Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61149R2
Full Title:	A Gnotobiotic System for Studying Microbiome Assembly in the Phyllosphere and in Vegetable Fermentation
Section/Category:	JoVE Biology
Keywords:	phyllosphere, cabbage, fermentation, germ-free, gnotobiotic, sterile vegetable extract, microbiome, microbial community
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Medford, MA, USA

TITLE:

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

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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[1–5]. In turn, the health of crops directly impacts food safety and quality[6,7]. 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[8]. 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[9,10]. The phyllosphere microbiome is an excellent experimental system for studying the ecology of microbiomes[11]. These communities are relatively simple and many of the community members can be grown on standard lab media[10,12,13].

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[14,15]. Lactic acid bacteria (LAB) are considered ubiquitous members of vegetable microbiomes, however they can be in low abundance in the phyllosphere[16]. 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[17]. 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 of Materials**) 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 of Materials**) 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 of Materials**) 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 of Materials**) 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 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 buffer 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 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[18,19].

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: Man, 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 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**). 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 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**).

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 3A**. The pH of each sample was also recorded throughout the fermentation (**Figure 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).

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of germ-free cabbage setup.

Figure 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 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.

Table 1: Microbial isolates inoculated on germ-free Napa cabbage.

Supplemental Figure 1: Growth of *Brassica rapa var pekinensis:* Bilko in germ-free conditions.

Supplemental Figure 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.

DISCUSSION:

Germ-free Napa cabbage plants have been used to study dispersal limitation of lactic acid bacteria in the Napa cabbage phyllosphere[17]. Germ-free Napa cabbages can also be used to test individual or pair-wise growth in the phyllosphere (**Figure 1**). 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**) show that LAB grow rapidly and acidify the media in the same way that would be anticipated in a ferment[17].

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[16]. 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 fermented 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 of Materials**) 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[20,21]. 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[22]. 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[13,23]. 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[24] or microbial colonization of maize root[25] 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[26]. 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[17]. 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.

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530 and legumes. *Antonie van Leeuwenhoek*. **49** (3), 337–348 (1983).

Figure 1

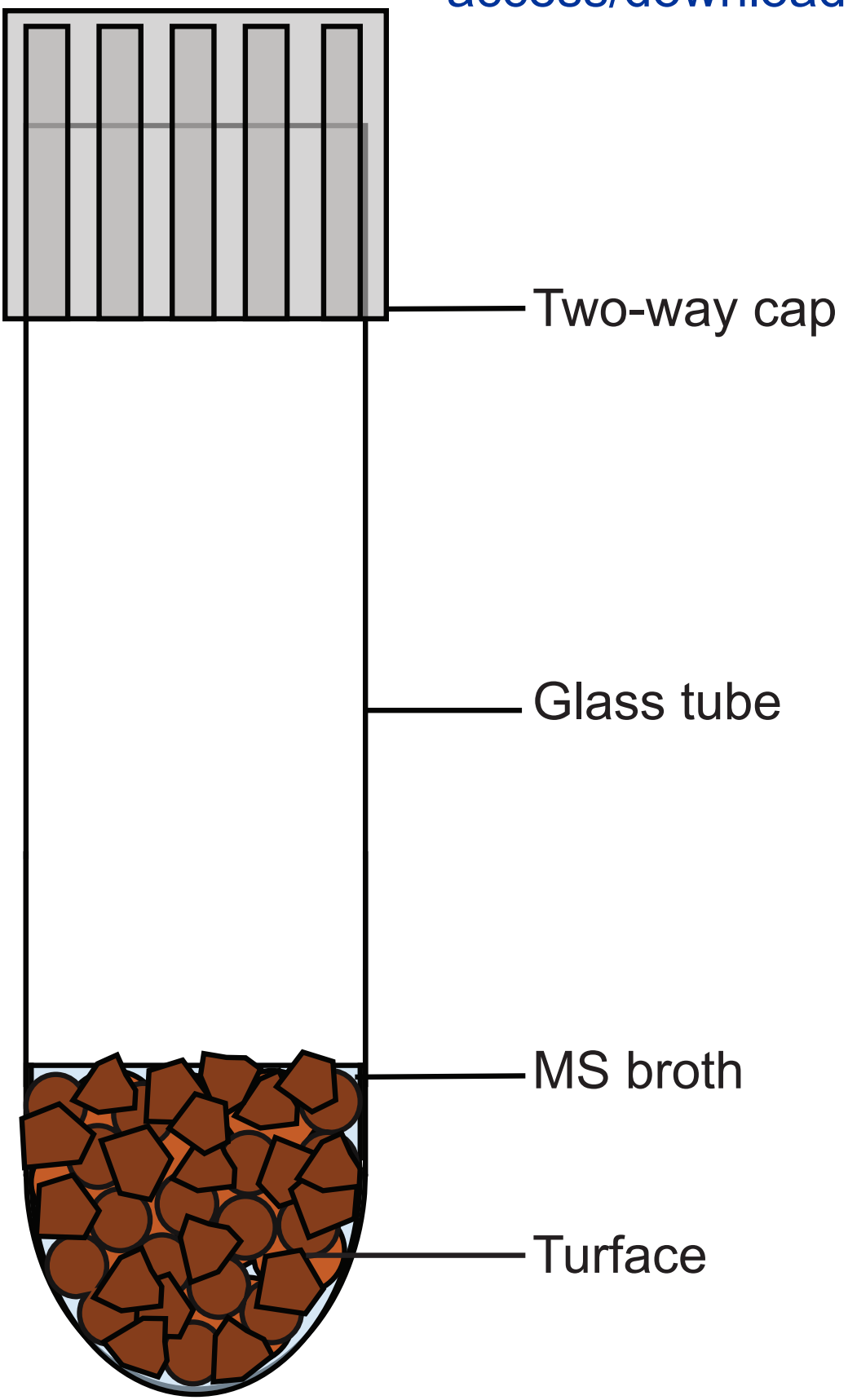


Figure 2

Click here to access/download;Figure;Figure2.pdf

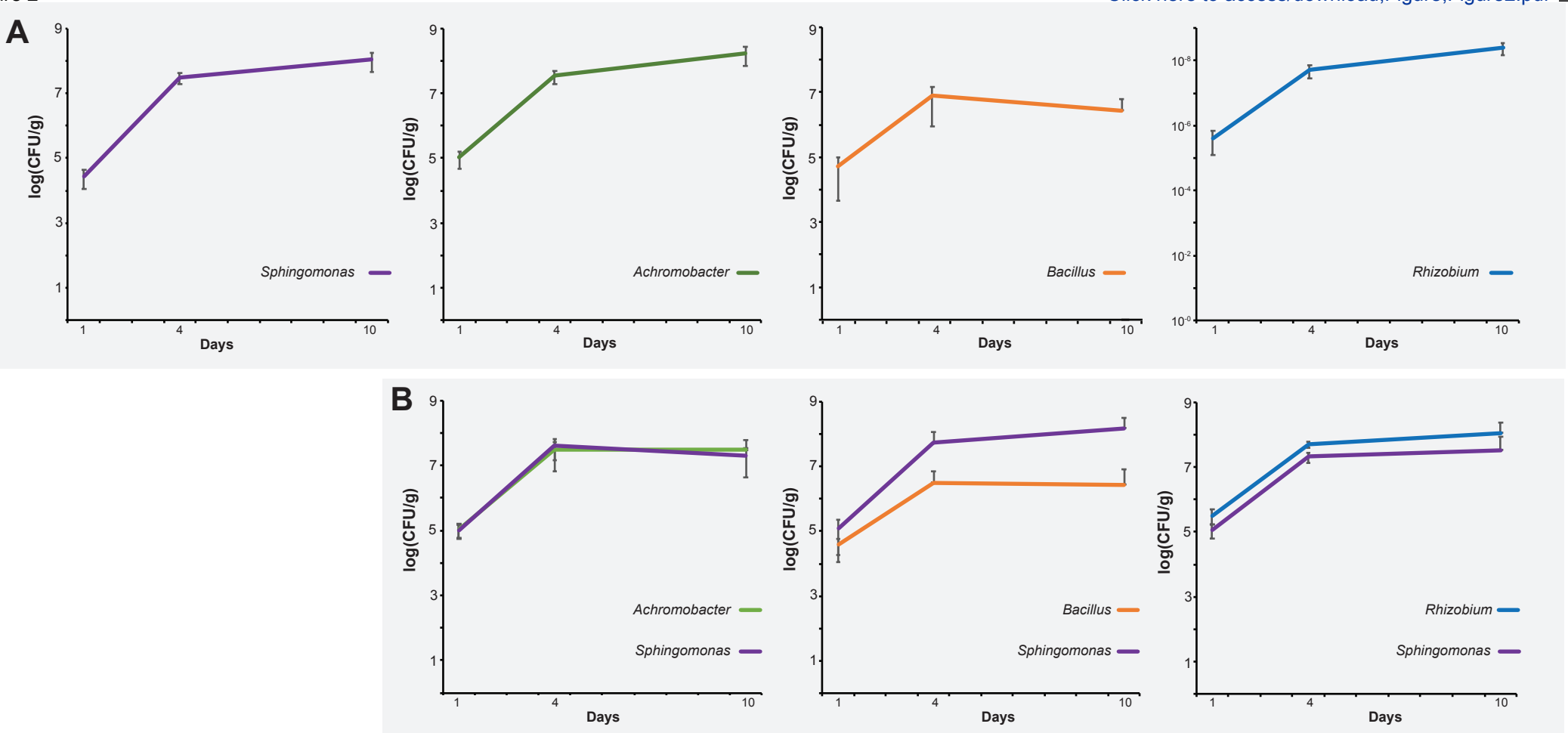
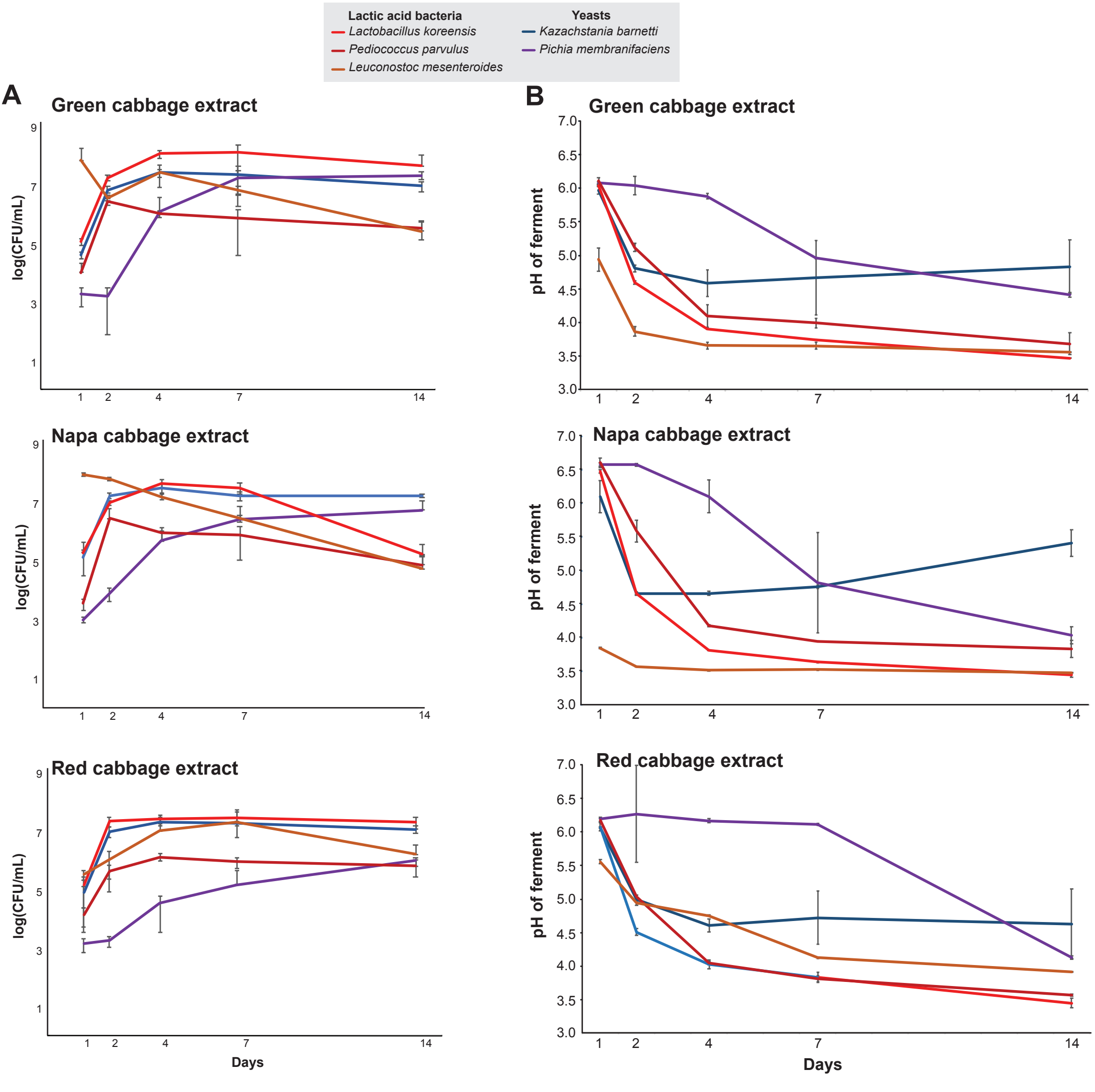


Figure 3

Click here to access/download;Figure;Figure3.pdf



Phyla/Genera of microbial inoculants	Source of microbe
Firmicutes *Bacillus*	Phyllosphere
Firmicutes *Lactobacillus*	Fermentation
Proteobacteria *Achromobacter*	Phyllosphere
Proteobacteria *Rhizobium*	Phyllosphere
Proteobacteria *Sphingomonas*	Phyllosphere

Name of Material/Equipment	Company	Catalog Number
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
Basket coffee filters	If you care	
Bleach (mercury-free)	Austin's	50-010-45
Borosilicate Glass tubes	VWR	47729-586
Calcined clay	Turface	MVP
Cuisinart blender	Cuisinart	
Dissection scissors	7-389-A	American Educational Products
Ethanol	VWR	89125-172
Forceps	Aven	18434
Glycerol	Fisher Scientific	56-81-5
KleenGuard M10	Kimberley-Clark	64240
Large plastic container	Rubbermaid	
Light racks	Gardner's Supply	39-357
Magenta tm 2-way caps	Millipore Sigma	C1934
Man, Rogosa, and Sharpe	Fisher Scientific	DF0881-17-5
Micro pH probe	Thermo Scientific	8220BNWP
Micropestle	Carolina	215828
MS nutrient broth	Millipore Sigma	M5519
NaCl	Sigma Aldrich	S9888
Napa cabbage seeds	Johnny's Select Seeds	2814G
Petri dish 100 mm x 15 mm	Fisher	FB0875712
Phosphate buffer saline	Fisher Scientific	50-842-941
Plant tissue culture box	Sigma	Magenta GA-7
Serologial pipettes	VWR	89130-900
Sterile dowel	Puritan	10805-018
Sterilizing 0.2 µm filter	Nalgene	974103
Tryptic soy agar	Fisher Scientific	DF0370-17-3

Wide orifice pipette tips	Rainin	17007102
Yeast, peptone and dextrose	Fisher Scientific	DF0428-17-5

Comments/Description
Ordered on Amazon.com from various suppliers
(unbleached paper) Purchased from Wholefoods
Ordered on Amazon.com from Root Naturally 6 Quart Bags. Particle size approximately 3-5 mm
Cuisinart Mini-Prep Plus Food Processor, 3-Cup
Ordered on Amazon.com
Ordered on Amazon.com
Ordered on Amazon.com
full-spectrum T5 fluorescent bulbs
This media is for broth and 15 g of agar is added to make plates
Also called Pellet Pestle
Murashige and Skoog Basal Medium
B. rapa var pekinensis (Bilko)
Used to make agar plates
Teknova
Autoclave before use to sterilize
This media is for broth and 15 g of agar is added to make plates

This media is suitable but media can also be made using yeast, peptone and dextrose, add 15 g of agar when making plates

JoVE61149 - Response to Reviews

We would like to thank the two reviewers for their comments and suggestions for improving this manuscript. Original comments are bold and italicized and our response to their comments has been added below each point.

Reviewers' comments:

Reviewer #1:

In this manuscript Miller et al. present a protocol for growing germ-free cabbages in the laboratory. These cabbages can then be inoculated with defined sets of microbes to study microbiome assembly and interactions in the phyllosphere. Overall the manuscript is well-written, and I believe that the methods are of interest to the community.

We are pleased that the reviewer considers this paper of interest to the community.

Minor Comments

I believe it is JoVE policy that the manuscript must be free of all commercial language and all commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We have removed all commercial language from the document and made sure that all materials are detailed in the Table of Materials

In Step 2.1.4 is the exact same tube containing the glycerol stock used both for enumerating colony counts and for inoculation onto the cabbages or are separate aliquots of the same stock used for each step?

After making a 15 mL tube of glycerol stock, 14 mL is retained for inoculating onto the cabbages and 1 mL is plated to determine the concentrations of this inoculating solution. We have edited point 2.1.4 to make this point clearer.

In Step 2.3.4 would it be more appropriate to report growth in terms of cells/g/mL of homogenate?

As the focus of this step is microbial growth on the cabbage leaf we report on CFUs/g of cabbage which takes into account variation in amount of cabbage.

In Step 3.6 is the weight of the 2% NaCl based on the plant weights measured in Step 3.3? Because this is a plant slurry, would it be more appropriate to indicate a final concentration of NaCl in % w/v?

We have changed this in the text.

In Step 4.4 are the plants incubated stationary or shaking?

The plants are stationary when growing.

Lines 364-7: Are you indicating here that the pump bottles can only be used a single time and then discarded or used a single time before they must be sterilized again?

We have had success with sterilizing and reusing pump bottles. However as they are relatively inexpensive we typically purchase new pump bottles for each experiment to reduce any risk of contamination. We do not reuse the sterile bottles when inoculating with *Bacillus* in experiments because we have found that spore-formers are especially problematic in terms of contamination.

It's not of immediate concern for this manuscript, but I am curious if the authors have ever germinated their cabbages in medium containing microbes to study potential interactions between the rhizosphere and the phyllosphere? Is this a potential application of the method?
We have not looked at rhizosphere interactions in this system, but it would be an interesting next step for this research.

Figure Comments
Figure S1 - Could the authors include an example of B. rapa var pekinese for comparison as I'm unable to determine what successful growth looks like in this system compared to these examples with limited success.
I have taken photos of successful *B. rapa var pekinese* to add to this figure.

Line Comments
60: Replace "cheap" with "inexpensive"
We have made this change.
77: What does MVP stand for?
This is part of the name of the calcined clay product which we recommend. We have removed the name from the manuscript and referred the reader to the Table of Materials.
145: Is growth of even a single colony indicative of contamination?
We have not used bottles with a single colony as we believe it could impact our results.
160: Insert a space between "1" and "mL"
We have made this change.
199: What is a "true leaf"? How do you distinguish a "true leaf" from another leaf?
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.
286: spp. shouldn't be italicized
We have made this change.
315: Why is natamycin added to the plates?
The natamycin is not needed in this instance. We apologize for this error. Natamycin is only needed to isolate bacteria from mixed bacteria and fungal communities.
335: Insert "and" between "rapidly" and "acidify"
We have made this change.

Reviewer #2:

Manuscript Summary:

This manuscript describes a protocol for the establishment and vetting of a system to evaluate the growth of microbial inocula in axenic cabbage plants as well as creation of media to evaluate fermentation and growth of these communities. Overall, I found the manuscript and protocols quite clear and I think they will certainly be of use to the larger community. Well done!

We are really glad that the reviewer found the methods to be clear and thinks that they will be useful.

Major Concerns:

None

Minor Concerns:

L7 and throughout: "germ-free" better as "gnotobiotic" or "axenic"

We regard gnotobiotic as having a defined community of microbes. Germ-free or axenic would be relevant terms for cabbages grown in our system.

L30-32: "the system is quick...assembly" feels like it could be rephrased in ways that are not as definitive or make assumptions. For example, instead of "cheap" it could say "relatively inexpensive and less time consuming".

"Easy" also seems a bit subjective here.

The text has been altered to “this system is relatively simple and inexpensive to set up in the lab”

L60: again, feels like there is a better term than "cheap" here

Changed to inexpensive

L80: would be good to have a relative number for the particle size here, in case "MVP" is changed in the future

Noted, approximate particle size added to the document.

L158: why mix into a 15mL solution if you only store 1mL?

We apologize for the confusion on this point. This text has been changed to reflect concerns by Reviewer 1 so hopefully this section is clearer. All 15 mL of the glycerol stock is stored but as a 14 mL inoculation solution and a 1 mL aliquot to determine the concentration of the inoculation solution.

L206: How will researchers know what constitutes 10^4 cells? Would be good to have a measurement step when freezing either through plating dilution series or OD600 or something

By thawing and plating the 1 mL aliquot of glycerol stock the researcher will be able to estimate the concentration of the 14 mL of inoculation solution. Hopefully changes in point 2.1.4 will make this clearer)

L367: would be good to suggest what steps could be implemented to carry out extra "caution" with Bacillus

Added “We do not reuse any pump bottles which have come into contact with *Bacillus* spp”

L377: can change in OD here be used as a quick metric for whether the fermentation is working then (if not for quantifying bacteria)?

We found that using OD to measure fermentation could be misleading as the sterile vegetable extract can clear as it ferments. Checking the pH of the sterile vegetable extract can also be used to check whether fermentation is proceeding.

L382: "a lot of interest" could be rephrased here to be less colloquial

Changed to “is topical as there is evidence that”

Figure S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);FigureS1.jpg

Bilko

B. rapa var pekinese

Figure S2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);FigureS2.jpg