Title: Study of microbial succession in fermenting cabbage

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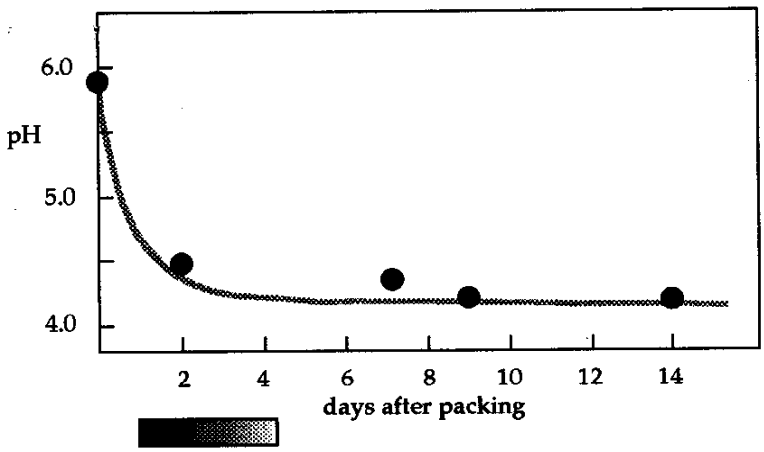
Abstract:

Sauerkraut is typically prepared by a natural fermentation resulting from microbial succession. Many introductory microbiology manuals describe a brief method for initiating this process. The fermentation is useful in beginning microbiology classes because it is slow (taking ~3 weeks), does not depend on good aseptic technique, and is not perturbed by regular sample–taking.

In my classes students take microbial and other samples from fermenting cabbage each class session, and isolate a few colony-forming organisms in pure culture from those they find present. The culture series, which can be enhanced by use of various differential media, visually demonstrates initial microbial diversity and the succession of different types that predominate later. Students study culture requirements and tolerances of the organisms they have isolated, and do other tests to try to identify one of the isolates. Using the information they obtain, students can speculate about the role each of the organisms they studied might have played in the microbial succession that turns raw cabbage into fragrant sauerkraut. Those wishing to meet a writing across the curriculum requirement write a paper describing their observations on the microbial succession and on the identification of one of the strains.

The series of experiments uses only methods typically taught in any introductory microbiology laboratory course, but provides a framework in which these methods can be integrated with each other and related to a familiar food. Through studying only a few organisms, at the end of the exercise students realize that they know, in principle, how to do a complete description of this microbial succession, down to identification of most of the bacterial strains that might be involved, and thus they know in principle how to study other microbial successions. Meanwhile they have developed a personal interest in "their" organism, and in microbiology.



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A Study of Microbial Succession in Sauerkraut
Figure 2

Study of microbial succession in fermenting cabbage

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Introduction

Introductory microbiology lab courses for undergraduates usually function as surveys of methods and protocols representing a variety of tasks encountered by working microbiologists. In the available laboratory manuals these are presented as independent brief exercises: to the student these can be a bewildering array of apparently unrelated material. One way to organize this material and make it more engaging for students is to apply groups of methods to a larger investigation that sets a context for their use. In microbiology this can be done easily by investigating the microbial succession that turns cabbage into sauerkraut.

The fermentation of cabbage into sauerkraut can be used at the beginning of the course with novice microbiologists, because a palatable product is usually obtained even when the preparation is opened and sampled twice/week by someone just beginning to learn aseptic technique. The students can practice various standard methods with organisms they themselves isolate from the fermenting preparation, so that experimental results are not predictable. Most students find this more interesting than to do the experiments with lab strains of bacteria that will give known results. Thus, students are engaged more deeply with the course material. And when all the data are collected, the class collectively will have a description of how this particular fermentation results from a microbial succession.

This module can be incorporated as part of the regular curriculum of any undergraduate laboratory course in introductory microbiology. It can be limited to a brief investigation of microbial succession, or widened to incorporate much of the curriculum typically included in an introductory microbiology lab course (as described here). Students can work independently or collaborate on the sequence of experiments, according to classroom resources. The only equipment needed is that already present in an introductory microbiology laboratory classroom. Project results are suitable for poster presentation or can form the basis of papers students write to satisfy writing–across–the–curriculum requirements, and the need to keep track of data for such purposes helps students learn laboratory notebook–keeping.

Observing microbial succession in fermenting cabbage

Cabbages, obtained from the supermarket, are cut into shreds, and packed with salt into jars of 125 ml to 250 ml capacity, capable of being sealed so as to exclude air completely. We pack the cabbage into the jars in 3% NaCl solution, but packing with dry sea or table salt (non–iodized) into beakers or crocks is also satisfactory[1]. Fermenting preparations are incubated at 20 to 25°, or at room temperature.

At each class visit (once/wk for 1 credit lab classes, twice/wk for 2 credit lab classes), students open the cabbage jars for sampling. First a loopful of the brine is streaked onto trypticase soy agar (TSA, Difco) or a differential medium such as eosin methylene blue agar with lactose (EMB, Difco). The medium can be tailored to the curricular emphasis chosen by the instructor. We prefer to use a glucose yeast extract medium with $CaCO_3$ (glcYE $CaCO_3$, contains per liter 5.0 g yeast extract, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 20.0 glucose, 10.0 g $CaCO_3$, 20.0 g agar), which is differential for fermenters producing a lot of acid from glucose. The calcium carbonate is insoluble in the medium as prepared, but solubilizes in halos around the colonies of copious acid producers. The medium is more satisfactory for the cabbage experiment than are differential media that depend on dyes, because its neutral color allows students to appreciate

the varied colony colors and morphologies of the microflora initially present on cabbage. The repetition of this sampling procedure allows student to become skilled at making streak plates.

As part of each sampling, pH of the brine is measured using pH paper and the students record the appearance of the cabbage, its texture is determined by eating a piece (if there is no evidence of spoilage), and the smell of the preparation is noted. Then the jar is topped up with 3% NaCl, to replace any lost during incubation or sampling, and the jar is resealed. This sampling continues for three weeks. It is also useful to titer the brine of the preparation on day 0 and at the close of the experiment. For titrations we use 3% NaCl as diluent and plate on the same medium used for streaking. All cultures are incubated at the same temperature used for incubating the fermenting cabbage.

The succession of microorganisms active in cabbage as it ferments is revealed by changes in the types of colonies seen on successive streak plates made from the brine (Figure 1). A day 0 culture on glcYE $CaCO_3$ medium is not shown because some colonies in these cultures are too mucoid to invert on the scanner. However, such cultures do show at least 10 visibly different colony morphologies in colors ranging from nearly transparent through white, yellow, orange, pink, and occasionally red or brown, in addition to having the occasional mold colony. Colony morphologies include mucoid, smooth, dry or wrinkled, and a variety of margin and elevation types. Thus, students become immediately interested in these cultures. Individual colonies are picked for isolation in pure culture by repeated restreaking. We ask students to purify two to four different isolates so that they can experience the variation in streaking technique that is needed to accommodate organisms of different properties.

Our experience (Table 1) is consistent with the successional pattern reported by Pederson[2]. Table 1 lists the most prominent types of genera and species we observe; however various types of organisms are typically observed on the increase before they become dominant and on the decrease afterwards, leading to class discussions of how this particular microbial succession might be working (see below). On day 0 plates, many various species are observed that do not occur in brine streaks made subsequently. Many of these organisms are obligate aerobes, others are acid intolerant (see below). That fermentation plays a role in the succession is suggested by observation of a precipitous drop in pH of the brine during the first few days, accompanied by gas production (Figure 2). The gas production forces fluid out of the jars (this is less likely to occur in crocks), which we catch by putting all the jars on an enameled pan.

Studying the microflora of fermenting cabbage

Having learned isolation of bacteria in pure culture by making streak subcultures of several isolates from the fermenting cabbage brine, students then use these cultures as the experimental materials for many of the standard methods routinely taught in introductory microbiology courses (Table 2). These methods provide data from which students may (a) understand the physiological bases for the microbial succession they have demonstrated, and (b) attempt to identify one of the bacterial strains they have isolated. Typically all the isolated strains are studied to understand the succession (Table 2 columns 1 and 2), but only one strain is chosen by each student for identification (Table 2 column 3).

The results usually correlate the day on which the isolate was planted on a brine streak plate with its physiological properties. For example, aerobes and acid-intolerant strains are found on day 0 plates, when sampling occurs immediately after packing the cabbage and before any

anaerobic incubation has occurred. Some of the more exotic soil bacteria are occasionally found on these plates, including *Corynebacterium, Arthrobacter, Nocardia, Streptomyces,* and others. Organisms found prominently on days 2 to 4 tend to be fermenters that have limited acid tolerance and little tolerance of lactic acid even when able to grow at pH 4.5 in the absence of this microbicide. The Gram positive cocci isolated days 4 to 7 (*Leuconostoc*) are moderately acid tolerant and produce so much acid fermentation product that on glcYE $CaCO_3$ plates their colonies are surrounded by halos (Figure 1). Gram positive rods (*Lactobacillus*) isolated after days 4 to 7 are even more acid tolerant and also tolerate lactic acid at least to 1%. Thus the early drop in pH appears to constrain the growth of acid intolerant species, the more acid tolerant *Leuconostoc* are seen to bloom later, but fade subsequently because they are sensitive to the lactic acid that is their own fermentation product, and *Lactobacillus* appear to dominate at the end because they have the highest tolerance of the lactic acid they, too, produce. If the brine is titered at these later stages, it can be seen that eventually the *Lactobacillus* are also killed.

Identification of these bacteria can be a challenge, especially recently with the rapid proliferation of newly described species. Nevertheless, the use of these wild strains provides a realistic view of the bacterial identification not available when the instructor simply assigns domesticated laboratory strains for the purpose[3].

Connections beyond the sauerkraut fermentation

Even students working next to each other at the lab bench often isolate different organisms, so we get to bring up the concept of microhabitat. The issues of food spoilage, use of organic acids as food preservatives, and use of cabbage as an antiscorbutic food by some early European navies[4] are mentioned in introducing the module to the class.

Staphylococcus is usually found by at least one person in the class, so we can point out that persons handling the cabbage on the farm, in shipping, or grocery handling might have contributed these. Students who get these cultures usually keep them to study later when skin bacteria are on the curriculum. Students who isolate *Bacillus* spp., which are very common in soil, can use them later as subjects for a spore staining exercise, since for purposes of the cabbage succession experiment we use Gram staining to identify spore–formers. Coliforms come up later in the term also, so cabbage isolates from this group are saved for comparison.

Conclusion

In this module students use the conventional curriculum of introductory experimental microbiology to do a real investigation of how a microbial succession works. No one student's data are exactly like anyone else's, since different sets of organisms are under investigation. Thus, collation of the results from the whole section can give a reasonably full overview of the succession.

Table 1: Dominant colony forming microflora in fermenting sauerkraut

Day 0	Day 1	Day 4	Day 7 & later
molds			
yeasts	yeasts		
Gram positive rods *Bacillus spp* *Corynebacterium spp* *Arthrobacter spp*	Gram positive rods *Bacillus spp*	Gram positive rods *Lactobacillus spp*	Gram positive rods *Lactobacillus spp*
Gram positive cocci *Micrococcus spp* *Staphylococci*	Gram positive cocci *Leuconostoc spp*	Gram positive cocci *Leuconostoc spp*	
Gram negative rods (facultative) coliforms *Erwinia spp*	Gram negative rods (facultative) coliforms *Erwinia spp*		
Gram negative rods (aerobic) *Pseudomonas spp*			

Table 2: Fermenting cabbage provides material for many methods and concepts typically taught in microbiology lab courses

Basic methods & observations used in microbiology:	Other methods & observations to study the roles individual isolates might have been playing in the microbial succession that occurred in the sauerkraut jars:	Additional methods and observations students can use to find the identity of bacterial species isolated:
Aseptic technique	O_2 requirements	Sucrose tolerance
Preparation of media	Nutritional requirements[5]	Oxidase & catalase tests
Streak plate	Temperature requirements	Fermentation tests
Serial dilutions	NaCl tolerance	Indole test
Viable counting	pH tolerance[6]	Starch, gelatin, & casein hydrolysis
Microscopy	Lactic acid tolerance*	Litmus milk
Gram staining	Study of molds	Use of citrate as sole source of carbon
Cell morphology		Nitrate reduction
Colony morphology		Ammonification
Capsule stain		H_2S production
Differential media		
Enrichment techniques		
Strain storage*		

*Except as noted, the methods listed are standard and may be found in any complete version undergraduate introductory microbiology laboratory manual[7]. All cultures are incubated at the temperature used for incubation of fermenting sauerkraut. In some cases we use bacterial strains suggested in the lab manual as positive and negative controls for comparison to the students' results with the unknowns. We study lactic acid tolerance by adding 0, 0.5, 1.0, or 1.5% sodium lactate to the broth routinely used to culture cabbage isolates, and titrating each medium to pH 4.5 before autoclaving. The ability of an organism to grow in these media is viewed with reference to results of the pH tolerance test. Strains are stored by mixing fresh broth cultures in cryotubes in sterile glycerol solution for a final concentration of 15% glycerol, and freezing at –70° C.

Figure legends

Figure 1. Microbial succession in fermenting sauerkraut, as displayed on simple and differential media. Trypticase soy agar (TSA) is an all purpose medium that supports the growth of many heterotrophic microbes. Eosin methylene blue agar with lactose (EMB) is selective against most Gram positive bacteria and differential as well. Organisms that ferment lactose make very deep purple colonies on this medium (day 4 EMB, white arrow). Neutral pH media that contain a large concentration of $CaCO_3$ make plates that are differential for high acid production. On agar plates of this type the $CaCO_3$ is insoluble, except in the vicinity of fermenters that make lots of acid by fermenting the glucose in this medium, where a zone of clearing is visible (day 4, glc yeast $CaCO_3$, black arrow). The sugars in the EMB and glc yeast $CaCO_3$ promote very mucoid growth of many of the colony–formers that are prominent in day 0 and day 1 cultures. The end stages of the succession are characterized by numerous, very small, difficult to see white colonies of *Lactobacillus* (day 7 cultures).

Figure 2: Evidence of fermentation in cabbage packed anaerobically with 3% NaCl. Evolution of gas in the period indicated by the bar pushes fluid out of the jar. The preparation is kept anaerobic by replenishing the salt solution that covers the cabbage leaves and seals the lid.

Acknowledgements

I am grateful to the late Dr. Moshe Shilo for suggesting the use of glucose yeast extract medium with $CaCO_3$ in classroom studies of the fermenting cabbage succession.

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[6] S. G. Kelley and F. J. Post, *Microbiology Techniques*, Star, 1991, Exercise 45 "pH", p. 257.

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Title: Isolation and characterization of new bacteriophages

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Abstract:

New bacterial viruses (bacteriophages) are easy to isolate in undergraduate laboratory classes, from such materials as soil, water, or sewage[1]. Standard virological methods can be used in a class setting to prepare microbiologically and biochemically purified stocks of these phages, characterize their growth cycles, and observe their morphology in the electron microscope.

In recent years there has been an increase in interest in characterization of new bacteriophages for such applications as study of virus genome evolution[2] (3) and as potential therapy for infectious diseases[3]. I have students form collaborative groups of three or four, to plan and carry out individual research projects aimed at further characterization of their wild phages. We plan projects that can be accomplished in three successive 4-hour class sessions. Typical projects include determination of genome nucleic acid type or isolation of mutants. I suggest a brief list of possible projects but encourage students to generate new project ideas. We also incorporate projects relating to ongoing research in departmental phage labs, when possible. Students prepare a sequence of documents to (i) propose a topic, (ii) suggest specific methods, and (iii) prepare a materials list for us so that we know what to provide for their experiments. At each of these stages we meet together to provide support and discuss feasibility and suitability of their plans. Finally, the students carry out their experiments. The sequence extends through the semester, and occupies perhaps 30% of the virology lab class curriculum. Students wishing to meet a writing across the curriculum requirement write a paper about the characterization of this new phage.

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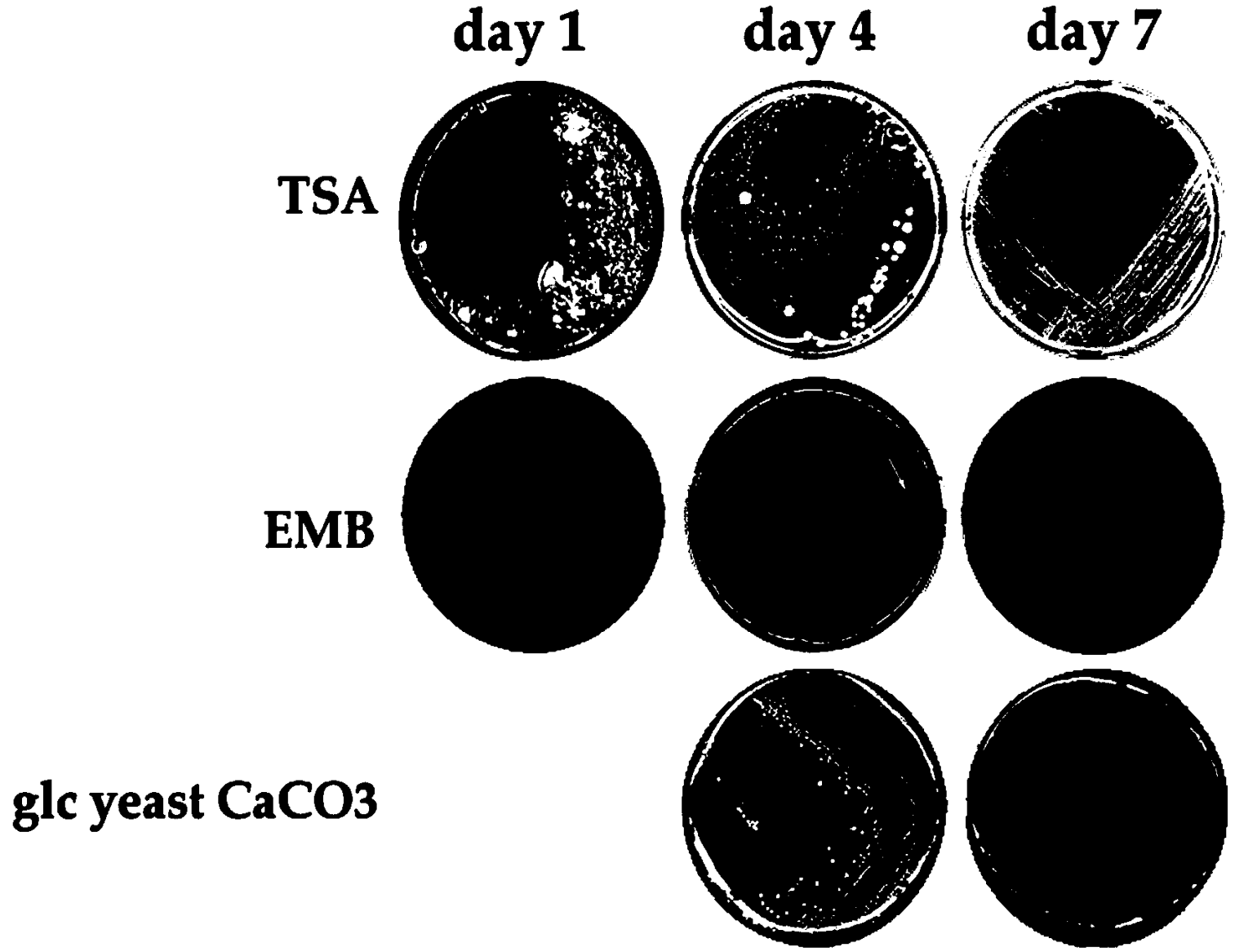
day 1
day 4
day 7
TSA
EMB
glc yeast CaCO3