoculated section of the rind might not have an even flavor profile. This could be a result of a lack of *Penicillium* metabolism in that area of the rind or it could be because other microbes might use *Penicillium* to disperse across the surface of the rind and were unable to disperse in its absence. Other problems from this uneven rind could result from a lack of biotic selection in the rind. *Penicillium* could provide establishment limitation in the rind that prevents undesirable microbes from growing. Each of these described scenarios arising from an uneven rind could have negative quality and consumer acceptance implications, suggesting that controlling small-scale dispersal in cheese rinds is essential. Although rapid spread of microbes across the cheese rind is desirable to create consistent products, small-scale dispersal could also result in the spread of undesirable spoilage or pathogenic organisms in the rind. For example, *Listeria monocytogenes* is a motile flagellated bacterium that can colonize cheeses (Quigley et al. 2013; O'Neil and Marquis 2006).

Medium-scale dispersal, that considers the movement of microbes between products or within the production environment, can have an important role in rind development. As cheese ages, filamentous fungi disperse from the cheese cave or from older cheeses already present in the cheese cave into the developing rind (Irlinger and Mounier 2009; Kure, et al. 2004; Lund, et al. 2003) (**Figure 5.2B**). Medium-scale dispersal can be particularly interesting to consider as it is an area where food producers readily interact with dispersal processes. For example, source pools of spoilage yeasts can exist in brine used to salt cheeses and on surfaces of production equipment. To prevent their dispersal into the ferment, disinfecting and cleaning programs should be implemented (Garnier, et al. 2017). Producers are also known to use medium-scale dispersal to inoculate developing rinds by aging young cheeses with aged cheeses that have desirable microbiomes. Investigating the proximity of these cheeses to one another or placing the aged cheese near to ventilation systems could influence dispersal rates. In a similar way, ensuring adequate separation of cheeses where medium-scale dispersal is not desirable is important. For this reason, bloomy-rind cheeses are often aged in separate caves to natural-rind cheeses.

Large-scale dispersal of microbes found in cheeses could happen across cheese production regions within countries or across entire continents. Reproducible cheese communities are found within cheeses made over large geographic distances (**Figure 5.2C**) suggesting high levels of community homogenization at large spatial scales (Wolfe et al. 2014). Mechanisms of large-scale dispersal of cheese microbes could be due to active dispersal of starter cultures by the dairy industry and distribution of milk or dairy products within and across countries. Passive dispersal may also play a role in large-scale dispersal when fungal spores move in air currents from one production facility to another (Garnier, et al. 2017).

Establishment limitation influences succession in cheese fermentation as initial colonization of the cheese by LAB acidifies the environment. A low pH (5.3) together with a high salt content (5-10% salt-in-moisture) and bacteriocins produced by LAB starter cultures limits some microbial growth (Irlinger et al. 2015; Fox, et al. 2017). This establishment limitation prevents *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, and *Mycobacterium paratuberculosis* which can be present in milk (at low level) from growing during cheese production (Fox, et al. 2017). Following acidification, the growth of acid tolerant yeasts and molds begin to deacidify the cheese enabling non-acid tolerant bacteria to grow (Irlinger et al. 2015; Cotter and Beresford 2017).

During ripening of cheese how the three cheese types: washed-rind, bloomy-rind and natural-rind cheese are created can all influence establishment limitation (McSweeney et al. 2017). For instance, inoculation of the rind with a fungal species such as *Penicillium camemberti* or *Geotrichum candidum* can alter the conditions of bloomy-rind cheese influencing whether a microbe that disperses into this rind can establish. There are low moisture levels in natural-rind cheeses compared to high moisture levels in washed-rind cheeses, which also influences the structure of the rind microbiome (Wolfe et al. 2014).

Considering all aspects of dispersal processes using the propagule limitation framework could help establish critical control points for cheese production. Microbial dispersal determines the composition of cheese rind microbiomes that in turn translate into specific flavors and aromas in a cheese. Uncontrolled dispersal processes can result in very serious pathogens such as *Listeria monocytogenes* establishing in the cheese (Quigley et al. 2013). Therefore cheese makers face a balance, where reliance on uncontrolled dispersal (such as dispersal from raw ingredients and the production facility) is essential for their cheese's flavor but could also results spoilage or even pathogenic microbes colonizing the cheese rind (Yeluri Jonnala et al. 2018; Garnier, et al. 2017). Improving our understanding of dispersal mechanisms in food systems can help develop strategies to increase beneficial dispersal in controlled ways.

Sourdough starters

Sourdough is composed of bacteria and yeasts that are serially passaged over time. Yeasts, such as *Saccharomyces cerevisiae*, provide the majority of the dough rise by giving off carbon dioxide as a waste product (Xu et al. 2019). LAB and acetic acid bacteria add flavors particularly sour notes, and additional rising (Yann and Pauline 2014). While the majority of the sourdough microbiome used to make bread originates from the starter culture, bacteria and yeasts present in the flour also contribute to the microbial community (De Vuyst and Neysens 2005). Flour contains many bacteria and yeast species that have been detected in the wheat phyllosphere and some LAB spp have even been traced to insect microbiomes (Gänzle and Ripari 2016). Traditional sourdough starter cultures are fed regularly with flour therefore, each successive feed is a potential to introduce more microbes. Only a portion of the serially passaged starter culture is used for making bread (Gänzle and Ripari 2016) and the remainder is maintained for future baking.

Small- and medium-scale dispersal have not been studied in the sourdough system but would provide an interesting research opportunity. Small-scale dispersal could be influenced by the viscosity of the starter and frequency with which the starter is fed. Feeding a sourdough starter requires thorough mixing of the community after more flour is added which would disperse the community throughout the freshly added flour and water. There are anecdotal reports of yeasts from bakery environments moving into sourdough communities, but this has not been a specific focus for research (Carbonetto et al. 2018).

As sourdough starter cultures are maintained by professional bakers as well as home bread makers across broad geographic areas, there is considerable potential for large-scale dispersal to influence the diversity of sourdough starters. This dispersal could occur as humans transport and share sourdough starters, or it could result from movement of sourdough ingredients, flour and water. For example, flour brands available over wide geographic areas might homogenize flour produced from different suppliers to create a uniform product. In this instance, large-scale dispersal of microbes would remove regional differences that might be present in sourdough microbiomes. Large-scale dispersal in sourdough has been studied as part of a large citizen-science project to survey sourdough samples collected from across the globe. Over 500 samples were collected and next-generation sequencing used to determine their microbiomes. In these samples there was no evidence of distance-decay relationships across the United States for either the bacteria or yeast communities, indicating that sourdough microbes are not dispersal limited and communities of LAB and yeast found in the sourdoughs have widespread distributions (ongoing research in the Wolfe lab, Landis et al. unpublished).

Inhibitory microbial interactions within the sourdough microbiome can be strong, resulting in many opportunities for establishment limitation. The rich mix of LAB in sourdough starters produces a range of organic acids, bacteriocins and even antibiotics (Messens and De Vuyst 2002). Lactic acid is produced by all LAB but additional organic acids such as acetic acid and propionic acid can be produced by heterofermentative LAB. Some LAB species are known to produce organic acids that can have a stronger antimicrobial effect than lactic acid for instance, *Lb. plantarum* produces phenyllactic acid and, *Lactobacillus sanfranciscensis* CB produces caproic, formic, butyric and *n*-valeric acids in addition to acetic and propionic (Chavan and Chavan 2011) Sourdough communities that produce a medley of organic acids have a stronger antimicrobial impact than communities that only produce a single type of organic acid (Corsetti, et al. 1996). Likewise, the variety of bacteriocins produced by the community can influence its structure. Bacteriocins are small microbial peptides produced by LAB that have antibacterial impacts; those produced in sourdough have been shown to be active against *Bacilli, Staphylococci* and *Listeria* (Messens and De Vuyst 2002). Sourdough starters containing *Lb. reuteri* have also been shown to produce the antibiotic reutericyclin which is active against gram positive bacteria (Gänzle 2004). Establishment limitation in the sourdough microbiome is predominantly driven by LAB species in the starter culture as they produce antimicrobials that limit the growth of spoilage organisms particularly filamentous fungi.

Fermented Vegetables

Fermented vegetables such as kimchi and sauerkraut are spontaneously fermented. In most cases no starter cultures are added as the fermentation proceeds due to the presence of LAB in the vegetable microbiome. A variety of different LAB species are needed as vegetable fermentation relies on a succession of a few species starting with heterofermenters. *Leuconostoc mesenteroides* is a heterofermenter that rapidly creates acidic conditions by producing lactic acid and acetic acid. In addition, *L. mesenteroides* produces carbon dioxide that forces out any remaining pockets of air in the ferment creating a completely anaerobic environment (Steinkraus 1983). The lower pH then enables populations of homofermenters such as *Lactobacillus plantarum* and *Pediococcus parvulus* to grow, these produce more lactic acid further lowering the pH. A successful ferment will have a pH of 4.2 or lower, which will be sufficient to prevent growth of vegetative pathogens in the ferment (IFT/FDA 2001)

Environmental species pools for vegetable microbiomes are not well understood; however, levels of LAB in plants can be variable, and levels are frequently very low in cabbage microbiomes (Miller et al. 2019; Yu, et al. 2019). Evaluation of two potential species pools for the phyllosphere of Napa cabbages, soil and leaves, found that LAB were not abundant (Miller et al. 2019). Perhaps other species pools could be potential reservoirs of LAB such as insects (Vásquez et al. 2012) or water (Lauzon and Ringø 2012).

There is no evidence for motility in the LAB isolated from vegetable ferments, therefore small-scale dispersal might only occur if there is mixing of liquids, perhaps as gases are produced by heterofermenters. Medium-scale dispersal where microbes move from ferment-to-ferment or disperse into the food from the production facility has not been detected in a facility that processes fermented vegetables. One study that looked at the microbiome of a sauerkraut factory did not find environmental similarities between the fermented vegetables and the production environment (Einson et al. 2018).

Sequencing fermented vegetable products across broad geographic areas can indicate whether there is evidence of a distance-decay relationship (evidence of dispersal-limitation) in the fermented vegetable microbial communities. While many of the same LAB are found in metagenomic surveys, one study identified regional differences in the viral communities between Korean fermented vegetables and Chinese fermented vegetables (Jung et al. 2018). There have been many observational studies of fermented vegetables but none specifically question the role of dispersal in fermented vegetable products (Lee, et al. 2015; Swain et al. 2014; Zabat et al. 2018; Xiong et al. 2012). Detecting the same LAB species in fermented vegetables produced in different locations, could suggest that these LAB are not dispersal limited.

Establishment limitation is likely to play a role in shaping the fermented vegetable microbiome. At the outset of fermentation abiotic selection results in the rapid growth of LAB. Conditions are anaerobic and high levels of salt are added, typically up to 3.5% w/v NaCl in Sauerkraut but can be 6-8% w/v in Asian products (Xiong et al. 2012). Growth of LAB results in the production of a number of organic acids that both lowers the pH and creates an environment that inhibits the growth of spoilage microbiomes (Steinkraus 1983). Many LAB produce bacteriocins that could play a role in establishment limitation. For example, *Lactobacillus sake* C2 isolated from fermented Chinese cabbage produced a bacteriocin that inhibited *Escherichia coli* ATCC and *Staphylococcus aureus* ATCC 63589 (Gao et al. 2010). LAB are excellent ecosystem engineers that can change fermented food environments by producing a range of inhibitory compounds that prevent growth of spoilage organisms.

Even in these three well-studied regularly consumed food systems our understanding of how ecological processes shape food microbiomes is predominantly based on observations that include sequencing and plating of food products. While successful food producers can link their manufacturing processes to successful fermentation, the cause of failed ferments is frequently unknown. We predict that in many cases a failed ferment might result from dispersal-limitation of beneficial microbes or an increase in dispersal of a spoilage organism. By moving from observations to experimental manipulation of dispersal rates in fermented foods, producers will be able to determine the role of dispersal in shaping the fermented food microbiome.

A number of fermented foods in addition to these three well-characterized food systems have been summarized according to the framework of propagule limitation in **Table 5.1.** This table shows the observational work that has been carried out and highlights areas that could be important for food producers to focus on, or areas where more research is needed.

Table

Table 5.1 Propagule Limitation in fermented foods. Examples where source limitation, dispersal limitation and establishment limitation have been noted in fermented foods. Boxes highlighted in grey indicate areas where more research is needed to understand dispersal processes in this food.

Fermented food	Origin of species pool[1]	Source limitation	Dispersal Limitation			Establishment limitation
			Small-scale (µm-mm)	Medium-scale (cm-m)	Large-scale (m-km)	
Fermented vegetables; (sauerkraut, kimchi)	Raw ingredients (Lee, et al. 2015; Einson et al. 2018)	Limited lactic acid bacteria in two potential source pools, soil and leaves (Miller et al. 2019)	Studies that measure within ferment dispersal are needed.	Production facility has a unique microbiome compared to fermented products (Einson et al. 2018)	Studies that measure distance-decay in fermented vegetable products are needed.	Low pH, organic acids and bacteriocins limit the growth of non-fermenting microbes (Zabat et al. 2018; Savard et al. 2002)

Surface-ripened cheese	Starter and adjunct cultures, raw ingredients, production environment and aging facility (McSweeney et al. 2017). Some raw milk cheeses are made without starter cultures (Alegría et al. 2009).	Using starter culture limits the impact of source limitation of fermenting organisms. During aging, members of the rind community could originate from wooden shelves, cheese cave walls or even other aging cheeses. (Yeluri Jonnala et al. 2018)	Movement of bacteria across the cheese surface (Zhang et al. 2018). More studies are need to understand the role of swarming in cheese rind development.	Within production facility dispersal of microbes (Bokulich et al. 2016). Production facility differences in cheese microbiomes (Bokulich and Mills 2013; Dugat-Bony et al. 2016)	Distance-decay relationship cross in cheeses rinds in Europe and the US (Wolfe et al. 2014)	During cheese fermentation: high salt concentrations, organic acids, and bacteriocins limit growth of non-fermenting microbes. During rind ripening: High salt, high moisture and the physical action of washing leads to establishment limitation in washed-rind cheese. Inoculation with a fungus shapes the community of bloomy-rind cheese though competitive inhibition. Dry conditions lead to establishment limitation in natural rind cheeses. (McSweeney et al. 2017).

Lambic, sour and wild-fermented beer	Starter culture, raw ingredients, and production facility (Spitaels et al. 2014; Van Oevelen, et al. 1976)	Many of the wild yeasts will be present on raw ingredients. Air and production facilities are sources of fermenting microbes (Bokulich et al. 2015). Seasonality of yeasts can impact the fermentation. (Traditional lambics are only made during the winter) (Spitaels et al. 2014)	Flocculation is likely to influence dispersal of yeasts in beers (Bokulich and Bamforth 2013)	Movement of wild yeasts and bacteria from production facility to wild fermented beer. Seasonal variation in beer microbiology. (Spitaels et al. 2014; Bokulich et al. 2015)	Global surveys of brewers yeasts have been carried out (Gallone et al. 2018) but there is no distance-decay of communities of wild beer ferments.	Low pH, high ethanol content, high CO_2 content, antimicrobial compounds from the addition of hops and an overall low availability of nutrients prevent growth of non-fermenting microbes (Bokulich and Bamforth 2013).

Sourdough	Starter culture and raw ingredients (Gobbetti and Gänzle 2013; Gänzle and Zheng 2019)	Once a sourdough community has been established the starter culture dominates the community. Source limitation might be important in setting up new sourdough ferments.	Sourdough is fed and mixed daily homogenizing the community. The viscosity of the starter might influence the ability of the microbes to disperse.	Production facility influences the LAB present in European sourdoughs (Scheirlinck et al. 2007)	There is no evidence of a distance-decay relationship in sourdough starters (Ongoing work in the Wolfe lab)	A low pH is created by LAB along with bacteriocins and organic acids. Although the pH and levels of antimicrobials are altered daily as more flour is added.

Fermented meat products	Starter cultures, raw ingredients, and production environment (Aquilanti et al. 2007)	LAB are present in the meat and are added as starter cultures (Krockel 2013; Ferrocino et al. 2018) Starter cultures containing bacteria, yeasts and molds are added to the ferment (Lücke 2015)	No reports of dispersal within the product. Studies are needed to determine the impact of microbial dispersal on the rind.	Little overlap between the microbial community of the production facility and sausage. (Hultman et al. 2015). More studies are needed to understand dispersal during aging of salami.	Studies are needed which measure distance-decay in fermented meat products. Surveys of natural meat products have been carried out but not as an area wide project (Aquilanti et al. 2007; Coppola et al. 2000).	Low pH and high salt content in the meat paste prevent growth of spoilage organisms (Kumar et al. 2017).

Wine	Wine yeasts are added as starter cultures, but wild yeasts and lactic acid bacteria also colonize wine. Yeasts can also come from the production environment. (Garde-Cerdán and Ancín-Azpilicueta 2006)	Starter cultures dominate the community. Other sources can be the wine yeasts and other microbes from the grapes and the production environment.	No reports of dispersal within the product. More studies are needed.	Microbes can be transferred from the production facility into the wines. There can even be a facility specific microbiome (Bokulich et al. 2013)	Distinct microbial communities are associated with vineyards (Bokulich et al. 2014) There is a suggestion that there are regional differences in subpopulations of wine yeasts in NZ (Gayevskiy and Goddard 2012) Dispersal of wine yeasts can be traced to their resistance to common pesticides (Marsit and Dequin 2015)	High alcohol content limits the growth of non-fermenting microbes.

Kombucha	Starter culture (SCOBY).	Source limitation is rare as community relies solely on a starter culture. There is the possibility that ingredients or production environment contribute to the microbiome. More studies are needed.	Spatial structuring of the community occurs during fermentation as bacteria produce a floating cellulose mat (May et al. 2019)	Studies that look at dispersal of microbes within production facilities are needed.	Studies that measure distance-decay in kombucha are needed.	Low pH and some production of ethanol could limit the growth of non-fermenting microbes.

[1] Origin of species pool refers to where we predict the majority of the fermenting microbiome originates from

Experimental approaches to measure dispersal processes in fermented food microbiomes

Experimentally testing the degree to which the structure of fermented food microbiomes is driven by dispersal processes would help producers develop mechanisms to control dispersal in food microbiomes. Although dispersal has not been extensively studied in all fermented foods, we can make predictions about how dispersal could be studied using the propagule limitation framework.

Source limitation. The first step in understanding the role of source limitation in fermented food microbiomes would be to find the species pools that feed into the fermented food microbiomes. While the species pools are typically defined in foods made with starter cultures, particularly when these foods are produced in sterile environments with closed fermentations. There might still be contributions from other unexpected sources such as ingredients, air flow, or workers. In studies where a fermented food has been contaminated by an undesirable species, tracing the spoilage organism in production environments has effectively been a study to find all potential species pools of the ferment microbiome. For instance, investigations into the contamination source of *Pseudomonas fluorescens* with a blue-fluorescent pigment in cheese microbiomes identified water sources as the contamination route. In another example, the source of a *Proteus* species that contaminated a washed-rind cheeses microbiome was identified and this information enabled the cheese producers to alter milk production to prevent further contamination (Martin et al. 2011; Kamelamela et al. 2018); (http://microbialfoods.org/curious-case-purple-cheese-rind/). These examples show that by tracing a contaminant, producers were able to identify all species pools for the cheese microbiome, which included water and milk. These examples also call for a systems-level approach to the study of ferment foods. An overview of the entire food system enables dispersal to be reviewed more efficiently than a narrowly focused study of one aspect of production.

Experimentally manipulating the rates of inoculation from different species pools could show the relative contribution of each species pool to the final food microbiome. For instance, comparisons of cheeses made with raw milk compared to pasteurized milk shows the degree to which the milk microbiome contributes to the cheese microbiome (Salazar et al. 2018; Beuvier et al. 1997). By considering the species pools that feed into fermented foods and their relative contributions, producers can develop strategies to limit invasion of the pathogens and spoilage organisms into the fermented food microbiomes. As well as limiting undesirable microbes, manipulating species pools could also be used to add beneficial microbes. For example, adding ingredients such as garlic (which has been found to contain high levels of LAB) to a vegetable ferment could improve the rates of fermentation (Lee, et al. 2015). If a microbe could be considered source limited in a ferment, then this highlights a situation where a starter culture could be added. In spontaneously fermented vegetables, starter cultures have been considered as a means to reduce the variability in ferment quality (Gardner et al. 2001; Zubaidah et al. 2020) this would reduce source limitation in the ferment. As well as using starter cultures, producers can reduce source limitation in their fermented foods by altering the microbiome of their production facility. This effectively creates a new species pools that can disperse into the fermenting food. An example of this can be seen in the production of some beers, aging the beer in casks inoculates the beer with microbes that reside in the wooden barrels (De Roos, et al. 2019).

Dispersal limitation. After identifying the species pools that contribute to the fermented food microbiome, researchers should investigate how microbes disperse from species pools into the ferment. To investigate small-scale dispersal, researchers could assess the quality of individual products to look at homogeneity of the microbiomes. For instance, pockets of unfermented vegetables or unmixed sourdough starter could be influenced by the physical mixing of communities by the producer during fermentation. In cheese, changing production conditions such as the moisture content of the paste could increase the level of dispersal. A higher moisture content would enable motile bacteria to spread across the rind. Researchers could also look to engineer starter communities for fermented foods which contained species that disperse well together, food producers could use these communities to help create uniform products. For example, if *Serratia* is desirable in a cheese rind community, pairing this species with *Mucor* would help it to spread quickly (Zhang et al. 2018). Small-scale movement of fungal spores can also be facilitated by cheese mites. Cheese mites live on the surface of cheese and are known to be positively attracted to *Penicillium camembe*rti (Thomas and Dicke 1971) and can transport fungal across the maturing cheese (Marcellino and Benson 2013). Studies of mites in the cheese rind can show how vectors influence small-scale dispersal.

Culture-dependent and -independent techniques could be used to measure medium-scale dispersal. For instance, culture-dependent methods could be used to determine fungal species present in a cheese aging facility. These cultures could then be compared with the species identified in a cheese rind pre- and post- aging in this facility. Identifying fungi present in aged cheese but not in the pre-aged cheese, could be used to show dispersal from the aging facility. Shotgun metagenomic sequencing techniques could be used to determine differences in community composition across ferments produced within a production facility. For example, aging cheeses in different cheese caves or locations of a cheese cave. In both scenarios distance-decay relationships can be measured where slight changes in the community composition might exist as a result of medium-scale dispersal. Small changes in community composition are more likely to be identified using metagenomic sequencing techniques than culture-dependent techniques. Complementing this study with sequencing swabs of the production facility could also identify species pools that could be the sources for this medium-scale dispersal. An example of this was carried out by Einson et al. (2018) in a sauerkraut production facility. Various ingredients and parts of the production environment were swabbed, and samples were sequenced for comparison to the ferment microbiome. Low levels of LAB were found throughout the facility, but there was a distinct overlap in the communities of the raw ingredients and the fermented foods. From this it was inferred that the main species pool for the ferment was the ingredients, showing that medium scale dispersal from the production facility was not important structuring the sauerkraut community.

Like medium-scale dispersal, large-scale dispersal would best be studied using sequencing surveys. Accurately identifying microbes using culture-dependent techniques can be challenging but by comparing the communities of fermented foods across large geographic areas the rate of microbial dispersal over long distances can be estimated (Wolfe et al. 2014; Martínez et al. 2004; Bokulich et al. 2014). One study that has specifically tested the role of dispersal in shaping microbiomes has been carried out in the UK in another model system, beech tea leaves. Using beech tree holes as sample sites changes in the composition of a synthetic microbial community were measured throughout the woods. A slight signal of distance-decay in the composition of the synthetic microbial communities was detected at the start of the experiment but this initial difference was lost over time as the microbiomes eventually converged (Bell 2010). This study was unique in that it experimentally manipulated dispersal in an ecologically relevant setting. Replicating this study in fermented foods could also be used to demonstrate the role of large-scale dispersal in fermented foods.

Establishment limitation. Adapting fermentation conditions to promote the growth of desirable organisms while still restricting the growth of pathogens is an important feature of good fermentations. Measuring the parameters of the ferment such as salt content and pH of the samples provide critical data on whether the ferment is progressing correctly. Experiments which manipulate these conditions and test the ability of the ferment to proceed help define accurate fermentation parameters. For instance, changing the levels of salt in a vegetable ferment might change the community succession or even enable pathogens to grow if levels are too low (Johanningsmeier et al. 2007). Unravelling components of establishment limitation in fermented foods to determine their impact on microbiome structure will help producers control the quality of their products.

There are very few scientific studies that have focused specifically on the role of dispersal in structuring microbiomes, but we consider that fermented foods would be excellent model systems to address how this process influences community structure. We believe that dispersal experiments using fermented foods will strike a balance between highly controlled artificial laboratory conditions and natural microbiomes (Wolfe and Dutton 2015). In **Table 1**, areas that could be important for studying fermented foods have been highlighted however while considering these gaps in our knowledge it is important to take into account some caveats.

Limitations to Measuring Microbial Diversity in Dispersal Studies

Many of the recent studies of fermented foods use next generation sequencing to analyze microbial community composition. If food producers use sequencing tools to study dispersal then there are a number of considerations that must be taken into account when analyzing the data such as the sequencing methods used, and how the data is processed after it is collected (Cao et al. 2017).

Some next-generation sequencing techniques, particularly 16S and ITS amplicon sequencing, can only occasionally identify organisms to the species level. It is much more common to detect microbial community members to the family or genus level of taxonomic grouping. This can give a very different result than a study with species level resolution

(Lu et al. 2016). The ability to distinguish between strains of microbes is particularly important in wine fermenting communities where *Saccharomyces cerevisia*e is found in many environments but can be functionally different across geographic areas producing unique flavors (Goddard et al. 2010). Shotgun metagenomic sequencing is more expensive than amplicon sequencing although it is able to detect the members of the microbial community to a great taxonomic resolution than amplicon sequencing. This results in a more detailed understanding of the complexity of fermenting microbiomes when they are analyzed by metagenomic sequencing. It also enables the researcher to ask questions about the function of a particular microbial community, perhaps providing evidence of a functional microbial terroir as opposed to a taxonomic microbial terroir (De Filippis, et al. 2017; Legras et al. 2007).

Setting up industry standards for analyzing and collecting next-generation sequencing would enable researchers to compare fermented food microbiomes across broad areas and might help scientists to understand mechanisms of dispersal by looking at distance-decay in food microbiomes. Sequencing studies could even look at the distributions of different strains of microorganisms by identifying functional traits differences, presence of antibiotic resistance genes, or the prevalence of CRISPR regions showing resistance to bacteriophage. This level of data collection would indicate potential regional strain level differences in food microbiomes that might contribute to a microbial terroir (Walsh et al. 2016; Kumar et al. 2019; Jung et al. 2011; Park et al. 2011). While the benefits of using metagenomic sequencing over traditional plating or gel electrophoresis techniques are apparent, there are still limitations to sequencing data.

Detection of a microbe in the sequencing data does not indicate that it is functioning in the community, as dead or dormant organisms are still detected in metagenomic sequencing data. In food systems where long-distance dispersal could be expected, (spontaneous fermented food systems), organisms capable of dormancy might be overrepresented in the data. Mechanisms that enable long-distance dispersal, i.e. the ability withstanding high levels of UV radiation and extreme desiccation in air currents, are also likely to be correlated with surviving long periods of dormancy (Lennon and Jones 2011). Dormancy has also been shown to influence distance-decay relationships in microbial datasets. As a result of dormancy many organisms can have wider distributions than their functional range, this dampens distance-decay signals between microbial datasets (Locey et al. 2020). This inability to sort dormant from active organisms using next-generation sequencing techniques could bias the sequencing data to spore-forming species that have no influence on the function of the microbiome.

Despite the limitations of collecting and analyzing sequencing data, it will still play an essential role in understanding how dispersal structures microbiomes. As costs of collecting data decrease and the ability to analyze the data becomes more streamlined, then metagenomic sequencing of fermented foods will become a common means to measure diversity of a fermented community. Collating data from fermented foods throughout a geographic area will enable patterns of diversity or distance-decay measurements at species or perhaps even sub-species levels to be collected. Overall this data will improve our understanding of the regional distribution of microbes.

Interactions between dispersal and other ecological/evolutionary processes

In this review, we focus on the importance of dispersal as an alternative process to selection however diversification and drift are also processes that influence microbiome structure. Diversification of microbes can result in phenotypic changes that can influence the dispersal ability of an organism. For instance, loss of spore forming (the primary dispersal propagule in many fungal species), or dormancy traits could prevent an organism from being able to disperse to a new habitat. An example can be seen in the cheese microbiome where the evolution of *Penicillium* to the cheese environment involved a loss of a number of traits including spores production (Bodinaku et al. 2019). This diversification directly impacts how the fungus disperses as the loss of spore forming ability will reduce its ability to disperse. In contrast, the ability to remain dormant for long periods of time or to withstand high levels of UV radiation will enable propagules to disperse long distances. It has also been shown in the dairy industry that pasteurization of milk can bias the milk microbiome to spore forming bacteria that can withstand and are even activated by the high pressure and temperature of pasteurization (Quigley et al. 2013).

Drift, another ecological process, is dependent on population sizes. If only small numbers of microbes disperse into a fermented food then drift might be an important force in shaping the community structure (Stegen et al. 2015). An example of drift in a fermented food might be important when considering the batch size of a spontaneous ferment. In a small batch, relying on the chance dispersal of fermenting organisms into the fermentation can result in a low diversity or a failed fermentation when compared to a large batch fermentation where there is a much greater probability that there are at least some fermenting microbes. Considering the population bottlenecks that fermenting organisms go through before rapid growth in a food can help producers make sure that they select appropriate numbers, strains or mixed communities of starter cultures to ensure a successful ferment.

How will understanding dispersal influence safety and quality of fermented foods?

Breaking dispersal into three areas outlined by propagule limitation (source limitation, dispersal limitation and establishment limitation) helps define areas of community assembly that can be influenced by the process of dispersal. This dissection of dispersal in fermented foods may seem like a purely academic exercise. But we strongly believe that this framework has the potential to provide fermented food producers with new microbiome management approaches.

Management of source limitation in fermented foods made with starter cultures could involve maintaining starter cultures at the correct temperatures and using them within an appropriate time frame to ensure that all microbes are viable when they are added. Source limitation in spontaneously fermented foods that rely on the presence of LAB, yeast, and other microbes in/on the raw ingredients will be harder to manage. In these foods it could be important for fermenters to consider seasonality or the weather conditions during crop growth or harvest. In many of these foods the presence of propagules in species pools is likely to be driven by large-scale dispersal. Large-spread dispersal could be seasonally impacted for example, an increase in precipitation and wind could increase microbial dispersal into species pools. Vegetable microbiomes can be altered following rainfall (Allard, et al. 2020) that in turn might affect fermentation. The connection between rainfall and fermentation has also been made in wine grapes, where seasonal rainfall has been linked to different yeast species (Longo et al. 1991).

Small-scale dispersal of microbes in fermented foods is generally found on the surface of solid foods or within liquids, while medium-scale dispersal relies on the movement of microbes from within the production facility, such as neighboring products, sequential batches, or even wooden shelves and barrels, into the ferment foods.

Implementing specific management techniques to control small-scale dispersal could be as simple as controlling the humidity of the environment to enable or prevent dispersal within a ferment. Medium-scale dispersal in fermented foods could be controlled by providing adequate spacing between shelves (in aging cheeses and salami) or vats (in beers), improving hygiene and cleaning routines, monitoring airflow and using filtration systems where appropriate. Workers have the potential to vector microbes into the production facility so making sure that staff have been appropriately trained to practice good hygiene is essential. Managing dispersal is not only about preventing addition of spoilage or pathogenic organisms but also considering how to promote dispersal of good microbes. Promoting good microbial dispersal could include inoculating microbes into foods or adding potential species pools i.e. foods containing desirable microbes to the aging facility.

A successful fermented food often has a predictable microbiome; however, producers often seek ways to obtain a unique set of microbes that will impart novel flavors. Relating dispersal in fermented foods whether from ingredients, starter cultures, aging on specific wood or even linking air flow or water sources to specific microbes will help producers understand how their product's microbial terroirs is developed.

Conclusions

To successfully manipulate microbiomes for improved human health and food production, we need to understand the processes involved in microbial community assembly (Mueller and Sachs 2015; Gopal, et al. 2013; Scheuring and Yu 2012). There has been a strong emphasis on the role of selection in shaping food microbiomes and dispersal has been considered less important. We show that dispersal can play an important role in determining the structure of fermented food microbiomes. To study dispersal, most studies rely on observations and calculate distance-decay values between spatially distinct communities and there are few studies that experimentally manipulate microbial communities to determine the role of dispersal in community structuring (Finkel et al. 2012; Archer et al. 2018; Bell 2010). Fermented foods are useful model systems for studying propagule limitation, as their microbiomes are experimentally tractable, can be easily reproduced, and strong selection is an inherent component of most fermentations. More research is needed on small-scale dispersal, medium-scale dispersal, and source limitation to fully understand the role of dispersal in these systems.

Box 1. Distance-decay One consistent principle in ecology is that two neighboring communities will be more similar in composition than two distant communities. This difference in composition over distance is termed distance-decay and is thought to come about as a combination of two processes; environmental selection and dispersal limitation. Over a geographic distance a concurrence gradient in environmental conditions affects which organisms can survive in a habitat. This environmental selection is in balance with dispersal limitation; how organisms disperse from a source. Organisms with a wide dispersal range can be found in more communities than those with limited dispersal range.

Chapter Six

Summary and Conclusions

One of the goals of microbial ecology is to determine how microbes build stable and reproducible communities. The evolutionary and ecological processes that influence the structure of microbiomes include selection, dispersal, diversification and drift (Nemergut et al. 2013; Hanson et al. 2012). While all of these processes are likely to play a part in structuring microbiomes, dispersal and selection are generally considered to be the main forces driving microbiome structuring (Fodelianakis et al. 2019). The extent to which these processes shape microbial communities has not been reported, and there is a lack of research considering the role of dispersal in shaping communities.

In this dissertation I use Napa cabbages and fermented vegetables as model systems to explore how patterns that are detected in phyllosphere and ferment microbiomes can be linked to specific ecological processes.

I show that dispersal can be considered as propagule limitation and broken into three components parts: source limitation, dispersal limitation, and establishment limitation (Miller et al. 2019; Turnbull et al. 2000; Moore and Elmendorf 2006). Each of these components parts can be considered in fermented food systems to determine how dispersal is structuring their microbiomes. If all aspects of the propagule limitation framework cannot be described in a fermented food, then further research in the food system could be required to fully understand how dispersal is shaping microbial community assembly.

In addition to emphasizing the role of dispersal as a process that shapes microbiomes, this dissertation also presents a new study system, the cabbage microbiome. I believe that this model system – gnotobiotic Napa cabbage and sterile vegetable extract could potentially be used to address many ecological questions in microbiology. But it is particularly suited to investigating dispersal, as vegetable fermentation is spontaneous relying on dispersal of microbes into the vegetable phyllosphere. This model system also provides a novel link between the structure and function of phyllosphere microbiomes and its reassembly in fermented vegetable microbiomes.

Exploring ecological questions in fermented foods is an exciting opportunity to link ecologists with food producers as many of the questions that pertain to the structuring of microbiomes can have important implications for the quality and safety of fermented food products.

Summary of findings

Chapter Two. I describe protocols for producing gnotobiotic Napa cabbages and sterile vegetable extract. These systems have been shown to be effective in measuring growth of bacteria and yeast in the Napa cabbage phyllosphere. Sterile vegetable extract can be used to measure growth of phyllosphere isolates under fermentation conditions. ***Future work:*** I believe this model system can be used to assess microbial community assembly in the phyllosphere. Microbial communities can impact the growth and safety of crop plants (Heaton and Jones 2008) and I believe gnotobiotic Napa cabbages could be used to experimentally explore how bacteria and yeast grow and interact in the phyllosphere. I use sterile vegetable extract in Chapter Four to determine how fermentation conditions influence the presence of yeast in fermentation. I believe this study could be expanded to explore biotic interactions during fermentation, for instance, *Leuconostoc mesenteroides* produces a range of organic acids that could influence community structure during fermentation. A combined approach with gnotobiotic cabbages and SVE, could also be used to explore the survival of spoilage or pathogenic species from the phyllosphere into a ferment (Dinu and Bach 2011).

Chapter Three. I determined that LAB are in low abundance in the Napa cabbage phyllosphere. Two species pools, soil and leaves, were tested as potential reservoirs for LAB. A field survey across the Northeastern United States found that LAB are also in low abundance in these species pools. Inoculation of LAB into the Napa cabbage phyllosphere did not result in high levels of these species in the phyllosphere as the initially high levels of LAB rapidly decreased shortly after inoculation. This showed that LAB are establishment limited in the Napa cabbage phyllosphere. ***Future work:*** I found that LAB are potentially source limited in the Napa cabbage phyllosphere, but there are other species pools that might be important for establishing the Napa cabbage phyllosphere. Literature and preliminary studies hint that LAB might be inoculated into the phyllosphere via insects. Growing cabbages in conditions that exclude insects and testing them for the presence of LAB and for their ability to ferment could test whether insects are a species pool for LAB.

Chapter Four. I determined the diversity and relative abundance of fermented vegetable microbiomes from across North America. Using metagenomic sequencing I found that these products are fermented by many of the same LAB species detected in kimchi and sauerkraut products in other parts of the world. Using culture-dependent techniques I found unexpectedly high amounts of yeast in many of the sampled products. How fermentation conditions influence the abundance of yeast in the samples was tested using *in vitro* fermentation experiments using SVE. These results showed that a warmer fermentation temperature (24ºC) resulted in a greater RA of yeast in the ferment. ***Future work:*** Exploring how biotic interactions in the fermentation environment can also alter the relative abundance of yeast in the ferment. High levels of *Leuconostoc* were detected at cooler temperatures, determining how *Leuconostoc* influences microbial structuring during fermentation could be essential for vegetable fermenters. Studying biotic interactions could be carried out by using leave-one-out experiments where each member of a synthetic community is removed sequentially to determine its role in microbial community structuring.

I acknowledge that some of the metagenomic data in this chapter remains unstudied. It is my goal to also determine the functional diversity of the fermented vegetable products using our metagenomic dataset. In this analysis I would focus my efforts into determining genes that metabolize carbohydrates using KEGG gene databases (Mitra et al. 2011). In addition, I would like to explore the taxonomic diversity of the viruses found in our samples using specific viral databases in Kaiju and by looking for the presence of bacteriophages using PHASTER (Arndt et al. 2016).

Chapter Five. This review summarizes what is known about dispersal in fermented food microbiomes with an emphasis on the cheese rind microbiome, sourdough, and sauerkraut. I advocate for the use of the propagule limitation framework to consider community assembly across all three fermented food systems. I hope that this review would be useful to food producers and will help them to consider the impact of ecological processes in food production. ***Future work:*** To explore practical applications of the propagule limitation framework, it would be valuable to work with one or more food producers to develop this framework to be a useful tool. For example, I could design and execute fermented food management plants that explicitly control microbial dispersal processes and monitor product quality over time.

Broader implications of this work

This dissertation demonstrates how fermented vegetable products can be used to study ecological processes, specifically dispersal, that structure microbiomes. The role of dispersal in microbiomes is interesting for ecologists to study but is essential for food producers to consider in order to control the microbiomes of their food products. I hope that this dissertation helps to establish links between vegetable fermenters and microbial ecologists. My research links the field ecology of LAB to their impact in vegetable fermentation, this provides a direct practical link between the work we do at Tufts University and how the sauerkraut you make in your kitchen is fermenting.

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