Fermented and Acidified Vegetables

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51.1 INTRODUCTION

Vegetables may be preserved by fermentation, direct acidification, or a combination of these along with other processing conditions and additives to yield products that are referred to as pickles. Pasteurization and refrigeration are used to assure stability of certain of these products. Organic acids and salt (sodium chloride) are primary preservatives for most types of pickles. Lactic acid is produced naturally in fermented products. Acetic acid (or vinegar) is the usual acid added to pasteurized, unfermented (fresh-pack) pickles. Acetic acid also is added to many products made from fermented (salt-stock) cucumbers. Other preservatives such as sodium benzoate, potassium sorbate, and sulfur dioxide may be added to finished products. Although the term "pickles" in the United States generally refers to pickled cucumbers, the term is used herein in a broader sense to refer to all vegetables that are preserved by fermentation or direct acidification. Cucumbers, cabbage, olives, and peppers account for the largest volume of vegetables and fruits commercially pickled. Lesser quantities of onions, tomatoes, cauliflower, carrots, melon rinds, okra, artichokes, beans, and other produce also are pickled.

The fermentation of vegetables is due primarily to the lactic acid bacteria, although yeasts and other microorganisms may be involved, depending on the salt concentration and other factors. Salt serves two primary roles in the preservation of fermented vegetables: It influences the type and extent of microbial activity, and it helps prevent softening of the vegetable tissue. Some vegetables are brined at such high salt concentrations as to greatly retard or preclude fermentation. Salt may be added in the dry form, as with cabbage, or as a brine solution, as with most other vegetables. The concentration of salt used varies widely among vegetables, depending on tendency of the vegetable to soften during brine storage. Softening of brined cucumbers can be reduced or prevented by adjusting the level of salt to inhibit pectinolytic enzymes.[3,4] Fermentation is an economical means for temporary preservation of produce such as cucumbers, cabbage, and olives. The produce is fermented and stored in large tanks until it is needed for further processing. After removal from brine storage, brined cucumbers may be desalted if needed before being finished into various products such as dills, sweets, sours, hamburger dill chips, mixed vegetables, and relishes.[50] Finished salt-stock dill cucumber pickles contain a minimum of 0.6% lactic acid, according to USDA grade standards.[137] The products may or may not be pasteurized, depending on the addition of sugar and other preservatives. Extensive reviews are available on the brining and fermentation of cabbage,[118,131] cucumbers,[48,53] and olives.[52,68,139,140,141]

Direct acidification with acetic acid (without pasteurization) has been a primary method for many years of preserving various pickles and sauces in the United Kingdom, where the products are referred to as acetic acid preserves. British researchers have determined that the minimum acetic acid concentration necessary to achieve satisfactory preservation of all pickles and sauces is 3.6%, calculated as a percentage of the volatile constituents of the product.[9] The high concentration of acid needed for preservation results in such a strong acid flavor, however, that the relative importance of this method of preservation has diminished. Milder acidic flavors are more in demand today, and use of acidification in combination with pasteurization has become more important. Nevertheless, some specialty products such as hot pepper sauce and sliced peppers still are preserved principally by high concentrations of acetic acid without pasteurization.

Fresh-pack cucumber pickles are preserved by mild acidification (0.5% to 1.1% acetic acid[137]) of fresh cucumbers, followed by heating to an internal product temperature of 74°C and holding for 15 minutes, according to the original recommendations of Etchells et al.[30,37,109] Such products are effectively pasteurized, since they are heated enough to inactivate microbial vegetative cells, and sufficient acid has been added to prevent outgrowth of bacterial spores. Although some packers still use this heat process, others now vary the times and temperatures, depending on product type and risk factors (for spoilage problems of non-health significance) acceptable to the packer. Fermented pickles, such as whole genuine dills and hamburger dill chips, may or may not be heated. If pasteurized, these products may be given a milder heat treatment than fresh-pack pickles, such as an internal product temperature of 71°C with no holding time. The fresh-pack process has been applied to peppers and other vegetables. Fresh-pack pickles are considered acidified foods for regulatory purposes. According to the U.S. Food and Drug Administration (FDA), "'Acidified foods' means low-acid foods to which acid(s) or acid food(s) are added; these foods include, but are not limited to, beans, cucumbers, cabbage, artichokes, cauliflower, puddings, peppers, tropical fruits, and fish, singly or in any combination. They have a water activity (a_w) greater than 0.85 and have

a finished equilibrium pH of 4.6 or below. These foods may be called, or may purport to be, 'pickles' or 'pickled'...."[136]

Refrigerated pickles may or may not be fermented before refrigeration. Also, they may or may not be acidified, although mild acidification is highly recommended.[49] Most commercially prepared and distributed refrigerated pickles sold today are not fermented, but are acidified and contain a preservative such as sodium benzoate.

Increasing environmental concerns related to waste disposal are influencing methods for preservation of pickled vegetables, particularly those involving use of salt for bulk storage. The U.S. Environmental Protection Agency (EPA) has proposed a maximum of 230 ppm of chloride in fresh waters,[29] a limit that may not be readily achievable by many vegetable briners who discharge chloride wastes into freshwater streams. Organic acids (lactic and acetic) in combination with calcium chloride and preservatives (e.g., sodium benzoate) are now used instead of sodium chloride for bulk storage of olives for "green-ripe" processing into canned black olives in California.[142] Salt is still used, however, for fermented olives. The use of calcium salts (chloride or acetate) has led to reduced levels of sodium chloride for bulk fermentation and storage of cucumbers. Calcium salts have been found to enhance firmness retention of cucumbers at reduced concentrations of sodium chloride.[14,61,65,84,135] Studies have revealed, however, that spoilage microorganisms may present a serious problem in fermented cucumbers if the salt concentration is too low.[68] Recently, the use of sulfite was proposed as a way to store cucumbers in the absence of salt, and the sulfite removed by reaction with hydrogen peroxide after storage and before conversion into finished products.[100]

51.2 NORMAL FLORA

Fresh produce contains a varied epiphytic microflora (Chapter 50). Pickling cucumbers were found to contain as high as 5.3×10^7 total aerobes, 1.9×10^4 aerobic spores, 9.8×10^5 total anaerobes, 5.4×10^2 anaerobic spores, 6.1×10^6 coliforms, 5.1×10^4 total acid formers, 4.6×10^3 molds, and 6.6×10^3 yeasts per g of fresh cucumber.[46] The numbers increased during storage at higher temperatures (21°C) and humidity (>70% relative humidity). Although some investigators have held that the interior of sound, fresh cucumbers is sterile, others have found microorganisms, mostly gram-negative rods, within the healthy fruit.[106,127] In cucumbers, bacteria were more often near the skin and less often in the central core; in tomatoes, their frequency was highest near the stem-scar and central core and decreased toward the skin.[127] Cabbage contains the greatest number of bacteria on the outer leaves and lower numbers toward the center of the head.[118]

The floral changes during natural fermentation of brined vegetables may be characterized into four stages: initiation, primary fermentation, secondary fermentation, and post-fermentation.[53] During initiation, the various gram-positive and gram-negative bacteria that were on the fresh vegetable compete for predominance. *Enterobacteriaceae*, aerobic spore-formers, lactic acid bacteria, and other bacteria may be active. Eventually, the lactic acid bacteria gain predominance by lowering the pH, and primary lactic fermentation occurs. During primary fermentation, five species of lactic acid-producing bacteria are active, listed in approximate order of their occurrence: *Streptococcus* (*Enterococcus*) *faecalis*, *Leuconostoc mesenteroides*, *Pediococcus cerevisiae* (probably *P. pentosaceous* and/or *P. acidilactici*, according to recent classification[130]), *Lactobacillus brevis*, and *Lactobacillus plantarum*. Although all five species are active during fermentation of sauerkraut,[118] which contains relatively low concentrations of salt (ca. 2.25%), only the latter three species predominate in fermentation of cucumbers, which contain higher concentrations of salt (ca. 5% to 8%).[48] *Lactobacillus plantarum* characteristically terminates the lactic fermentation, apparently because of its greater acid tolerance.[121]

During fermentation of brined cucumbers, lactic acid bacteria may grow within the cucumber tissue as well as the brine.[22] Gas composition of the cucumbers at the time of brining greatly influences the ratio of bacterial growth in the cucumbers and the brine.[25] Yeasts were found not to grow within the cucumber tissue, presumably because of their larger size, which prevented their entry through stomata of the cucumber skin.

Green olives contain inhibitors of lactic acid bacteria,[55,57,87] which are thought to influence fermentation of Spanish-type green olives.[44,88] Yeasts are not inhibited and predominate in the fermentation when the olives are neither properly lye treated nor heat shocked before brining.[44]

Various species of fermentative yeasts also are active during primary fermentation. If fermentable sugars remain after primary fermentation, these sugars may give rise to secondary fermentation dominated essentially by yeasts. Fermentative yeasts grow as long as fermentable sugars are available; this may result in severe gaseous spoilage (bloater formation).[32,39,88] During post-fermentation, growth of oxidative yeasts, molds, and bacteria may occur on brine surfaces of open tanks that are not exposed to ultraviolet radiation or sunlight.[33, 112] Vegetable brining tanks are typically uncovered and are held outdoors to allow sunlight to reduce or prevent surface growth. Surface growth does not occur in fermented and anaerobically stored green olives.[140] Attempts have been made to develop a suitable anaerobic tank for the cucumber-brining industry.[63,66,85]

Lactic starter cultures have been used commercially on a limited basis in sauerkraut, olives, cucumbers, and other products.[54] *Pediococcus cerevisiae* and *L. plantarum* have been used in pure culture or controlled fermentations of cucumbers,[43,47] and olives.[44] Although starter cultures have been used on a limited commercial scale for fermenting cucumbers over the past 10 years, they are not widely used.

51.3 FLORA CHANGES IN SPOILAGE

51.31 *Fermented Vegetables*

Production of CO_2 in the cover brine of fermenting cucumbers by various bacteria, including heterofermentative lactic acid bacteria and fermentative species of yeasts, is associated with bloater spoilage. Even homofermentative lactic acid bacteria such as *L. plantarum* and *P. cerevisiae* produce sufficient CO_2, when combined with CO_2 from cucumber tissue, to cause bloater formation in brined cucumbers.[56] The major source of CO_2 production by homofermentative lactic acid bacteria is decarboxylation of malic acid, a natural constituent of pickling cucumbers.[103] It has been demonstrated that cultures that do not degrade malic acid will ferment cucumbers with reduced bloater damage.[104] Procedures have been developed to produce and isolate non-malate-decarboxylating mutants of *L. plantarum*.[24,26] Purging fermenting cucumber brines with nitrogen has been shown to be effective in preventing bloater formation.[20,47,56,60] Purging is now widely used by the pickle industry. Air purging also is effective in preventing bloater formation,[20,60] but can result in cucumber softening due to mold growth,[21,60,75] reduced brine acidity due to yeast growth,[123] and off-colors and -flavors unless the purging regimen is care-

fully controlled. In fact, air is most commonly used today by the pickle industry, with intermittent purging regimes and addition of potassium sorbate to prevent growth and spoilage by yeasts and molds. Bloater formation has been attributed to growth of gas-forming microorganisms in the brine surrounding the cucumbers[45] or within the cucumber.[22,126]

Softening of brined vegetables may be caused by pectinolytic enzymes of plant or microbial origin. Growth of film yeasts on brine surfaces may occur and result in loss of brine acidity. Accompanying mold growth on the brine surface can cause softening of sauerkraut, cucumbers, or olives. Heavy scum yeast and/or mold growth is usually the result of neglecting brined material during extended storage. It is important for the brine surface of cucumber tanks to be exposed to direct sunlight to inhibit yeast and mold growth. Sauerkraut tanks are usually held indoors, and a properly seated plastic cover weighted down with water or brine is necessary to prohibit aerobiosis and thereby prevent surface growth of yeasts and molds. Softening of brined cucumbers may also result from mold polygalacturonases that accompany the cucumbers, especially cucumbers with flowers attached,[6,42] into the brine tank. This problem was reduced in former times by draining and rebrining of the tank ca. 36 hours after initial brining,[41] but this solution is not normally used today because of environmental concerns with salt disposal. Recycled brine may be treated to inactivate softening enzymes.[76,102] Adding calcium chloride can slow down the rate of enzymatic softening of fermenting cucumbers.[15] However, this should not be relied upon to eliminate enzymatic softening problems. Care must be taken to minimize contamination of cucumbers, particularly the small fruit, with flowers and plant debris, which can be a source of contamination by pectinolytic molds.

Butyric acid spoilage of brined olives has been attributed to two distinct types of microbial action. In one type, *Clostridium butyricum* and a closely related group of clostridia produce butyric acid from sugars during the primary stage of fermentation.[78] In a second type of malodorous olive fermentation, "zapatera" spoilage results from decomposition of organic acids at a time when little or no sugar is present and the lactic acid fermentation stops before the pH has decreased below pH 4.5.[90] Propionibacteria were isolated from brined olives with indications of "zapatera" spoilage and were hypothesized to grow and cause a rise in pH because of degradation of lactic acid, thus permitting subsequent growth by *Clostridium* species.[10,80,122]

Butyric acid spoilage of brined cucumbers was found to occur after an apparently normal primary fermentation by lactic acid bacteria.[68] *Clostridium tertium* was identified as contributing to the spoilage. Evidence from end products indicated that unidentified bacteria, possibly propionibacteria species, degraded lactic acid, causing a rise in pH that allowed C. *tertium* to grow. This problem, particularly acute when cucumbers are stored at relatively low salt concentration, can be reduced/avoided by assuring a final brine pH of 3.5 or lower after fermentation.[70]

51.32 Finished Pickle Products from Salt-stock Vegetables (Not Pasteurized)

Fully cured salt-stock vegetables are made into various types of finished pickle products by a series of operations involving leaching out most of the salt, souring with vinegar, and then sweetening with sugar if desired. Preservation of these products depends on sufficient amounts of vinegar alone (for sour pickles), or a combination of vinegar and sugar with or without sodium benzoate (for sweet pickles).[2] If the concentration of these ingredients is inadequate and the product is not pasteurized, fermentation usually takes place. Osmotolerant yeasts are the principal spoilage organisms in such products.[2] Molds and film yeasts may grow on the surface of the liquid chiefly as the result of faulty jar closure. Lactic acid bacteria, propionibacteria and butyric acid bacteria also may cause spoilage in unpasteurized fermented vegetables that do not contain adequate concentrations of acetic acid or other preservatives.

51.33 Acetic Acid Preserves

The 3.6% acetic acid (as a percentage of volatile constituents) required for preservation of pickles and sauces[9] is similar to, but slightly lower than the concentration found necessary by Bell and Etchells[2] to prevent yeast spoilage in finished pickles from salt-stock cucumbers. Microbial spoilage apparently occurs in acetic acid preserves when the concentration of acid is marginal. Spoilage microorganisms include yeasts[27] and lactobacilli, particularly the heterofermentative *Lactobacillus fructivorans*.[28]

51.34 Pasteurized Pickle Products

Spoilage usually occurs in these products when they are improperly pasteurized or improperly acidified so that an equilibrated brine product of pH 3.8 to 4.0 is not achieved. Spoilage is due chiefly to acid-forming bacteria and, to a lesser extent, yeasts that survive faulty heat treatment, or butyric acid bacteria when the product is not acidified adequately at the outset. Molds and film yeasts are factors in cases of poor jar closure.

51.35 Refrigerated Pickle Products

51.351 Fermented

A wide array of fermented, refrigerated cucumber pickle products are prepared as specialty products.[49] Examples of such products include overnight dills, half-sour dills, genuine kosher dills, kosher new dills, sour garlic pickles, half-sour new pickles, fresh-packed half-sour pickles, new half-sours, home-style new pickles, half-sour kosher new dills, and the like. The cover brine may or may not be acidified. The products are held in barrels for a few days or longer at room temperature and then refrigerated at 2° to 5°C. They may be distributed in bulk or in consumer-size glass containers. In some cases, they may be initially brined, held, and distributed in consumer-size containers. Under such conditions and at equilibrated brine strengths of 10 to 12 salometer (1 salometer = 0.264% salt by weight), microbial growth (chiefly coliforms, gas-forming and non-gas-forming lactics, and fermentative yeasts) and enzymatic activity (pectinolytic and cellulolytic) together with the curing process continue at a slow rate.[45] Gaseous spoilage of the product is caused chiefly by the gas-forming microbial groups mentioned earlier. Gas production may be sufficient to reach 15 psi pressure within the container.

Softening problems may be even greater than for salt-stock cucumbers since these products are held at much lower concentrations of salt. Fresh, whole garlic cloves and other spices are normally added to such products. These spices may contain high activities of softening enzymes that increase softening problems. In a few months, the stored pickles may have lost much of their characteristic flavor, texture, and color and also may be bloated because of gaseous fermentation by the principal gas-forming microbial groups present.

Whether these pickles are made in bulk or in the retail jar, the very nature of the product makes it difficult to maintain good

quality for any reasonable length of time. The barreled product reaches the good manufacturing practices (GMP)-recommended brine pH of 4.6 or below for acidified foods, usually before refrigeration or shortly thereafter, and then slowly continues acid development. This recommended condition for brine-product pH cannot be assured for the product made in the retail jar because there is no uniform process accepted by packers wherein the product is acidified at the outset or where it is deliberately incubated for development of natural lactic acid fermentation.

Sauerkraut marketed in plastic bags in refrigerated display cases is preserved by the addition of sodium benzoate and bisulfite.[133] The shelf life of such products is influenced by chemical changes that may result in discoloration (browning) and objectionable flavor formation.

51.352 Not Fermented

Most of these products for national distribution are acidified with vinegar to an equilibrium pH well below 4.6, contain 2% to 3% NaCl, and are immediately refrigerated upon packing.[49] They may contain sodium benzoate or other preservatives. Like the fermented refrigerated product, the cucumbers are not heated either before or after packing. If properly acidified, refrigerated, and preserved, the products will maintain acceptable quality for several months and do not present a public health concern. Recipes that do not contain vinegar or other acid in the initial cover liquor, however, should be viewed with caution.

51.4 PATHOGENIC MICROORGANISMS

We know of no authenticated reports of pathogenic microorganisms associated with standard commercial pickle products prepared under "good manufacturing practices" of acid, salt, and sugar content (and combinations thereof) from brined, salted, and pickled vegetable brine-stock, including cucumbers. The Commissioner of the FDA stated that "No instances of illness as the result of contamination of commercially processed fermented foods with *Clostridium botulinum* have been reported in the United States."[136] Even so, certain types of microorganisms that may cause spoilage of the product may, at times, be encountered, such as molds, yeasts, and acid-tolerant lactic acid bacteria. These organisms, usually under conditions associated with neglect, may reduce the quality of the texture and flavor of the product—whether prepared in bulk or retail container—and render it unusable. However, these organisms are not considered human pathogens.

Essentially the same pattern of consumer safety applies to fresh-pack (pasteurized) pickle products. These products have continued to increase in popularity until they now use over 40% of the annual cucumber crop in the United States. These pickles usually are prepared from raw cucumbers, but may include other vegetables in a mixture; also, vegetables other than cucumbers may be packed, such as various types of peppers, okra, carrots, green beans, and tomatoes. The process calls for the packed product to be acidified at the outset with a sufficient amount of food-grade organic acid, e. g., vinegar, acetic acid, or lactic acid, to result in an equilibrated brine product pH of 4.0 or below (preferably 3.8). Vinegar (acetic acid) is usually the acidulant of industry choice for cucumber pickle products. The basic pasteurization procedure, with acidified product heated to an internal temperature of 74°C and held for 15 minutes, has been used successfully by industry since ca. 1940.[30,35,37] Insufficient acidification of pasteurized pickles can result in butyric acid-type spoilage, possibly involving public health concerns.

Listeria monocytogenes, a food-borne pathogen, has become a major concern to the food industry over the past 15 years. The bacterium is commonly found in the environment and has been isolated from various plant materials, including silage,[51] soybeans, corn,[144,145] and cabbage.[128] Beuchat et al.[8] showed that *L. monocytogenes* was able to grow on raw cabbage and in cabbage juice. Conner et al.[19] found death of *L. monocytogenes* (one strain tested, LCDC 81-861, is a pathogen isolated from coleslaw) to occur in cabbage juice adjusted to pH ≤ 4.6 with lactic acid and incubated at 30°C; at 5°C, the death rate was slower than at 30°C. However, two strains tested grew well at pH values of 5.0 to 6.1. In a nutrient medium acidified with hydrochloric acid, the minimum pH values at which growth of *L. monocytogenes* was detected at 30°, 20°, 10°, 7°, and 4°C were, respectively, 4.39, 4.39, 4.62, 4.62, and 5.23.[77] Johnson et al.[86] demonstrated that *Listeria* could be recovered from fermented sausage made with beef intentionally contaminated with the bacterium. To our knowledge, *Listeria* has not been reported in fermented vegetables. However, the observation that *Listeria* can be isolated from fermented materials (silage and sausage), coupled with the bacterium's ability to tolerate moderately low pH[51,19,77,86] and high salt concentrations (growth in complex media at 10% salt[129]) suggests that *Listeria* may pose a concern for mildly acidified or fermented vegetables.

While pathogenic bacteria have not been reported in commercially fermented vegetable products, the potential for the survival and growth of some pathogens in acidic environments has been investigated.[83,124] These studies suggest that *Escherichia coli*, *L. monocytogenes*, *Salmonella* species, and others may potentially grow in mildly acidified or fermented vegetables. Outbreaks of *E. coli* O157:H7 in unpasteurized apple juice or cider, which typically has a pH between 3.5 and 4, have resulted in over 100 reported illnesses and at least 1 death.[7,16,17] Researchers have found that adaptation to acidic conditions can be induced in some pathogenic bacteria,[72,89,92,94] and may even increase virulence.[113] Acid adaptation, or acid tolerance response, in bacteria typically involves an initial sub-lethal acid shock, which results in changes in gene expression analogous to the response observed with heat shock,[96] and the resulting physiological changes allow the treated cells to survive for extended periods in normally lethal acid conditions. A number of acid shock proteins and pH regulated genes in *Listeria*, *Salmonella*, and other bacteria, have been identified.[1,71,81,82,114,116] It has been shown that acid adaptation can enhance the survival of *Salmonella* and *Listeria* in fermented dairy products,[74,95] and may be part of a more general phenomenon called stress hardening, which results in increased resistance of bacteria to a variety of environmental stresses.[97,115,124] The significance of acid tolerance/adaptation of food pathogens in fermented and acidified vegetables is yet to be revealed.

51.5 RECOMMENDED METHODS

51.51 Collection and Storage of Brine Samples

In examination of pickle products, brine or pickle liquor covering the vegetable material is required. The size of container to be sampled may range from a small jar of pickles to a 1,000-bu tank of fermented brine stock. Brine samples from containers such as tanks and barrels should be taken for bacteriological analysis as follows:

For large brine tanks, insert a suitable length of 3/16-in stainless steel tubing, sealed at one end with lead or solder and perfo-

rated with several 1/16-in holes for a distance of 6 to 8 in from the sealed end, through an opening between the wooden boards composing the false head down into the brine toward the mid-depth of the vegetable material. Withdraw brine through a sanitized, attached piece of flexible tubing into a sample container. The length of the steel sampling tube is governed by the depth of the container to be sampled. Withdraw and discard approximately 100 mL of brine before taking the final sample, about 10 mL, into a sterile test tube. If microbial changes during the fermentation are to be followed, start sampling at the time the material is salted or brined and continue at regular intervals of 1 to 2 days during active fermentation. After each sampling, wash the whole assembly thoroughly.

For tightly headed barrels such as those used for genuine dills and salted vegetables for non-pickle use, take the sample through the top or side bung. For smaller containers, such as jars or cans of pickle products, shake thoroughly and take the sample from the center of the material by means of a sterile pipette. Wash the tops of the metal cans with alcohol, flame, and puncture. If the containers show evidence of gas pressure, carefully release gas by puncturing the sanitized top with a flamed ice pick. Containers under heavy gas pressure may be refrigerated overnight to reduce the gas pressure prior to sampling.

Brine samples from actively fermenting material should be examined as promptly as possible after collection to prevent changes in the microbial flora. The same is true for samples of packaged pickle products. If it is necessary to ship or store samples, this should be done so under refrigerated conditions; the elapsed time from collection to examination should not exceed 24 hours. When shipment by air is required, samples are collected in sterile, 16 × 105-mm tubes fitted with plastic screw caps having rubber liners. Pulp and oil liners, or plastic liners such as Teflon, may leak because of changes in air pressure.

Brine samples may be preserved for subsequent chemical determinations by the addition of one to two drops of toluene or Merthiolate (1% aqueous solution) per 10 mL of sample. Samples preserved with the above chemicals are unfit for human consumption and should be so marked.

Many techniques have been developed for sample preparation and storage for ascorbic acid analyses. For fermented and acidified vegetables, quickly mixing a sample with at least four volumes of 3% wt/vol metaphosphoric acid is a good sample preparation procedure. Little or no ascorbic acid is lost after 24 hours of storage in the refrigerator. Metaphosphoric acid stabilizes ascorbic acid much better than sulfuric acid or oxalic acid.

51.52 Comminution of Whole Vegetables and Particulates

To enumerate microflora of whole or particulate vegetables, approximately 300 grams of tissue are homogenized aseptically with an equal weight of sterile saline (0.85% NaCl). The samples are homogenized in a heavy duty commercial blender (e.g., Waring Blendor model 31BL46, Waring Products, New Hartford, CT). With a 1 liter blender jar homogenize for 1 minute at maximum RPM. To initiate blending, it may be necessary to cut whole or large pieces of vegetables in the jar using a sterile knife. Approximately 100 mL of the vegetable slurry is immediately removed after blending for further processing in a stomacher apparatus (e.g., Stomacher 400 homogenizer, Spiral Biotech, Inc., Bethesda, MD). The slurry is dispensed into a stomacher bag containing a filter (Stomacher 400 filter bags, Spiral Biotech) and processed using the maximum force setting for 1 minute. The filtrate removed from these bags should contain particles approximately 40 microns in diameter or less and can be used in a spiral plater or plated directly on agar petri plates. For sauerkraut in particular, as well as vegetables in general, the Robot Coupe blender model RSI 2YI (Robot Coupe USA, Inc., Ridgeland, MS) has proven to be very useful for preparing samples for chemical analyses. This model will accommodate up to 1000 grams of tissue and does not require additional liquid for homogenization. The sample is blended for 3–4 minutes, which results in a homogenous slurry. This slurry may then be filtered or centrifuged prior to chemical analyses.

51.53 Microscopic Examination

Microscopic examination of brine and vegetable samples for bacteria and yeasts is helpful at times, particularly when carried out in conjunction with plate count observations.

51.531 Bacteria

Make direct counts for bacteria according to the following procedures:

1. Place 0.01 mL amounts of liquid on slides using a calibrated pipette or loop and spread evenly over a 1 cm^2 area; fix with heat.
2. Stain according to the Kopeloff and Cohen modification of the gram stain.[91] Count according to the Wang[143] modification of the Breed[11] technique.
3. Report results as "numbers of different morphological types of gram-positive and gram-negative bacterial cells per mL of brine."
4. To determine the number of bacteria within brined vegetable tissue, blend the tissue to a homogeneous slurry and filter through coarse filter paper (Reeve Angel 202, Whatman Laboratory Products, Inc., Clifton, NJ). Bacteria within the filtrate are then enumerated with a Petroff-Hauser counting chamber at a magnification of about 500×.[25]

51.532 Yeasts

Use the microscopic technique for determining yeast populations in fermenting vegetable brines and various types of finished pickle products undergoing gaseous spoilage by the organisms, particularly where populations are in excess of 10^4 cells per mL of sample and where yeast colonies are not required for isolation and study. The use of a vital stain permits differentiation of yeast population into viable and non-viable cells and increases the usefulness of the direct counting technique.

The counting procedure is essentially the method of Mills[108] as modified by Bell and Etchells[2] for counting yeasts in high salt content brines and in high sugar content liquors:

1. Add 1 mL of brine or pickle liquor sample to 1 mL of 1:5,000 (0.02%) erythrosin stain.
2. Shake the sample stain mixture to obtain an even suspension.
3. Using a 3-mm diameter platinum loop, transfer enough of the mixture to the area under the cover glass of an improved Neubauer double-ruled hemacytometer to fill the chamber in one operation.
4. Allow cells to settle for approximately 5 minutes, and count the yeast cells using a microscope equipped with a 4-mm objective and 15× oculars.
5. Record cells stained pink as "dead yeast cells" and unstained cells as "live yeast cells."

The number of yeast cells per mL of brine or pickle liquor may be calculated thus:

$$\frac{\text{Number of yeast cells counted} \times \text{dilutions} \times 250{,}000}{\text{number of large squares counted}} = \text{Numbers per mL}$$

If only one side of the hemacytometer counting chamber is used (25 large squares), the lowest yeast count obtainable is 20,000 per mL, while, if both sides are counted (50 large squares), a population as low as 10,000 per mL can be counted.

Report yeast count as "total yeast cells," "live yeast cells," and "dead yeast cells" per mL of sample.

51.54 Enumeration of Viable Microorganisms

In addition to the procedures described in this section, we have found certain alternative methods such as use of Petri Film (3M Microbiology Products, St. Paul, MN) to be particularly useful for field studies (see Chapter 10 for an array of rapid methods). Also, the use of a spiral plater (Spiral Biotech, Inc., Bethesda, MD) has become highly useful in our laboratory for microbial enumeration (see Chapter 12 for automated methods).

51.541 Aerobic Plate Count

Use plate count agar or nutrient agar and incubate at 30°C for 18 to 24 hours. For longer incubation, overlay the solidified, plated samples with about 8 to 10 mL of the same medium to prevent or minimize spreaders.

51.542 Lactic Acid Bacteria

Selective enumeration of lactic acid bacteria may be carried out using MRS agar containing 0.02% sodium azide and incubating for 1 to 4 days at 30°C.[23] Lactic acid bacteria of the genus *Lactobacillus* or *Pediococcus* may be enumerated with *Lactobacillus* selective medium (LBS), supplemented with 1% fructose[132] and 200 ppm cycloheximide to inhibit yeasts. Bromcresol green (or brilliant green, as in Chapter 63), 0.0075%, may be added to aid in colony counting, but may restrict the growth of some lactic acid bacteria. To differentially enumerate all species of lactic acid bacteria associated with vegetable fermentations, plate fermenting samples on a non-selective medium such as tryptone-glucose-yeast extract agar.[120] After incubation at 30°C for 24–48 hours, isolate colonies for later identification on the basis of cell morphology, acid and gas production, and mucoid growth;[119] other reactions may be used. For incubation of agar plates longer than 24 hours, place the petri plates in a sealed plastic bag (it is convenient to use the sterile plastic sleeve in which the petri plates come packaged), or in a humidified incubator to prevent desiccation of the medium.

Several kinds of differential and selective media are available for the characterization of lactic acid bacteria. HHD medium (see Chapter 63 for composition) is used for the differential enumeration of homofermentative and heterofermentative lactic acid bacteria.[98] This medium incorporates fructose, which is reduced to mannitol by heterofermentative but not homofermentative lactic acid bacteria. In agar medium, homofermentative colonies of lactic acid bacteria are blue to green, while heterofermentative colonies are white. MD medium (see Chapter 63 for composition) may be used to differentiate malate-decarboxylating (MDC^+) and malate-non-decarboxylating (MDC^-) lactic acid bacteria. The decarboxylation of malic acid is undesirable in cucumber fermentations because of the CO_2 produced. The differential reaction is based upon pH changes in the medium caused by malate decarboxylation. A pH decline (MDC^-) is shown by a color change from blue to green, whereas no color change indicates an MDC^+ reaction. HHD and MD media are non-selective for lactic acid bacteria. MS agar medium may be used to select for MDC^+ bacteria.[12] This medium contains malate and has a low initial pH 4.0. MDC^+ bacteria are capable of raising the pH via the malolactic fermentation and initiating growth, while MDC^- bacteria cannot initiate growth.

51.543 *Total* Enterobacteriaceae *and Coliform Bacteria*

Add 1% glucose to violet red bile agar, which is referred to as MacConkey glucose agar[110] or VRBG agar. Incubate for 18–24 hours at 30°C. For coliform bacteria, use violet red bile agar without added glucose, and count all purplish red colonies surrounded by a reddish zone of precipitated bile, 0.5 mm in diameter or larger. In addition to these methods, a number of rapid methods for enumeration of *Enterobacteriaceae*, such as *E. coli* O157:H7 or *Salmonella* species, are available (see Chapters 8 and 10).

51.544 Yeasts and Molds

Yeast and mold populations may be enumerated on YM agar (Difco Laboratories, Detroit, MI or see Chapter 20) supplemented with 20 mL/L of an antibiotic solution containing 0.5% chlortetracycline and 0.5% chloramphenicol. We have found it desirable to use both antibiotics to preclude growth of bacteria. Alternatively, acidify sterile tempered molten dextrose agar (at 45°C) with 10% tartaric acid, usually 5% by volume, to achieve a final pH 3.5. Incubate YM or acidified dextrose agar for 24 to 48 hours at 30°C. Small colonies of lactic acid bacteria may appear on the acidified medium, but are suppressed in the antibiotic medium.

Mold colonies are filamentous and, thus, are distinguished readily from yeasts on acidified dextrose agar. Differentiation of subsurface yeasts and film yeasts presents more difficulty. Surface colonies of the common film-forming yeasts associated with pickle products and vegetable brines, i.e., species of *Debaryomyces, Endomycopsis, Candida,* and *Pichia,*[33,111] are generally dull and very rough, as contrasted to the usual round, raised, white, glistening colonies of the fermentative, subsurface yeasts, i.e., species of *Torulopsis, Brettanomyces, Hansenula, Saccharomyces,* and *Torulaspora.*[36,39,40] However, even when distinguishing colony characteristics of the two yeast groups exist, they are not considered sufficiently clear-cut for separation. Because of this, the procedure outlined under Section 51.545 should be used. Film yeasts rapidly form a heavy wrinkled surface film at one or both salt concentrations. Certain species, such as *Saccharomyces halomembranis,* form heavier films at 10% salt than at 5%.[31,36,38,111]

51.545 Film Yeasts

For an estimate, pick representative filamentous colonies from the yeast plates into tubes of dextrose broth containing 5% and 10% salt. Incubate 3 to 5 days at 32°C and look for heavy surface film. Two salt concentrations are suggested because some species develop heavier films at the lower salt strength (5%), whereas, with other species, the reverse is true.

51.546 Obligate Halophiles

Use tubes of liver broth plus salt (Chapter 63). Prepare decimal dilutions, seal with sterilized, melted petroleum jelly, and incubate 7 days at 32°C. Record positive tubes daily by noting the raising of the petroleum seal caused by gas production and the absence of any distinctive odor.

This medium has proved satisfactory for detecting obligate halophiles sometimes found in brined and dry-salted vegetables. The salt content of the medium should approximate that of the sample. No growth of coliforms or yeasts has been encountered in this medium. This is probably due to the inability of either group to initiate satisfactory early growth in laboratory media even at moderately high salt concentrations in competition with the very fast-growing obligate halophiles.

51.547 Butyric Acid-forming Bacteria

Neutralize the brine sample with an excess of sterile calcium carbonate. Heat a 50- to 100-mL sample in a water bath for 20 minutes at 80°C to kill vegetative cells. Prepare decimal dilutions and inoculate previously heated and cooled tubes of liver broth medium. Seal with melted petroleum jelly and incubate 7 days at 32°C. Examine tubes daily for production of gas and a strong butyric acid odor.

51.55 Chemical Analyses

51.551 Titratable Acidity and pH

Determine titratable acidity of a 10-mL sample of the fermentation brine or finished pickle liquor (liquid of the final product) by diluting the sample with 30 to 50 mL of distilled water; titrate with 0.1N NaOH using phenolphthalein as the indicator. Alternatively, samples may be titrated to pH 8.2 with a pH meter. Report values for fermented, brined samples as g of lactic acid per 100 mL of sample, and for finished pickle liquor samples as g of acetic acid per 100 mL of sample.

For a 10-mL sample, use the following calculations:

a. mL of 0.1N alkali used × 0.090 = g of lactic acid per 100 mL.
b. mL of 0.1N alkali used × 0.060 = g of acetic acid per 100 mL.

When only a small amount of the original sample is available, use a 2-mL amount for titration purposes. Such small samples are not recommended. For the 2-mL sample, multiply the mL of 0.1N alkali by 5, then by the above number for lactic or acetic acid.

Carry out pH determinations of the samples with a pH meter, checking the instrument frequently with a standard buffer in the pH range of the sample under test.

51.552 Chloride and Calcium

It is often helpful to know the approximate salt content in performing microbiological examination of brines. Use a salometer, and test about 200 mL of brine. A chemical test for salt is required for small amounts of sample or when a higher degree of accuracy is desired than that obtainable with the salometer.

The following method is recommended. Transfer 1 mL of sample to a flask, and dilute with 15 to 20 mL of distilled water. Titrate with 0.171N silver nitrate solution, 29.063 g per liter, using 3 to 5 drops of 0.5% dichlorofluorescein as the indicator. Agitate to keep the precipitate broken up until a light salmon pink color develops. Report as "g of sodium chloride per 100 mL of the sample." When 1 mL of sample is titrated, each mL of silver nitrate solution is equal to 1 g of sodium chloride per 100 mL. A chloride test strip is commercially available from Environmental Test Systems (http://www.etsstrips.com/water.html).

A rapid colorimetric procedure based upon calcium binding by methylthymol blue can be used to measure the calcium content of brines or blended tissue.[79] Samples are mixed with an equal volume of 4% wt/vol trichloroacetic acid solution. Acidified solution, containing 50 to 600 µg calcium, is added to a test tube, and reagent solution is added. An immediate absorbance change at 612 nm occurs. The relationship between calcium concentration and absorbance is hyperbolic. A standard curve can be constructed by hand, or the data may be fitted to a hyperbola using nonlinear regression. There are also test strips available for calcium and some other inorganic components of brines such as sulfite and nitrate. One supplier of test strips is EM Science (http://www.emscience.com).

51.553 Fermentation Substrates and Products

To determine whether the intended fermentation occurred or to determine the nature of an off-fermentation, it is important to measure both the substrates and products of a fermentation process. High-performance liquid chromatography (HPLC) procedures to measure all major substrates and products of both heterolactic acid and homolactic acid fermentations have been developed using refractive index detection.[105] Reversed phase C_{18} columns gradually lose resolution of organic acids and need to be replaced periodically. More recently, a procedure to determine all these compounds in a single HPLC injection has been developed. For this method, a conductivity detector is used to measure organic acids and an electrochemical detector connected in series is used to detect sugars and alcohols.[99] Resin columns in the H^+ form are extremely stable and reproducible in their separations of compounds in fermented vegetable samples. We have used a single column (Bio-Rad HPX-87H) for over 3 years without loss of performance. Sucrose degrades during chromatography on resin columns in the H^+ form and, if mannitol is present as occurs in heterolactic fermentations such as sauerkraut, it will not separate adequately from fructose to analyze either compound. All major sugars and sugar alcohols involved in vegetable fermentation can be separated using a Dionex Carbopak PA1 column with dilute NaOH as the eluant.[117] Due to low analyte capacity on columns of this type, electrochemical detection of sugars is preferred over a refractive index detector. For the analysis of organic acids, reversed-phase columns gradually lose resolution and need to be replaced periodically. Also, not all C_{18} columns adequately separate malic acid, lactic acid, acetic acid, and ethanol. Resin columns in the H^+ form are extremely stable and reproducible for organic acid analysis using 0.02N sulfuric acid as the eluant. One problem with this procedure for cucumber pickle analysis is that fructose and malic acid coelute. The coelution problem has been solved in two ways using the same column as cited above. Lazaro et al.[93] developed equations to quantify fructose and malic acid differentially based on peak heights obtained from ultraviolet and refractive index detectors connected in series. Frayne[73] actually resolved malic acid and fructose by connecting two of the HPLC columns in series.

Though HPLC is today the method of choice for analysis of fermentations, it has the disadvantage that an expensive instrument is required. An alternative approach for analysis of many fermentation substrates and products is enzymatic analysis using commercially available kits. The analysis can be done manually with an inexpensive visible colorimeter or spectrophotometer. The main disadvantage is that only a single compound can be analyzed at a time. Compounds for which kits are available include glucose, fructose, malic acid, L-lactic acid, acetic acid, ethanol, and CO_2. Commercial sources for such kits include Boehringer Mannheim Biochemicals (www://biochem.boehringer.com) and Sigma Chemical Company (http://www.sigma-aldrich.com). Enzymatic analysis is the only routine way to measure the L-isomer of lactic acid specifically.

It is important for the stability of fermented vegetables that all fermentable sugars be metabolized by the end of the fermentation process. For products like cucumbers that have little or no sucrose, colorimetric measurement of reducing sugars is a simple, rapid quality control procedure to assess the completion of sugar utilization. The dinitrosalicylic acid (DNS) procedure[107,134] is recommended. The reagent is stable at room temperature for many months. The assay can be reliably performed with an inexpensive colorimeter. A fermentation can be considered complete if the brine contains less than 0.05% reducing sugar and acid shows no increase for several days.

51.554 Softening Enzyme Activity

Softening enzymes in brines of fermenting cucumbers and other vegetables may be determined by the highly sensitive viscometric method of Bell et al.[5] The procedure, which has been widely used in the pickle industry for many years, is based on viscosity loss of a buffered polypectate solution. Brine samples, 25 mL, are dialyzed in running water for 3 hours and distilled water for 1 hour. One mL of the dialyzed sample is added to 5 mL of 1.2% sodium polypectate, which is dissolved in 0.018 M, pH 5.0 citrate buffer in an Ostwald-Fenske no. 300 viscometer. A drop of toluene is added to the sample to prevent microbial growth during incubation. The flow time of the pectate solution is measured after sample addition and at 20 hours. The viscosity loss is calculated according to the following equation:

$$\text{Percent loss in viscosity} = \frac{A - B}{A - W} \times 100 \qquad (1)$$

where A is the initial flow time in seconds, B the flow time at 20 hours, and W is the flow time for water. Bell et al.[5] provide a table that relates loss in viscosity to the units of pectate depolymerizing activity. A less than 9% loss of viscosity in 20 hours is considered to represent weak to negative activity in brine samples. Buescher and Burgin[13] described a diffusion plate assay that is in common use in the pickle industry to determine if there is polygalacturonase activity in brines before they are recycled.

Refer to Chapter 18 for isolation of pectinolytic organisms and characterization of pectinolytic enzymes.

51.555 Dissolved Carbon Dioxide

The advent of purging to remove CO_2 from fermenting cucumber brines and, thereby preventing bloater formation, has created a need to determine the concentration of dissolved CO_2 in the brine. For the highly accurate determinations that may be required for research purposes, dissolved CO_2 is determined by the micro distillation procedure.[59] A 10-mL brine sample is injected by syringe into a capped jar containing a phosphoric acid solution. A small vial containing 5 mL of 0.200N NaOH placed inside the jar traps the CO_2 as it distills from the acidified solution. After 24 hours at 37°C, the vial is removed, 5 mL of 0.2 M $BaCl_2$ is added, and the remaining base is titrated to the phenolphthalein end-point with 0.100N HCL. Values are expressed as mg CO_2 per 100 mL brine. When exact amounts and concentrations of acid and base specified in the method are adhered to, the following equation may be used:

$$\text{mg}CO_2/100\text{ mL brine} = (1.000 - \text{mL 0.1N HCl}) \times 220$$

For quick estimates that may be required for quality control tank monitoring, dissolved CO_2 is determined with a micro CO_2 apparatus. Adaptation of this instrument for the determination of CO_2 in fermenting cucumber brines has been described.[58] This is a gasometric method based on the classical Van Slyke procedure. A 1-mL brine sample is placed in the instrument vial, a volumetric syringe is clamped into place, an acid solution is added, the apparatus and sample vial are shaken, and the gas volume displacement is read on the calibrated syringe scale. Carbon dioxide in the brine sample is calculated from scale readings of the brine compared to a CO_2 solution of known concentration and is expressed as mg CO_2 per 100 mL brine. It is suggested that brine samples be taken from brine tanks through a siphon tube (see Section 50.51) and 8.5 mL injected by syringe through a needle into a Vacutainer tube (10 mL draw, Becton-Dickinson, containing 0.5 mL of ca. 3N NaOH) to minimize CO_2 loss. The samples are then equilibrated to the same temperature as the known solution before analysis.

In both methods, the total CO_2 content of the solution is determined and is expressed as mg CO_2 per 100 mL brine, or as percent saturation.[62]

51.556 Ascorbic Acid

Brine samples should be stored for ascorbic acid analysis as described in section 51.51. The HPLC procedure of Vanderslice and Higgs,[138] which involves separation of ascorbic acid on a reversed phase polymer column with pH 2.14 phosphate buffer as the eluant solution followed by fluorometric detection, has been shown to give accurate ascorbic acid and dehydroascorbic acid analysis in a wide variety of food products. It has been used in this laboratory to measure ascorbic acid in fermented cucumber samples using an electrochemical detector instead of a fluorometric detector (Zhou et al., unpublished).

51.6 INTERPRETATION OF DATA

51.61 Fermented Vegetables

Proper record-keeping of salting procedures and chemical and microbiological data can greatly aid the commercial briner in assessing causes for success or failure in preserving the quality of brined vegetables. Records of chemical determinations of salt, titratable acidity, pH, fermentable sugars, dissolved CO_2, and softening enzyme activity are very useful in such assessments, depending on the particular commodity. A recordkeeping system for brined cucumbers has been published.[34]

In fermented vegetables, it is important that the lactic acid fermentation become established early to preclude growth by spoilage bacteria. Acidity and pH data provide this information. Salt concentrations above 8% for cucumbers and olives or above 2.5% for cabbage may prevent or retard a desirable lactic fermentation. Unusually low salt concentrations may result in softening of the brined vegetables.

If the dissolved CO_2 concentration in the brine of fermenting cucumbers is allowed to exceed about 50% saturation (equals 54 mg per 100 mL at 21°C and 6.6% NaCl) at any time during brine storage, bloater damage may result. Maintaining the brine CO_2 concentration below 50% saturation will greatly aid in reducing bloater damage.[62] Sporadic bloater damage may occur even in effectively purged brine-stock cucumbers. Such damage may be due to growth of bacteria within the brined fruit.[22] Since brines must be purged as long as fermentation occurs, it is important to monitor the level of fermentable sugars in the brine. When fermentable sugars are not detected and acid development has ceased, the fermentation is considered to be complete, and purging can be safely discontinued.

Microbial softening enzyme activity of brines may indicate the cause of soft brine-stock pickles, especially if the cucumbers are held at relatively low brine strengths (5% to 8% NaCl). Higher salt concentrations will prevent softening by these enzymes,[3] but high salt levels present disposal problems, in addition to affecting the lactic fermentation adversely. Studies have indicated that calcium chloride, ca. 0.2% to 0.4%, and other salts of calcium may inhibit the action of softening enzymes.[14] Calcium chloride is now being added to commercial cucumber brines. The extent of protection against softening offered by calcium has not been fully assessed.

The absence of softening enzyme activity in older brine-stock pickles does not necessarily mean that such activity did not cause the softening. Softening enzymes that accompany the cucumbers and attached flowers into the brine tank may exert their influence early in brine storage and then be dissipated or inactivated so as not to be detectable later.

Softening in the seed area of large cucumbers, commonly termed "soft centers," is thought to be due to natural polygalacturonase of overly mature cucumbers,[141] not to microorganisms.

The advent of reliable HPLC procedures to measure changes in substrates and products of fermentations has made it practical to assess the balance between substrate utilization and product formation in complex food fermentations. Carbon recovery of <100% indicates that some fermentation products have been missed in the analysis, while recovery of >100% suggests that unknown substrates have been fermented. Examples of fermentation balances done on complex fermentations have been published.[18,66,64,67,101] The determination of fermentation end products was extremely useful in recent efforts to determine microorganisms responsible for spoilage of fermented cucumbers.[68]

51.62 Finished Pickle Products from Salt-Stock Vegetables

These products normally contain a few thousand microorganisms per mL. These counts may be composed chiefly of spores of aerobic bacteria that remain inactive in the acid medium and tend to decrease during storage. Fermentative yeasts and lactic acid bacteria may cause vigorous gas production, which causes the pickle liquor to become highly charged with gas and to possess a tang when tasted. Viable microorganisms, normally latent in properly fermented and preserved products, may cause gaseous spoilage in improperly finished products. Gaseous spoilage and cloudy cover brine may be the result in hamburger dill chips, genuine dill pickles, Spanish-style green olives, and similar products, if residual sugar remains.

51.63 Pasteurized Pickle Products

Properly acidified, packaged, and pasteurized pickle products are not subject to microbial spoilage. When spoilage occurs, it is usually due to underpasteurization. Some commercial packers minimize heat processing in order to maintain greater product quality. Minimal processing is done at the risk of spoilage. Spoilage results in recall of the product at the packer's expense. No public health problem exists in pasteurized pickle products that have been properly acidified. After spoilage occurs, however, as evidenced by gas pressure and brine turbidity, there is no way to ensure that the product was properly acidified initially. Lactic acid bacteria are normally found in such products. The spoiled product usually contains acid, but it is not known at that point if the original product, particularly if it was fresh produce, was sufficiently acidified to prevent growth of *Clostridium* before growth of the lactic acid bacteria.

Improper acidification can also be a source of spoilage with potential public health significance, as discussed in Section 51.4. Improper closure can result in growth of aerobic microorganisms on the surface of the brine and a reduction in acidity.

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COMPENDIUM OF METHODS FOR THE

MICROBIOLOGICAL EXAMINATION OF FOODS

FOURTH EDITION

EDITED BY
FRANCES POUCH DOWNES
KEITH ITO

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Washington, DC 20001-3710

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10 M 3/01
Library of Congress Catalog Card Number: 99-073697

ISBN: 0-87553-175-x

Printed and bound in the United States of America.
Cover Design: Sam Dixon Design
Typesetting: Susan Westrate, Joseph R. Loehle
Set in: Palatino and Universe
Printing and Binding: Sheridan Books, Inc.