CHAPTER 51

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 pasteurization or refrigeration and selected additives, to yield products with an extended shelf life and enhanced safety. Organic acids such as lactic, acetic, sorbic, and benzoic acids, as well as sodium chloride (NaCl) are used as the primary preservatives for most types of products. Lactic acid is produced naturally in fermented vegetables. Acetic acid is typically added in the form of vinegar to acidify unfermented pickle products, better known as fresh-pack, which are also pasteurized. This acid is also occasionally added at the beginning of cucumber fermentations for flavoring purposes. A description of the chemical composition of selected preserved products is presented in Tables 51.1 and 51.2. Cucumbers, cabbage, olives, onions, and peppers account for the largest volume of vegetables and fruits that are commercially processed worldwide. Lesser quantities of tomatoes, cauliflower, carrots, melon rinds, okra, artichokes, beans, and other produce also are pickled.

The traditional vegetable fermentation process requires salting. Modifications to this centuries-old process, such as acidification with vinegar, have resulted in a variety of modern alternative processing methods yielding a wide array of finished commercial products. The simultaneous presence in the market of preserved vegetables manufactured using traditional and modern methods has generated a need to redefine the terms fermented, acidified, and pickled. It is therefore convenient to combine scientific information and tradition for a rational classification of the existing products. The following definitions should be considered for the application of specific terms throughout this chapter.

Fermented vegetables: This term is used here to refer to all vegetables that are preserved by fermentation, and is defined as follows: (a) low-acid vegetables subject to the action of acid-producing microorganisms that will naturally achieve and maintain a pH of 4.6 or lower, *regardless of whether acid is added;* (b) the primary acidulent(s) in the product are the acids naturally produced by the action of microorganisms. If the fermentation proceeds to completion and good manufacturing practices are applied, spoilage organisms capable of raising the pH above 4.6 are prevented from growing in the product, and pathogens of public health significance are destroyed during the process, thus making the final product safe for consumption.

Acidified vegetables: This term is used to refer to products in which an acid is directly added to preserve any nonfermented vegetable with an initial pH above 4.6, so that the final product pH is maintained below that initial pH, *regardless of whether acetic acid is used for acidification.*

Pickled and/or pickles: This term is applied here specifically to refer to any fermented or acidified vegetable covered with a solution that contains vinegar (acetic acid) as the major acidifying agent.

The above definitions are simultaneously scientifically accurate and in agreement with the traditional and global association of the term pickles with a vinegary flavor, and the use of acetic acid as the primary acidulent in finished processed vegetables.

51.2 GENERAL CONSIDERATIONS

The fermentation of vegetables is due primarily to the activity of naturally occurring lactic acid bacteria (LAB). However, yeasts and other microorganisms may also be involved in the process, depending on the salt concentration and other environmental factors.[4] Salt may be added in vegetable fermentations in the dry form, or as a brine solution in variable concentrations depending on the type of vegetable to be processed and the desired final product. Salt, primarily NaCl, serves four major roles in the preservation of fermented vegetables: (a) it influences the type and extent of microbial activity; (b) it helps prevent softening of the vegetable tissue; (c) it determines the flavor of the final product; and (d) it assists in rupturing the fruit membranes, allowing the diffusion of various components into the cover brine solutions used by microbes for growth

Table 51.1. Range of typical chemical components in fermented cucumbers, shredded cabbage and olives (brine stock)*

Brine Stock	pH	Acidity (% lactic acid)	Sodium Chloride (%)	Calcium (%)	Sugar (% glucose)	Potassium Sorbate (ppm)
Cucumbers (pickles)	3.2–3.6	0.8–1.2	6–10	0.10–0.3	0–0.05	0–500
Cabbage (sauerkraut)	3.2–3.4	1.5–2.5	2–3	0.05–0.2	0–0.05	0
Olives	3.6–4.2	0.8–1.2	5–8	0.01–0.1	0–0.05	0–350

*Survey of commercial fermented cucumber and sauerkraut brine stock in the United States between the years 2001 and 2010 by Dr. Ron W. Buescher at the Department of Food Science, University of Arkansas, USA. Data for fermented table olives was adapted.[97]
ppm=parts per million

and metabolic activities. Some vegetables are brined at such high salt concentrations as to greatly retard or preclude fermentation. Softening of brined cucumbers and olives can be reduced or prevented by adjusting the salt level to retard pectinolytic enzymes derived from the fruits and/or pectinolytic yeasts.[11,13]

Fermentation is an economic means of preservation and bulk storage of produce and the production of unique flavor and other quality characteristics. Fermented produce are stored in large tanks until needed for further processing. Cucumber cuts or whole fruits, washed olives, and shredded and dry salted or brined cabbage are added into tanks with capacities of 10–25 tons.

The initial pH of brined cucumbers and cabbage is between 5.0 and 6.5. However, the initial pH of brine olives may be above 7.0, depending on how much washing was done after the required sodium hydroxide (NaOH) treatment to debitter the fruits *via* the degradation of oleuropein.[108] The initial pH of brine vegetables may be further reduced by purging with carbon dioxide (CO_2), as is done with olives, and by adding acetic acid in the form of vinegar. Lower initial pH values help in releasing the excess CO_2 formed during the fermentation, in selecting for the growth of LAB, and in inhibiting the growth of the acid-sensitive enterobacteria.

Cucumber brining tanks are typically uncovered and are held outdoors to allow the ultraviolet waves from sunlight to reduce or prevent surface yeast growth.[54,150] Air purging may be used to remove excess CO_2 formed during the fermentation and prevent gas pockets forming in the fruit.[77] However, such air purging may lead to the growth of oxidative yeasts, which consume lactic acid, resulting in elevated pH and spoilage problems. Attempts have been made to develop a suitable anaerobic tank for the cucumber-brining industry.[78,81,111] The fermentation of cucumbers in the absence of oxygen demands a blanching step to control fermentative yeast growth and preserve quality. After fermentation and removal from brine storage, cucumbers may be desalted, if needed, before being finished into various products such as dills, sweets, sours, hamburger dill chips, mixed vegetables, and relishes.[70] Finished fermented dill cucumber pickles contain a maximum of 0.35% (w/v) lactic acid (Table 51.2). The products may or may not be pasteurized, depending on the addition of sugar and other preservatives.

Fermented cabbage contains mannitol, acetic acid (about 1% each), and lactic acid in excess of 2% as the available sugar is not a limiting factor. For most manufacturers in the United States, sauerkraut may be stored for up to 1 year in fermentation tanks until it is processed for foodservice or retail sale. Although bulk storage is economical, the products may become very sour as lactic acid accumulates. European manufacturers typically package sauerkraut at the end of the heterolactic fermentation stage (about 1 week after the start of fermentation) to obtain a product with a mild acid flavor.[164,189] Spices, wines, and other ingredients may be added to the sauerkraut to augment its flavor. Unlike sauerkraut, the preparation of kimchi requires half-cuts or quartered cabbage soaked in 5–10% NaCl brine, then washed and drained prior to fermentation. Ground red pepper, garlic, ginger, green onions and a highly salted (20% NaCl) anchovy product are usually mixed with the wilted cabbage. After packing the vegetable mixture into jars or pouches, the incubation stage proceeds at 18°C for a few days, followed by very cold refrigeration at 1–2°C). This procedure allows the initial heterolactic stage of fermentation to occur and delays the onset of the homolactic stage, keeping kimchi from becoming too sour before consumption.

The principal types of fermented olive products include Spanish-style green, Greek-style naturally black, and California-style black ripe. Green table olives are treated with lye (NaOH) to debitter them by degrading oleuropein,[18,108] and then washed prior to being brined and fermented to create the so called Spanish-style product. Following fermentation, they may be pitted and stuffed prior to sale. The lye or NaOH treatment also contributes to reduce the antimicrobial activity of the phenolic components derived from the olives[90,91,114] and makes the skin of the olives more permeable, thereby aiding with sugar diffusion during fermentation. To stabilize fermented green olives after the main fermentation, the NaCl concentration must be raised above 8.5% to prevent the purported fourth fermentation phase, which may lead to a rise in pH and the development of spoilage (zapatera).[101] This process, which also yields the most popular fermented olive product, also offers adequate characteristics for the preparation of many commercial presentations, including plain, pitted, stuffed with many different fillings, and sliced olives. The physicochemical conditions in the final products differ with markets, but the concentration of NaCl is usually around 4–8% and the final acidity in the range of 0.30–1.00% lactic acid.[97] According to the trade standard for table olives,[32] minimum NaCl concentrations and maximum pH depend on preservation procedures. Fermented olive products intended to have a specific chemical composition or packed under modified atmospheric conditions must contain 5% NaCl and have a final stable pH of 4.0 or lower.

Table 51.2. Range of typical chemical components in brines of commercial retail fermented, acidified and pickled products*

Retail Product	pH	Acetic acid (%)	Lactic acid (%)	Sodium chloride (%)	Calcium (ppm)	Aluminum (ppm)	Sorbate (ppm)	Benzoate (ppm)	Brix	Sulfite (ppm)
Fermented cucumber pickles										
Whole dills	3.09–3.50	0.59–0.97	0.15–0.35	2.74–4.86	160–1310	0–100	0–446	0–1051	–	0
Hamburger slices	3.14–3.58	0.62–1.00	0.10–0.30	2.74–4.70	285–1234	0–200	0–200	0–1045	–	0
Processed sweet	2.68–3.41	0.94–1.73	0.03–0.30	1.16–2.59	139–889	0–152	0	0–1123	25.5–35.5	0
Relish	2.75–3.40	0.85–2.55	0.02–0.30	1.03–4.07	192–820	0–115	0–1093	0–1057	22.9–36.8	0
Pasteurized fresh pack pickles										
Whole dills	3.26–4.05	0.65–1.02	0	1.30–3.41	132–985	0	0–440	0–1199	–	0
Kosher dill spears	3.61–3.92	0.52–0.89	0	1.00–3.66	150–1120	0	0–113	0–1160	–	0
Sandwich dill slices	3.61–3.85	0.57–0.77	0	1.42–3.34	382–944	0	0	0–1182	–	0
Bread and butter vertical slices	3.51–3.75	0.72–1.32	0	1.00–1.88	300–736	0	0	0–1132	18.5–30.0	0
Bread and butter chips	3.28–3.73	0.75–1.53	0	0.80–2.74	104–830	0	0	0–1027	24.0–35–0	0
Refrigerated fresh pack pickles										
Whole dills	3.68–4.02	0.38–0.66	0	2.33–3.15	362–630	0	0	648–1005	–	0
Spears	3.76–4.01	0.35–0.59	0	2.42–3.31	257–516	0	0	522–917	–	0
Peppers										
Banana pepper slices	2.56–3.42	1.99–3.67	0	2.62–4.90	302–840	0	0	0–1115	–	0–530
Sauerkraut (fermented)**										
Glass jar	2.87–3.60	–	1.06–1.87	1.05–3.33	194–750	0	0	0–1122	–	0–928
Canned	3.23–3.47	–	0.96–2.26	1.16–1.58	234–745	0	0	0	–	0
Olives ***										
Dehydrated	3.6–5.1	–	—	5.6–10.2	—	–	0–500	0–1000	–	0
Fermented	3.2–4.3	–	0.4–1.0	2.0–8.3	50–350	0	0–400	0–950	–	0
Acidified	3.3–4.3	0.3–0.8	0.3–1.2	4.0–7.0	50–150	0	0–500	0–1000	–	0
Filled	3.3–4.2	–	0.3–1.2	4.5–8.0	50–170	0	0–500	0–1000	–	0

*Survey of commercial products in the United States between the years 2001 and 2010 by Dr. Ron W. Buescher at the Department of Food Science, University of Arkansas, USA. Data for olive products with the exception of fermented olive products is provided by the Instituto de la Grasa, Sevilla, Spain. Data for dehydrated olives refer to olive juice and those for acidified to seasoned green olives.
**Residual sugars in fermented sauerkraut are mainly glucose and fructose, which range between 0.02% and 0.5%, and 0.15% and 0.19%, respectively.
***Residual sugars in fermented olives are mainly fructose and mannitol, which range between 0.02% and sto 0.6%.
ppm=parts per million

Final fermented olive products containing preservatives or distributed under refrigeration may be prepared with 4% NaCl, but must still achieve the pH 4.0 standard. However, pasteurized products are not subjected to NaCl limits, and the pH should be below 4.3. Most of the final products from Spanish-style green olives contain lactic acid produced naturally during fermentation as the primary acidulent, but citric and ascorbic acids are also frequently added as antioxidants. Acetic acid is used in seasoned olive products that are mainly sold in local markets.[97]

Most of the Spanish-style fermented green table olives are traditionally and scientifically considered as fermented vegetables owing to the natural production of lactic acid and the subsequent decrease of pH below 4.6. Stuffed olives are also considered fermented products, given that the proportion of the stuffing material to fruits is usually low (approx. 6%, w/w) and the stuffing material is itself an acidified product. However, finished products characterized by physicochemical conditions, such as partial fermentation, high combined acidity, and/or other physicochemical conditions, lack microbial stability and must be heat treated.[97]

Natural black olives are also prepared by fermentation, but do not receive NaOH treatment prior to brining. Black olives are picked in a ripened state and are characterized by the natural black color and a softer texture than green table olives. Fermentation is a much slower process in black olives because of the lack of NaOH treatment. Antimicrobial phenolic compounds diffuse into the brine, which retards fermentation, and sugar diffusion is also reduced compared to the NaOH-treated green olives. As a consequence, the fermentation may take months to complete. It uses a reduced rate of air (0.1–0.2 L/h/L capacity) with continuous or alternating application. Flux control is a critical issue, since an excess may cause an excessive growth of oxidative yeasts, with a possible increase in softening. The process improves color, reduces the debittering period and, when properly applied, reduces losses and costs.[97] Fermented black olives may also be packed in diverse commercial presentations, but the limits for pH and NaCl are always 4.3% and 6%, respectively. The exception to this standard pertains to pasteurized products, in which limits may be defined according to good manufacturing practice. The acid of choice when packing black olives may be lactic acid (olives in brine). However, vinegar is frequently added to specific specialties such as Kalamata olives or similar products. A small proportion of virgin olive oil is also added to improve brightness, particularly in combination with vinegar.[97]

Some specialties of table olives, particularly seasoned olives, are packed in a cover brine solution that characteristically contains vinegar. Such specialty products or pickles have pH values below 4.6 and are difficult to stabilize. Preservation is usually achieved by the addition of potassium sorbate, sodium benzoate or a mixture of both, although they may also be subjected to mild pasteurization, which must be carefully estimated in order to maintain their sensorial peculiarities.[97]

Commercial 'ripe black olives' are also prepared from green or semi-ripened olives that have been brined without an initial NaOH treatment. Following storage in brine under either anaerobic or aerobic conditions, or in acidified solutions with either acetic acid, lactic acid, or a combination of both for several months, the olives are usually subjected to one or more vigorous oxidation treatments with pressurized air after treatment with 1–2% NaOH. Such treatment blackens the olives, which are then washed with water to remove the NaOH and bring the pH down to around pH 7.0 or less. The olives are then canned in a 1–3% NaCl brine and processed in a retort to sterilize the fruit. Sterilization is nececcary for these black olives to prevent botulism due to the relatively high final pH, which is frequently above 4.6. Iron gluconate or iron lactate is commonly added to the final products to stabilize the black color of the olives obtained after oxidation.[115] The final products with olives processed in this way will not be considered in the rest of this chapter, given that their final pH is above that targeted in fermented and acidified products.

For many years direct acidification with acetic acid (without pasteurization) has been a primary method 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 concentrations to achieve satisfactory preservation of all pickles and sauces is 3.6%, when calculated as a percentage of the volatile constituents of the product.[17] The strong acid flavor resulting from the addition of the high concentration of acid needed for preservation has postponed the adoption of this method. Milder acidic flavors are favored today, and the use of acidification in combination with pasteurization has become common. Nevertheless, some specialty products, such as hot pepper sauce and sliced peppers, are still preserved principally by high concentrations of acetic acid without pasteurization.

Fresh-pack cucumber pickles are preserved by mild acidification of fresh cucumbers in 0.5–1.1% acetic acid, 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.*[51,69,148] Growth of microorganisms in these products is prevented because of the heat step capable of inactivating microbial vegetative cells, and the supplementation of the product with sufficient acid to prevent the germination of bacterial spores. Although some packers use such a heat process, time and temperature combinations vary depending on the product type and risk factors, primarily related to the development of spoilage by organisms of non-public health significance. Products containing a high final sugar content, such as sweet pickles or relish, may receive a milder heat treatment than for fresh-pack pickles, such as an internal product temperature of 71°C with no holding time. The fresh-pack process has also 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, puddings, cabbage, artichokes, cauliflower, peppers, tropical fruits, and fish, singly or in any combination'. They have a water activity (α_ω) greater than 0.85 and a final equilibrium pH of 4.6 or below. These foods may be called, or may purport to be, 'pickles' or 'pickled' when acidified with vinegar (or acetic acid)'. (US Code of Federal Regulations, Part 114.3).

Refrigerated pickles may or may not be fermented before refrigeration. Likewise, they may or may not be acidified, although mild acidification is highly recommended.[59,196] Most commercially prepared and distributed refrigerated pickles sold today are not fermented, but are acidified with vinegar (or acetic acid) and contain a preservative, typically sodium benzoate.

Growing environmental concerns related to waste disposal are influencing methods of preserving fermented, acidified or pickled vegetables, particularly those involving the use of salt brines for bulk storage. In general, fermented brine stock vegetables frequently have salt concentrations that are too high to be used in products for human consumption. Prior to packing and distribution, the salt is reduced by washing with water, or by a gradual conditioning throughout the operations to the levels described in Tables 51.1 and 51.2. An alternative way of reducing the sodium in olive and cucumber fermentations or the final commercial products is the partial replacement of NaCl with other chloride salts, such as potassium chloride (KCl), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$).[8,105] Regardless of the processing method, the waste stream generated has high concentrations of salt and a high biological oxygen demand owing to the organic components derived from the vegetables. To reduce the environmental impact, cucumber fermentation brines are usually recycled and may be used in subsequent fermentations.[99,139] Prior to recycling, cucumber fermentation brines are commonly assayed for polygalacturonase activity[23] and, if necessary, treated with a protein-adsorbing clay[24] to reduce the softening potential caused by enzymatic degradation of pectic substances in the cucumber cell walls. Although brines are not recycled in Spanish-style olive processing, a portion of the olive fermentation brines is used during the conditioning operation or packing.[97] Additionally, the volume of wastewater generated in the green olive industry is reduced by minimizing the number of post-treatment washings, and by treating the lye solutions and washing waters with ozone to reduce the content of polyphenols and ensure the progression of fermentations upon recycling.[97,183,184] Extensive reviews are available on the brining and fermentation of cabbage,[164,189] cucumbers,[75,79] and olives.[73,198,199,200]

In 1987, the U.S. Environmental Protection Agency (EPA) proposed a maximum of 230 ppm of chloride in fresh water,[49] a limit that could not readily be achieved by many vegetable briners who discharge chloride wastes into freshwater streams. More recently, the EPA proposed reducing such limits to 170 ppm. The situation is similar in other countries, such as Spain, which is one of the major producers of olives.[172] The use of organic acids, for instance lactic and acetic acids, in combination with calcium chloride and traditional preservatives (e.g., sodium benzoate) in cover brine solutions, in lieu of the high salt concentrations that have been traditionally used, has been applied as a strategy to eliminate NaCl from waste streams. Calcium salts have been found to enhance firmness retention in cucumbers at reduced concentrations of NaCl.[25,26,83,84,110,194,201] Studies have revealed, however, that if the salt concentration is too low, spoilage microorganisms may cause serious problems in fermented cucumbers.[76] The use of sulfite has been proposed as a way to store cucumbers in the absence of salt. Sulfite may be subsequently removed by its reaction with added hydrogen peroxide after storage, before preparation of the finished products.[136] However, the use of metabisulfite as a preservative for the storage of vegetables – primarily table olives – is controversial.[32,153] Combinations of calcium chloride with ~1% acetic acid and either sodium benzoate or more natural preservatives, such as fumaric acid (0.3%) and plant-derived compounds (*i.e.* allyl isothiocyanate), have also been proposed and evaluated for the long-term preservation of cucumbers.[144,165,166] However, the application of traditional preservatives to improve table olive storage is uncommon, with the exception of the NaCl-free bulk storage of green-ripe olives for canning of black olives in California, USA. Still, preservatives are widely used to stabilize the finished packed product.

51.3 NORMAL FLORA

Fresh produce contains a varied epiphytic microbiota (see chapter on Fruits and Vegetables). Average microbial counts for fresh and fermented pickling cucumbers, cabbage, and olives are presented in Table 51.3. However, microbial counts for fresh produce vary with season, stage of maturity, environmental humidity and temperature, and the use of pesticides, among other factors. Generally, microbial counts increase during storage at higher temperatures (21°C) and humidity (>70% relative humidity). Although the majority of the microorganisms colonize the exterior of the fresh fruits, a representative number of them, mostly Gram-negative rods, have been found to colonize the outmost interior flesh.[132,179] In cucumbers, bacteria were more often near the skin and less often in the central core.[38,132] In tomatoes, their frequency was highest near the stem-scar and central core and decreased towards the skin.[145] Cabbage contains the greatest numbers of bacteria on the outer leaves and lower numbers toward the center of the head.[164,189] The viable cell numbers of microorganisms associated with fresh olives are generally minimal (Table 51.3).[2,90,91]

Microbial growth during natural fermentation of brined vegetables may be characterized into four stages: initiation, primary fermentation, secondary fermentation, and post-fermentation spoilage.[97,164,170,198] During initiation, the various Gram-positive and Gram-negative bacteria that colonize the fresh vegetable, and are present in the processing water and the fermentation environment, compete for predominance. *Enterobacteriaceae*, aerobic spore-formers, LAB, and other groups of bacteria and yeasts may be active for several days or weeks, depending on environmental factors such as temperature and salt concentration. Eventually, the LAB gain predominance by lowering the pH as primary lactic acid fermentation occurs. During primary fermentation, five species of lactic acid-producing bacteria may be actively present, and are listed in approximate order of their possible occurrence: *Enterococcus faecalis, Leuconostoc mesenteroides, Pediococcus cerevisiae* (probably *Pediococcus pentosaceous* and/or *Pediococcus acidilactici*, according to recent classification[187]), *Lactobacillus brevis*, and *Lactobacillus plantarum* or *Lactobacillus pentosus*. Recent studies suggest that *Weisella* spp. are also present during the early stage of sauerkraut fermentation.[170]

Table 51.3. Average microbial counts for fresh and fermented vegetables expressed in $\log_{10}$ of colony-forming units/gram

	Total Aerobic Counts	Yeast and Mold Counts	Lactic Acid Bacteria	*Enterobacteriaceae*
Fresh Produce				
Cucumbers	5.16 ± 0.76	2.82 ± 0.95	3.84 ± 1.21	4.58 ± 0.98
Cabbage	4.84 ± 0.26	2.87 ± 0.79	3.18 ± 0.33	4.36 ± 0.06
Olives (Aloreña)	1.90 ± 0.50	0.6 ± 1.00	Not Detected	Not Detected
Non-Pasteurized Fermented Products				
Cucumber Pickles	7.45 ± 0.23	4.15 ± 0.68	6.91 ± 1.02	Not Detected
Table Olives	6.90 ± 1.31	5.1 ± 0.86	6.75 ± 1.60	Not Detected

Although all six species are active during the fermentation of sauerkraut,[164] which contains relatively low concentrations of salt (*ca*. 2.25%), only *L. plantarum* and *L. brevis* predominate in cucumber fermentations, which contain higher concentrations of salt (*ca*. 5–8%; Table 51.1).[64] Only lactic cocci such as *Pediococcus* and *Leuconostoc* were traditionally found during the primary fermentation of table olives.[5] However, recent studies suggest that the use of bulk tanks for table olive fermentations is favoring the predominance of *L. plantarum* or *L. pentosus* instead of the cocci. Such rod-shaped bacteria frequently terminate lactic acid production, presumably because of their greater acid tolerance and homofermentative metabolism.[133,163]

In sauerkraut, *Lc. mesenteroides* converts the vegetable sugars, typically fructose and glucose, to lactic and acetic acids and carbon dioxide. The carbon dioxide produced replaces air and provides anaerobic conditions favorable for the stabilization of ascorbic acid and the natural colors of the cabbage. Heterofermentative LAB such as *Lc. mesenteroides* also use fructose as an electron acceptor, reducing it to mannitol.[138] The lower pH resulting from the metabolic activity of *Lc. mesenteroides* and other heterofermentative LAB during the primary fermentation favors the growth and predominance of acid-resistant homofermentative LAB such as *L. plantarum*.[133] The homofermentative stage of sauerkraut making is commonly known as the secondary fermentation. In general, adequate progression of the primary and secondary fermentations is sufficient to stabilize and preserve the fermented vegetables and prevent spoilage, as described below.

Most commercial cucumber, olive, and cabbage fermentations rely on the growth of the LAB that are naturally present on the surface of the fruits or present in the industrial environment.[80] However, a limited number of processors choose to use starter cultures to enhance product consistency. *P. cerevisiae* and *L. plantarum* have been used in pure culture or controlled fermentations of cucumbers[55,62] and olives[202]; and *Lc. mesenteroides* has been proposed as a starter culture for low-salt sauerkraut fermentations.[112] Olive fermentation proceeds satisfactorily if inoculated with *L. plantarum* LPCO10 2 days after brining in a solution with NaCl below 4.0% and a controlled pH between 4.5 and 6.5.[61,149,202] It has also been demonstrated that sequential inoculation of table olives with *Enterococcus casseliflavus* cc45 and *L. pentosus* 5138A could be useful for a fast acidification.[45] Selected strains of *L. pentosus* have also been used as starter culture for Greek olives (*Conservolea* cv.).[161] In general, the use of a starter culture for vegetable fermentations has the potential to increase the rate of death of *Escherichia coli* O157:H7 with respect to spontaneous fermentation.[188] A method for the preparation of *L. plantarum* and other LAB starter cultures that meets kosher requirements is currently available to processors.[167]

In addition to lactic acid, the LAB isolated from vegetable fermentations produce a range of antimicrobial substances, which can be divided into two main groups: the non-bacteriocin antimicrobials, with molecular mass below 1 kiloDalton (kDa) (hydrogen peroxide, ethanol, formic acid, diacetyl, and reuterin, among others); and the high molecular mass substances above 1 kDa, such as bacteriocins.[185,193] Bacteriocins produced by LAB have been reported to have a limited range of sensitive hosts (attack only strains closely related to the producing strain), whereas others are effective against a variety of Gram-positive[117] microorganisms. A study conducted recently revealed a time-dependent emergence of mesentericin, pediocin, and plantaricin A, which was associated with the succession of the fermentative microbes present in a total of 30 cucumber fermentation samples.[186] The challenges associated with the potential application of bacteriocins to control fermentation have recently been considered.[96,185] However, attention must be paid to the fact that there are many limiting factors influencing the efficacy of bacteriocins in foods, particularly in vegetable fermentations. Environmental variables such as temperature, pH, salt concentration, and inoculum size are among the factors influencing the production of bacteriocins by LAB species isolated from olive fermentations.[46,47,120] A bacteriocin-producing starter culture, *L. plantarum* LPCO10, isolated from an olive fermentation was capable of outnumbering the other naturally occurring LAB populations, but did not completely eliminate the indigenous microbiota.[176]

Bacteriophages that infect LAB are suspected to have a significant role in the natural succession of fermentative microorganisms in fermented vegetables. More than 100 bacteriophages have been isolated and characterized from cucumber and cabbage fermentations.[7,128,129,130,205,206] Isolates from the primary and secondary fermentation stages include phages from the *Myoviridae*, *Siphoviridae* and *Podoviridae* families. Lytic phages active against *L. plantarum* were isolated up to 60 days after the start of sauerkraut fermentation, when the pH was below 4.0. Interestingly, the host-range data showed that some phages were capable of attacking more than one species. Genome sequence analysis has been performed for phages from both cucumber and sauerkraut fermentations.[127,128] Genomic analysis of a sauerkraut phage active against *Lc. mesenteroides* has shown a

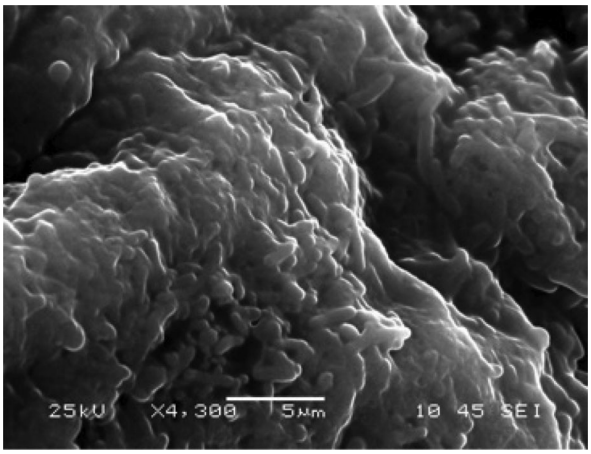

Figure 51.1. Mixed biofilm formed by LAB and yeasts obtained directly by electron microscope on olive surface (courtesy of Dr. Rufino Jiménez Díaz, Instituto de la Grasa, Sevilla, Spain).

similar pattern of genome organization to sequenced dairy phage, but phage protein sequences had little similarity to dairy phage.[127] The full impact of phages on fermentation ecology remains unclear and demands further research.

Various species of fermentative yeasts are also typically present during both primary and secondary fermentation of vegetables. Yeasts were found not to grow within cucumber tissue, presumably because of their larger size, which prevented entry through the stomata of the cucumber skin. Fermentative yeasts grow as long as fermentable sugars are available, and may induce the development of severe gaseous spoilage.[53,54] Yeasts propagate and predominate in olive fermentations (up to 10^6 CFU/mL) if the fruits are neither properly lye-treated nor heat-shocked before brining.[150] Yeasts present in olives and cucumbers include species of the genera *Candida, Pichia, Debaryomyces, Saccharomyces,* and others,[4,54,56,63,151] and may contribute desirable flavor characteristics to the final olive products. Yeasts naturally present in olives may form mixed biofilms with LAB, growing vigorously in fermentation brines as shown in Figure 51.1, suggesting that probiotic LAB may be delivered to the human digestive tract by the inclusion of olives in a regular diet.[44] A review on the effect of yeasts in table olive fermentations and packaging is currently available.[4]

51.4 MICROBIOTA CHANGES IN SPOILAGE

The production of CO_2 in the cover brine of fermenting vegetables by heterofermentative LAB and fermentative species of yeasts has been associated with the formation of gas pockets inside the cucumber and olive fruits, known as bloater formation (Figure 51.2). Homofermentative LAB capable of decarboxylating malic acid, such as *L. plantarum,* may produce sufficient CO_2 to cause bloating when combined with the CO_2 formed from the respiring vegetable tissues.[39,60,88,142,143] Purging fermenting cucumber and olive brines with nitrogen or air has been effective in preventing bloater formation.[34,77,97] Air purging has to be carefully controlled as it may result in fruit softening due to mold growth,[35,86,98,171] reduced brine acidity due to yeast growth,[54] and off-colors and flavors. The addition of

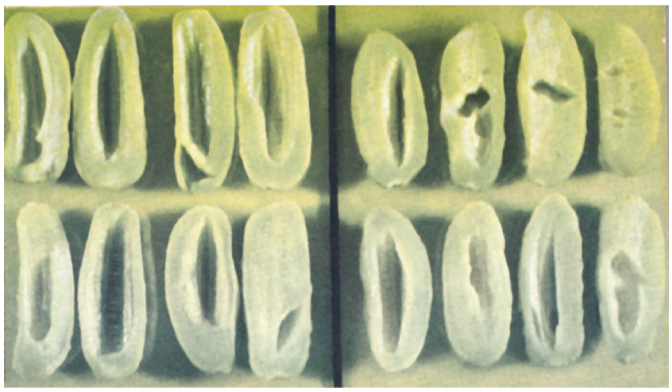

Figure 51.2. Cucumber bloater defect caused by carbon dioxide microbiologically produced during fermentation by either yeasts or LAB. [74]

potassium sorbate to fermentation brines, including spray applications to the surfaces of brines, is commonly used to reduce yeast growth and CO_2 production.

Oxidative yeasts may induce development of malodorous spoilage of fermented olives and cucumbers. These microorganisms can consume the lactic acid produced during fermentation, with a subsequent increase in pH which facilitates the growth of spoilage microorganisms.[97] Malodorous olive fermentation, also known as 'zapatera' spoilage, results from the decomposition of organic acids at a time when little or no sugar is present. The spoilage commonly proceeds after the lactic acid fermentation stops before the pH has fallen below pH 4.5.[101] *Propionibacteria* were isolated from brined olives with indications of 'zapatera' spoilage and were hypothesized to grow and cause a rise in pH owing to the degradation of lactic acid, permitting subsequent growth by *Clostridium* species[103,116,169] capable of producing butyric acid.[101] In cucumbers, yeasts of the genera *Pichia* and *Issatchenkia* are capable of catabolizing lactic acid produced during the primary fermentation, causing an increase in pH.

Softening of brined vegetables may be caused by pectinolytic enzymes derived from the plant material or

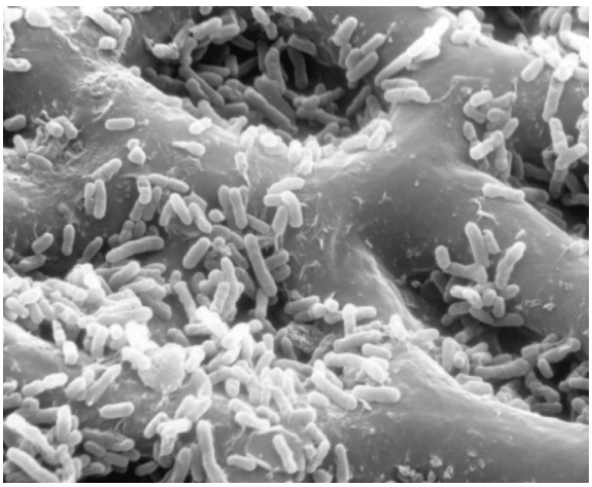

Figure 51.3. *Lactobacillus plantarum* cells colonizing the cucumber tissue as seen by electron micrograph.[173]

microbes. Mold growth accompanying film-forming yeast growth on the brine surface can cause softening of sauerkraut, cucumbers, and olives. Heavy scum yeast and/or mold growth is usually the result of neglecting brined material during extended storage in the absence of sunlight and the presence of minimal amounts of oxygen.[52,54] Sauerkraut tanks are usually held indoors, with a seated plastic cover weighted down with water or brine to maintain anaerobic conditions and limit the growth of surface yeasts and molds. Softening of brined cucumbers may also result from mold polygalacturonases that are associated with the cucumber flowers.[12,58] This problem may be reduced by draining and rebrining of the tank *ca*. 36 hours after initial brining.[57] However, this solution is not normally used today because of environmental concerns about salt disposal. Instead, recycled brines are treated to inactivate the softening enzymes, as needed.[24,27,57] The addition of calcium chloride can slow down the rate of enzymatic softening of fermenting cucumbers.[27] However, this should not be relied upon to eliminate enzymatic softening problems. Care must be taken to minimize the contamination of cucumbers, particularly the small fruit, with flowers and plant debris, which can be a source of contamination by pectinolytic molds. Softening is not a very serious problem in bulk Spanish-style olive fermentation owing to the reduced amount of brine surface in contact with air with respect to the total volume. However, yeasts and/or molds present on the plastic drums used during the conditioning operations (sizing, grading, pitting, stuffing, *etc.*) may induce softening.[97]

Non-pasteurized fermented cucumbers are prepared by desalting, followed by the addition of cover liquor often containing acetic acid and preservatives. For sweet pickles, sugar is added at concentrations up to 40%. Osmotolerant yeasts are the principal spoilage organisms in such products, and a preservation prediction chart, based on the concentration of acid and sugar needed for shelf stability, has been developed.[10] Aerobic molds and film yeasts may grow on the surface of the liquid, chiefly as the result of faulty jar closure. Spoilage microorganisms in sweet pickles include yeasts[42] and lactobacilli, particularly the heterofermentative *Lactobacillus fructivorans*.[43]

Non-fermented pickle products in which acetic acid is added to fresh cucumbers (known as fresh-pack pickles) are pasteurized to prevent the growth of LAB and yeasts. Recommended procedures include 165°F (74°C) for 15 minutes, as described by Etchells *et al*.[66,68] Spoilage of pasteurized pickle products usually occurs due to improper processing (insufficient heat to pasteurize) and/or improper acidification, so that an equilibrated brine product of pH 3.8 to 4.0 is not achieved. As with sweet pickles, molds and film yeasts are factors in cases of poor jar closure, where oxygen is introduced into the container. This can lead to a potentially dangerous situation caused by the pH increasing as organic acids are consumed by the spoilage microorganisms. If the pH rises above 4.6, germination of *Clostridium botulinum* spores may occur.

Refrigerated nonacidified products are commercially sold under a variety of names, including 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, *etc.*[59] These brined cucumbers may be held in barrels for a few days or longer at room temperature and then refrigerated at 2–5°C to allow fermentation to occur. Under refrigeration conditions, microbial growth, enzymatic activity, and the curing process continue at a slow rate.[59] Gaseous spoilage of the product is caused primarily by the gas-forming microbial groups mentioned earlier. Softening problems in refrigerated–fermented products may develop owing to the much lower concentrations of salt added to these product types. Fresh, whole garlic cloves and other spices are normally added to such products. These spices may contain softening enzymes.

Whether the half-sour products are made in bulk or in the retail jar, the very nature of the product makes it difficult to maintain good quality for more than a few weeks. 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 acid development slowly continues. This recommended condition for brine-product pH cannot be ensured for the product made in a retail jar, because there is no uniform process adopted by packers wherein the product is acidified at the outset, or where it is deliberately incubated for the development of a natural lactic acid fermentation.

The refrigerated fresh-pack (non-fermented) products are acidified with vinegar to an equilibrated pH of around 3.7, contain 2–3% NaCl, and occasionally sodium benzoate or other preservatives.[59] Like the half-sour pickles, 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. Recipes that do not contain vinegar or other acid in the initial cover liquor, however, should be viewed with caution.

Quality assurance of sauerkraut products starts with the removal of the outer leaves and woody core of the cabbage. In addition to its undesirable texture, the core contains sucrose, which can lead to the formation of dextran by *Lc. mesenteroides,* resulting in a slimy or stringy texture. Sauerkraut marketed under refrigerated conditions is preserved by the addition of sodium benzoate and metabisulfite.[191] The shelf life of such products is influenced by chemical changes that may result in discoloration (browning) and the formation of objectionable flavors. The growth of yeasts naturally present in cabbage may be the result of uneven salting during preparation of the sauerkraut, and can induce pink coloration and softening of the vegetable.

Spanish style-olives were formerly preserved by fermentation in cover solutions containing relatively high salt concentrations. However, it has been demonstrated that an appropriate combination of low pH (≤3.5), combined acidity (~0.025 milliequivalents (mEq)/L) and moderate proportions of acid (≥0.4%) and salt (≥5.0%) is also able to preserve well-cured olives.[2,174] Pasteurization has been progressively used to allow the commercialization of incompletely cured olives, or those with characteristics that are outside the ranges necessary for complete stabilization without heat treatment.[97] In some cases,

particularly when pasteurization is not recommended (plastic bags, seasoned olives, *etc.*), producers used authorized preservatives such as potassium sorbate or sodium benzoate.[2,32]

51.5 PATHOGENIC MICROORGANISMS

There are no authenticated reports of pathogenic microorganisms associated with commercially fermented or acidified vegetable products prepared under GMP from brined, salted, and pickled vegetable brine stock. In 1979, 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.'[196] Certain types of microorganisms that may cause product spoilage, such as molds, yeasts, enterobacteria and acid-tolerant LAB, may be encountered at times, usually under conditions associated with neglect. These organisms may reduce the quality of the texture and flavor of the product (whether prepared in bulk or retail container) and render it unacceptable. However, microorganisms capable of spoiling processed vegetable products are not considered human pathogens.

Although 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.[15,29,30,33,93,109,122,175,181] Outbreaks of *Escherichia coli* O157:H7 in unpasteurized apple juice or cider, which typically has a pH between 3.5 and 4.0, have resulted in over 100 reported illnesses and at least one death.[29,30,175] Extended survival of *E. coli* O157:H7, the adaptation of diverse strains of *Enterobacter cloacae* to resist low pH and high salt concentrations, and the presence of *Staphylococcus aureus,* have been documented in a variety of fermented olive products to date.[16,168,188] Researchers have found that adaptation to acidic conditions can be induced in some pathogenic bacteria,[9,95,109,118,121,122,154] and may even increase their virulence.[154] Acid adaptation, or acid tolerance response, in bacteria typically involves an initial sublethal acid shock, which results in changes in gene expression analogous to the response observed with heat shock.[95,123] 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 have been identified in *Listeria, Salmonella,* and other bacteria.[9,92,106,107,155,160] It has been shown that acid adaptation can enhance the survival of *Salmonella* and *Listeria* in fermented dairy products,[95,121] 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.[126,159,175] The significance of acid tolerance/adaptation of food-borne pathogens in fermented and acidified vegetables is yet to be revealed.

Listeria monocytogenes, a food-borne pathogen, has become a major concern to the food industry over the past 15 years, mainly for refrigerated and ready-to-eat products. The bacterium is commonly found in the environment and has been isolated from various plant materials, including silage,[72] soybeans, corn,[203,204] and cabbage.[15,182] Beuchat *et al.*[15] showed that *L. monocytogenes* was able to grow on raw cabbage and in cabbage juice. Conner *et al.*[33] found that death of *L. monocytogenes* (one strain tested, LCDC 81-861, is a pathogen isolated from coleslaw) occurred in cabbage juice adjusted to pH ≤ 4.6 with lactic acid and incubated at 30°C. The death rate of this pathogen was slower at 5°C than at 30°C. However, two tested strains 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, 4.39, 4.39, 4.62, 4.62, and 5.23, respectively.[100] Johnson *et al.*[113] demonstrated that *Listeria* could be recovered from fermented sausage made with beef intentionally contaminated with the bacterium. Caggia *et al.*[28] showed that *L. monocytogenes* can survive and grow during the processing of green olives. More recently, *Pseudomonas* and *Listeria* spp. were detected using molecular methods in stored acidified olives.[1] The times and temperatures needed to achieve a 5-log reduction of pathogens in acidified vegetables have been reported for both pasteurized and non-pasteurized acidified vegetable products.[20,21,22]

51.6 SAMPLING REQUIREMENTS

In the examination of vegetable products, brine or pickle liquor is commonly used to cover the vegetable material. The size of container to be sampled may range from a small jar of pickles to a 40 000 L tank of tightly packed fermented brine stock. In olives, the volume of fermentation vessels/storage tanks is typically about 15 000 L, with a volume of about 6000 L brine and 9000 kg of fruit. Brine samples from containers, such as tanks and barrels, should be taken for microbiological or physicochemical analysis as follows:

For large cucumber brine tanks, sampling is done after mixing the content by purging with air. Olive brining tanks are mixed using circulation created by pumps. A suitable length of $^{3}/_{16}$ inch stainless steel pipe sealed at one end with lead or solder, and perforated with several $^{1}/_{16}$ inch holes at a distance of 6 to 8 inches from the sealed end, is inserted through an opening between the wooden cover boards to the center of the fermentation vessel. Alternatively, the stainless steel pipe may be inserted through the purging for consistency tube in the cucumber brining tanks. Brine may then be withdrawn using a sanitized tube that deposits the sample into a sterile sample container. The length of the steel sampling tube is governed by the depth of the container to be sampled. Approximately 100 mL of brine are withdrawn and discarded before taking the final sample of 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 1–2 days during active fermentation. After each sampling, wash and sterilize the whole assembly by immersing in a hypochlorite solution or ethanol.

For smaller containers, such as jars or cans of products, shake thoroughly and take the sample from the center of the material by means of a sterile pipette. 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 vegetable products. If it is necessary

to ship or store samples, this should be done under refrigerated conditions (4–8°C); the elapsed time from collection to examination should not exceed 24 hours. When shipment by air is required, samples are collected in sterile, 1.6 x 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 spinning, decanting and freezing the supernatant until use. A headspace of about one-quarter of the volume of the container to be frozen should be maintained to allow for expansion during freezing.

51.7 RECOMMENDED METHODS

To enumerate the microbiota of whole or particulate vegetables, approximately 300 g of plant tissue are homogenized aseptically with an equal weight of sterile saline solution (0.85% NaCl). The samples are homogenized in a heavy-duty commercial blender (*e.g.*, Waring Blender model 31BL46, Waring Products, New Hartford, CT) with a 1 L sterilized blender jar 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 and aseptic techniques. Approximately 100 mL of the vegetable slurry is removed immediately after blending for further processing in a stomacher (*e.g.*, Stomacher 400 homogenizer, Spiral Biotech, Inc., Bethesda, MD). The slurry is dispensed into a stomacher bag containing a filter on the side (Stomacher 400 filter bags, Spiral Biotech) and processed using the maximum force setting for 1 minute. The filtrate removed from these bags should only contain particles of approximately 40 μm or less in diameter, and can be used in a spiral plater or plated directly onto agar plates. For samples with low numbers of bacteria (200 CFU/mL or less), 1 mL of brine can be added to 25 mL of agar at 45°C prior to pouring into plates. The filtrate could also be spun by centrifugation and the supernatant maintained at –20 °C for future chemical analysis.

Occasionally it may be necessary to determine the microbiota attached to the surfaces of fresh or fermented vegetables. The microbiota may be composed of yeasts and bacteria, as depicted in Figures 51.1 and 51.3. Cucumber or cabbage skins may be sampled by forcefully – but carefully – swabbing 1 cm^2 areas with sterile swabs and resuspending the collected microbes in 0.85% saline solution. Aseptic techniques should be used during sample collection and handling, prior to swabbing. For the analysis of olive samples it is recommended to dry the brined fruits by aeration, then weigh and transfer the fruits to a sterile saline solution (0.85% NaCl) where they can be vigorously shaken for a period of approximately 2 hours at room temperature. The saline solutions may then be spread on the culture medium of choice.

51.7.1 Bacteria

Several methods may be used to determine the number of bacteria in brined vegetable tissue. For a total cell count using microscopic methods, the vegetable filtrate obtained as described above is enumerated with a Petroff–Hauser counting chamber at a magnification of about 500x.[37] To count total aerobic bacteria, the filtrate may be analyzed by plating onto plate count agar or nutrient agar incubated at 30°C for 18–24 hours. For longer incubation, overlay the solidified plated samples with about 8–10 mL of the same medium to prevent or minimize spreaders. Methods, including the use of Petrifilm plates, to obtain counts of aerobic microbes may be particularly useful for field studies (see chapter on Rapid Methods for Detection Identification and Enumeration and Labor Savings and Automation for an array of rapid methods).

Selective enumeration of LAB may be carried out using *Lactobacilli* deMan Rogosa and Sharpe (MRS) agar combined with an incubation period of 1–4 days at 30°C. MRS agar should be supplemented with 1% cycloheximide or 0.02% sodium azide[41] to inhibit the growth of yeasts. However, sodium azide may inhibit the growth of some LAB. Incubation of MRS plates under anaerobic conditions will foster the growth of LAB and inhibit yeast growth. This is especially helpful when dealing with spoilage samples containing osmotolerant yeast species. LAB of the genus *Lactobacillus* or *Pediococcus* may be enumerated with *Lactobacillus* selective medium (LBS), supplemented with 1% fructose and 1% cycloheximide. Bromocresol green (or brilliant green, as in the chapter on Media, Reagents and Stains), 0.0075% (w/v), may be added to aid in colony counting, but may restrict the growth of some LAB. This dye may help in the differentiation of heterofermentative and homofermentative LAB species.[19] For the incubation of agar plates over more 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.

Several kinds of differential media are available for the characterization of LAB. The Heterofermentative and Homofermentative Differential (HHD) medium (see the chapter Media, Reagents and Stains for composition) is used for the differential enumeration of homofermentative and heterofermentative LAB species.[134] This medium incorporates fructose, which is reduced to mannitol by heterofermentative – but not homofermentative – LAB. In agar medium, homofermentative colonies of LAB are blue to green, whereas heterofermentative colonies are white. The Malate Decarboxylating (MD) medium (see chapter on Media, Reagents and Stains for composition) may be used to differentiate malate-decarboxylating (MDC^+) and malate-non-decarboxylating (MDC) LAB. The decarboxylation of malic acid is undesirable in cucumber fermentations because of the CO_2 produced.[40] The differential reaction is based on 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.[19]

After incubation at 30°C for 24–48 hours, isolated colonies may be analyzed to determine cell morphology, acid and gas production, and mucoid growth;[41,190] other reactions, such as the assimilation of different substrates, are also commonly used. In the case of differentiation of *L. pentosus, L. plantarum* and *Lactobacillus paraplantarum,* molecular identification can be carried out based on the primers designed by Torriani *et al.*[195] for the amplification of *recA*. A combination of the random amplification of polymorphic DNA (RAPD) technique with primer OPL5 is widely used for the molecular characterization of LAB to the strain level.

Counts for *Enterobacteriaceae* or coliforms may be obtained by plating on violet red bile agar supplemented with 1% glucose, which is referred to as MacConkey glucose agar[150] or VRBG agar, and incubated 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. It is recommended practice to add an enrichment step for the detection of *Enterobacteriaceae* from brine samples with a pH lower than 4.2 prior to plating on the selected medium. The Enterics Enrichment Mossel broth containing glucose will allow the detection of *Salmonella* species and other lactose-negative bacteria. Lactose broth is also used occasionally to enrich the population of enterobacteria. A 1% inoculation in the enrichment broth is recommended, followed by an incubation period of up to 24 hours. 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 on Enterobacteriaceae, Coliforms, and *Escherichia coli* as Quality and Safety Indicators and Rapid Methods for Detection Identification and Enumeration and Labor Savings and Automation).

Culturing butyric acid bacteria requires neutralization of the brine sample with an excess of sterile calcium carbonate. Heat a 50–100-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 on liver broth medium. Seal with melted petroleum jelly and incubate for 7 days at 32°C. Examine tubes daily for the production of gas and a strong butyric acid odor. If butyric acid production by *Clostridium* species is suspected, the differential reinforced clostridial medium may be used to culture heat-shock samples using the pour-plating technique. Sulfite-reducing *Clostridium* species will develop a black colony on plates after 24–36 hours of incubation under anaerobic conditions.

51.7.2 Yeasts

Use the microscopic technique hemacytometry to determine yeast populations in fermenting vegetable brines and various types of finished pickle products undergoing gaseous spoilage, particularly where populations are in excess of 10^4 cells/mL of sample, and where yeast colonies are not required for isolation and study. The use of a methylene blue stain along with hemacytometry permits the differentiation of yeast populations into viable and nonviable cells and increases the usefulness of the direct counting technique. The percentage of viable cells may be calculated based on the total counts and the number of blue-colored dead cells.

The counting procedure developed by Mills[147] and modified by Bell and Etchells[10] for counting yeasts in high salt-content brines and in high sugar-content liquors, uses supplementation of 1 mL of brine or pickle liquor with 1 mL of 1:5000 (0.02%) erythrosine stain. Pink dead yeast cells and colorless live yeast cells are recorded and calculations performed using the following equation:

$$\frac{Number\ of\ yeast\ cells\ counted\ x\ dilutions\ x\ 250000}{Number\ of\ large\ squares\ counted} = Numbers\ per\ mL$$

If only one side of the hemacytometer counting chamber is used (25 large squares), the lowest yeast count obtainable is 20 000/mL, whereas if both sides are counted (50 large squares), a population as low as 10 000/mL can be counted. Report the results as total dead and live yeast cells per mL of sample. The technology for automated yeast counts using fluorescent dyes is also currently available.

Yeast and mold populations may be enumerated on Yeasts and Molds agar (see chapter Yeast and Molds) supplemented with 20 mL/L of an antibiotic solution containing 0.5% chlortetracycline and 0.5% chloramphenicol (YMA). We have found it desirable to use both antibiotics to preclude the growth of bacteria, although gentamicin sulfate and oxytetracycline (0.005%) can also be used as selective agent for yeasts. Alternatively, acidify sterile tempered molten dextrose agar (at 45°C) with 10% tartaric acid, usually 5% by volume, to achieve a final pH of 3.5. Incubate inoculated YMA or acidified dextrose agar for 24–48 hours at 30°C. Small colonies of LAB may appear on the acidified medium, but are suppressed in the antibiotic medium. Commercially available Petrifilms may also be used for the enumeration of yeasts and molds.

Mold colonies are filamentous and therefore readily distinguishable from yeasts on acidified dextrose agar and YMA 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*,[52,54,56] are generally dull and very rough, in contrast to the usual round, raised, white, glistening colonies of the fermentative subsurface yeasts, *i.e.*, species of *Torulopsis, Brettanomyces, Hansenula, Saccharomyces,* and *Torulaspora*.[52,53,56,63,67] However, even when distinguishable colony characteristics of the two yeast groups exist, they are not considered sufficiently clear-cut for separation. Because of this, the procedure outlined above for yeast counts should be used. For an estimate of film yeasts, pick representative filamentous colonies from the yeast plates into tubes of dextrose broth containing 5% and 10% salt. Incubate for 3–5 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. Certain species, such as *Saccharomyces halomembranis,* form heavier films at 10% salt than at 5%.[13,67]

Selection of the osmotolerant and acid- and preservative-resistant spoilage yeasts, *Zygosaccharomyces* spp.,[10] may be achieved by using acidified tryptone–glucose–yeast extract agar.[131] The sterilized and tempered medium may be acidified using glacial acetic acid (17.4 N, 5.0 mL/L) before pouring it into Petri plates.

Since 2006, molecular methods have been used for the identification of yeasts associated with vegetable fermentations.[3,36] They are based on restriction fragment length polymorphism (RFLP) of the 5.8S rRNA and the two

ribosomal internal transcribed spacers,[50] and sequencing of D1 and D2 domains of the 26S rRNA gene with primers NL1 and NL4, which confer greater reliability than classic biochemical tests.

51.7.3 Obligate Halophiles

The use of liver broth plus salt (see chapter on Media, Reagents and Stains) has proved satisfactory for detecting the 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. Prepare decimal dilutions, seal with sterilized, melted petroleum jelly, and incubate for 7 days at 32°C. Observe and record growth in the tubes daily by noting the raising of the petroleum seal caused by gas production, and the absence of any distinctive odor.

51.7.4 Bacteriophages

For the isolation of lytic bacteriophages, brine samples from fermenting vegetables are collected, transported on ice, and processed within 24 hours. The brine samples are spun in 15 mL plastic screw-cap tubes at 3000 *g* for 10 minutes (Sorvall model RC5-B, with a SS-34 rotor; Sorvall Products, Newtown, CT, USA) in order to remove the suspended material. As a phage enrichment step, 1 mL of the supernatant is added to 10 mL of fresh MRS broth along with 0.1 mL of 1.0 M $CaCl_2$ and 0.5 mL of an early-log-phase culture of LAB as a host with an optical density of 0.3 to 0.4 (approximately 10^7 CFU/mL). To identify host LAB strains, individual colonies can be picked from MRS agar plates containing mixed fermentation microflora. A 96-well microtiter plate method for testing potential host–phage combinations has been described.[130] The phage enrichment medium is incubated overnight at 30°C or 37°C. The enriched samples are spun at 3000 *g* for 10 minutes, followed by filtration using a 0.45 μm sterile filter. The potential phage-containing filtrate (about 9 mL) is treated with 0.1 mL of chloroform (optional) and stored at 4°C until use. A plaque assay may be performed by mixing an aliquot of 0.1 mL of the phage lysate with 3 mL of MRS soft agar (top agar) kept at 50°C, 0.1 mL of 100 mM $CaCl_2$, and 0.1 mL of a mid-log culture of the host bacterium, and poured over MRS agar plates (bottom agar). Plates are incubated overnight at the optimal growth temperature of the host bacterium. Once plaques appear on the turbid lawn of the host bacterium (Figure 51.4), single phages may be isolated by physically selecting the plaques, using a sterile pipet tip to inoculate the actively grown host in MRS broth, followed by overnight incubation. After centrifugation, tenfold dilutions of the supernatant are prepared and 10 μL of the diluted preparations are spotted on the MRS soft agar containing the actively growing host (LAB).[130,205,206]

51.7.5 Bacteriocins

The presence of bacteriocin producers in brine fermentations may be noticed while culturing the brine samples on MRS agar plates as shown in Figure 51.5. After purification of the strains that produce a clear halo of inhibition around the colony, the bacteriocin should be tested against other LAB strains or pathogenic bacteria to determine its spectrum of action. To determine that the inhibition is exclusively due to the action of bacteriocins instead of lactic acid, samples are generally treated with proteinase K. DNA primers useful for the identification of selected bacteriocin-encoding genes from fermented cucumber brine samples using a polymerase chain reaction (PCR) assay or real-time PCR are currently available.[186]

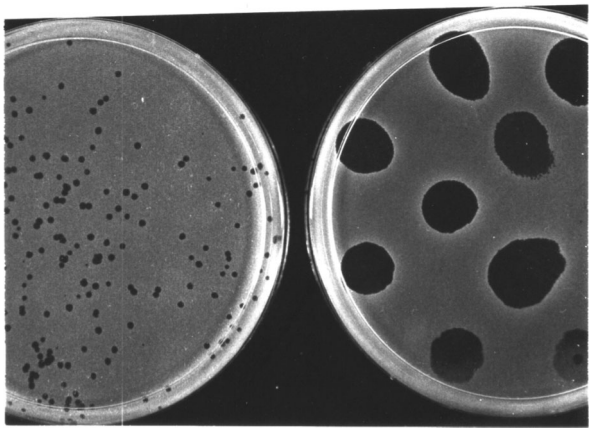

Figure 51.4. Plaque morphology (left) and spotted phage lysate (right) of LAB bacteriophage isolated from fermenting sauerkraut brine.[205]

51.7.6 Chemical Analyses

Chemical analyses of fermented and acidified vegetables typically include pH, titratable acidity, activity of softening enzymes, and concentrations of acid, sugar and salt. Titratable acidity and pH are routinely measured to assess the progress and completion of fermentation of cucumbers, cabbage, and olives by LAB, or to determine whether direct acidification of vegetables has been used correctly. The salt concentration in cucumber fermentation brines is routinely determined during storage, and salt levels are adjusted as needed to ensure stability of the brine stock. Residual sugars are also commonly assayed, as they affect the microbiological stability of fermented brine stock during storage. Analysis of polygalacturonase activity during fermentation, and prior to reusing spent brines, is frequently done. The calcium added to improve texture, and the sorbate used as a processing aid to inhibit yeast growth, are also routinely measured. Aluminum, benzoate, and sulfite content may also be assayed in finished products if they were included in brine stock or added during processing. Although CO_2 in brines was routinely measured during fermentations to determine the rate of purging that brines needed to prevent pickle bloating and hence damage,[55,77,85,89,192] it is now rarely assayed, as purging rates and schedules, based on established data, have proved effective in preventing pickle damage due to excessive CO_2 accumulation.

For the analysis of chemical components during bulk fermentation and storage of brine stock, samples of filtrates

prepared as described above may be used. Sample pH should be determined using a pH meter calibrated with certified buffer solutions that bracket the range of the sample pH values. Buffers commonly used as standards for testing the pH of fermented and acidified vegetable products are pH 7.00, 4.00 and 2.00, and are sold commercially. Electrodes for measuring pH that are encased in glass or other breakable material, or that contain toxic chemicals, should not be used to directly measure the pH of fermentation tanks or process brines within the tank, as electrolyte leakage occurs and breakage could result in fragments ending up in the food product. Instead, samples should be isolated for analysis to avoid contamination of the foods.

Temperature has a significant influence on pH measurements, therefore a combination electrode that incorporates a temperature sensing probe should be used, or measurements should be taken as close to 25°C as possible. Temperature compensation is an automatic feature of many types of pH meters. If it is not, standardizing the temperature of calibration buffers and samples will avoid errors caused by temperature differences. After several measurements the pH meters should be recalibrated before further use.

Although much less accurate than pH meters, various types of commercially available pH test papers, indicators, or solutions can be used for a rapid pH measurement of fermentation samples by visualizing their color changes. Comprehensive information has been published on pH and titratable acidity measurements in foods.[177]

Titratable acidity is routinely measured to estimate the amount of free acid in fermenting and fermented or acidified products using a known amount of sample in distilled water. The sample is titrated with a standard solution of dilute alkali such as NaOH. The concentration of NaOH solutions should be verified and protected from atmospheric CO_2, since sodium carbonate is readily formed, causing a decrease in NaOH concentration that will inflate titration values. AOAC Official Method 936.16 provides details for the determination of NaOH standards.[158] The pH indicator phenolphthalein is used in colorless or slightly colored samples to indicate the titration end-point at pH 8.2, whereas a pH meter is used for highly colored samples[158] (AOAC Official Method 942.15). Sample size and alkali concentration are established based on the expected acid concentration of the samples. For cucumber, cabbage, and olive fermentations, 10 mL of sample diluted with 40 mL of neutral water is conveniently titrated with standardized 0.1 N NaOH until a light pink color of the phenolpthlalein indicator is maintained for 30 seconds. Titratable acidity is usually reported as the concentration of the predominant acid in the sample, which would be lactic acid for fermented cucumbers, cabbage, and olives, or acetic acid for finished products processed with vinegar. For a 10-mL sample titrated with 0.1 N alkali, the following calculations may be used:

Fermented brine stock: (mL of 0.1 N alkali titrant) x 0.0901 = g of lactic acid per 100 mL

Finished products: (mL of 0.1 N alkali titrant) x 0.0601 = 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 0.1 N alkali by 5, then by 0.0901 for lactic acid or 0.0601 for acetic acid. Considering that other organic acids may be produced by fermentative microorganisms, and that the acidity of many fermented and acidified vegetable products is contributed to by a combination of acids, analysis using HPLC methods is more informative than titratable acidity.

The combined acidity of table olive brines plays an important role in stabilizing and maintaining pH. The term 'combined acidity' refers to the buffer capacity of the brine containing multiple acids in the form of organic acid salts, mainly sodium lactate and sodium acetate. Combined acidity is usually expressed as mEq of overall organic salts present per liter. In packed products, values above 25 mEq/L are recommended; otherwise it is very difficult to reach a final pH that can properly preserve the products. This parameter is obtained by potentiometric titration of the brines with hydrochloric acid to pH 2.6, which is the average minimum pH value of the buffering curve. For its determination, introduce 25 mL of brine in a 50–100 mL beaker and titrate it with standardized hydrochloric acid solution; add the acid slowly and shake simultaneously until pH 2.6 is reached as measured by a potentiometer provided with glass electrode and calomel electrode as reference. Results are expressed as the normality of hydrochloric acid, although it is also common to express the results as mEq/L.

It is important for the stability of fermented vegetables that all fermentable sugars be metabolized at the end of the fermentation process. For products such as cucumbers, which 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 is recommended.[146] The reagent

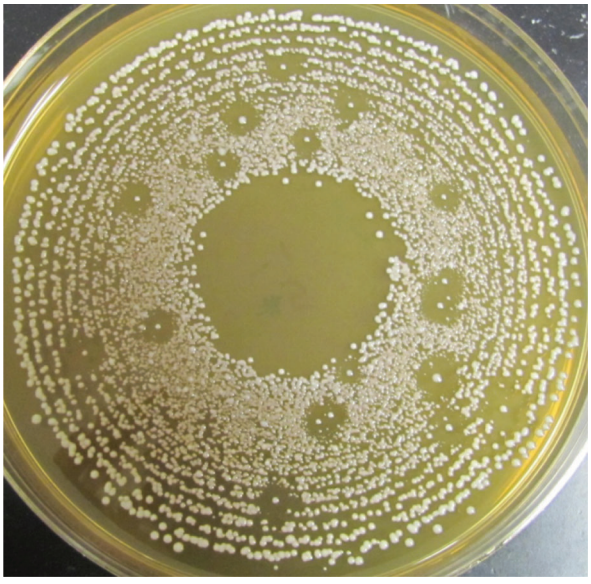

Figure 51.5. Visualization of bacteriocin production by LAB in MRS agar plates supplemented with 1% cycloheximide after 48 h of incubation at 30°C under anaerobic conditions. An automated spiral plater was used to deposit brine samples on the MRS agar plate.

is stable at room temperature for many months, and the assay can be reliably performed with an inexpensive colorimeter. Fermentation can be considered complete if the brine contains less than 0.05% reducing sugar and acidity remains stable. Test strips for estimating glucose based on coupled reactions of glucose oxidase products are sometimes used for quick checks of residual sugars; however, these are unreliable for determining the completion of fermentation, as other fermentable sugars could be present.

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 the products of a fermentation process. Using high-performance liquid chromatography (HPLC), the components of interest in the fermentation and spoilage of processed vegetables can be quantified using an H+ resin column at 65°C with 0.8 mL/min 0.02 N sulfuric acid (H_2SO_4) eluent for separation, followed by refractive index and diode array detectors in series for the detection of sugars and alcohols and acids, respectively, as described by McFeeters and Barish.[135,137,162,164] This analytical system is used routinely to quantify glucose, fructose, glycerol, ethanol, propanol, 1,2-propanediol, and malic, succinic, lactic, acetic, propionic, and butyric acids in a single 30-minute analysis, with minimal sample preparation required. However, there are a few caveats regarding this method that should be considered with regard to fermented and acidified vegetable products. 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 may not separate adequately from fructose to permit analysis of either compound. Another potential problem with the single injection procedure for cucumber pickle analysis is that fructose and malic acid coelute.[135] The coelution problem has been solved in a number of ways using the same column. Lazaro *et al.*[119] developed equations to quantify fructose and malic acid differentially based on peak heights obtained from ultraviolet and refractive index detectors connected in series. Frayne[94] actually resolved malic acid and fructose by connecting two of the HPLC columns in series. Recently, it has been demonstrated that malic acid can be separated from glucose and fructose by simply altering the elution conditions to 0.6 mL/min of 0.0015 N H_2SO_4 eluent at a column temperature of 80°C.[125] Additionally, it has been found that column temperature plays a key role in separating propionic acid, a spoilage metabolite in fermented cucumbers, from an unknown peak component that is sometimes present in non-spoiled fermented cucumbers. In this case, it is desirable to use a column temperature of 37°C and a flow rate of 0.6 mL/min at the cost of a longer analysis time, to avoid mistakenly presuming the presence of propionic acid or overestimating its quantity. Also, if samples are expected to contain benzoate or sorbate additives, the 65°C column temperature is recommended and the sample analysis time should be extended to 60 minutes to avoid peak interference in subsequent sample chromatograms.

All major sugars and sugar alcohols involved in vegetable fermentations can be separated using a Dionex Carbopak PAl column with dilute NaOH as the eluent. Owing to the low analyte capacity on columns of this type, electrochemical detection of sugars is preferred over a refractive index detector, and organic acids would require a separate analysis.

Although HPLC is today the method of choice for analysis of fermentations, it requires an expensive instrument. An alternative approach for the 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. However, enzymatic analysis is the only routine way to specifically measure the L--isomer of lactic acid. Kits are available for glucose, fructose, malic acid, D- and L-lactic acid, acetic acid, ethanol, and CO_2.

The sugar content of sweetened pickle products and their process brines is typically estimated by hydrometers calibrated in either degree Brix or percent sugar, or by refractometers that determine the refractive index of the solution, which is scaled to percent soluble solids or sugars. Although salt and other dissolved substances contribute to specific gravity and refractive index, these rapid methods provide reasonable approximations for solutions with high ($\geq$20%) sugar content, such as sweet pickles or relish.

Salt analysis and adjustment are essential for the satisfactory fermentation of cucumbers, cabbage, and olives and the manufacturing of pickled food products. Also, it is important to know the approximate calcium and sodium salt content of samples when performing microbiological examinations of brines. Hydrometers similar to those used for estimating sugar content based on the specific gravity of a solution are commonly used for estimating the salt content of fermentation brines. These hydrometers, called Salometers, are calibrated on a scale from 0 to 100 based on the percent saturation of aqueous solutions of NaCl at 15.5°C. For example, a NaCl solution measuring 50°S (degrees Salometer) at 15.5°C would be 50% saturated and contain 13.2% NaCl based on the weight of the brine. As with all specific gravity values, corrections for measurements made at different temperatures are required. Instruments called salt meters are available which use light refraction or electrical conductivity to estimate salt content. These may provide temperature compensation, but dilution of brine samples is usually required. Conductivity meters calibrated for NaCl and titration of chlorides are commonly used for quality control. Titrations of acidified salt solutions for chloride content using standard silver or mercuric nitrate solutions with dichlorofluorescein[6,71] or diphenylcarbazone[180] as color indicators are frequently used for chemical estimation of salt content. The indicators form colored products with silver or mercuric ions after all chlorides are complexed by the metal ions, which is similar to the principles of AOAC Official Method 960.29 and 973.51.[157,158] For example, 1 mL of sample is mixed with 10 mL of deionized water, 4 drops of 0.33 N H_2SO_4 and 2 drops of diphenylcarbazone (2.5 mg/mL of methanol). The mixture is titrated with standard 0.141 N mercury nitrate until a faint violet color emerges, which is the point of total formation of mercury chloride with a slight excess of the titrant in the solution. Based on

the molecular weight and normality of the titrant, the percent NaCl is estimated from the mL of titrant x 0.83. A similar analysis is titration of 1 mL of sample diluted with 15–20 mL distilled water containing 0.5% dichlorofluorescein, as the color indicator, against 0.171 N silver nitrate. This titration allows a direct estimation of NaCl, as each mL of titrant represents 1 g of NaCl/100 mL.[71]

Chloride test strips are less accurate, and most would require the dilution of brine samples owing to their limited detection range. Although chloride analyses are useful for estimating NaCl content, other chloride salts, such as calcium chloride, are often present in significant amounts. Adjustment of the values obtained by the analysis may be necessary to avoid overestimating the amount of NaCl. For specific analysis of sodium as well as other minerals, such as calcium and aluminum, atomic absorption spectroscopy or inductive couple plasma technologies are required. Specific ion electrodes similar to those used for measuring pH may be useful for certain analyses, but they are prone to errors owing to interference from other elements.

Calcium chloride may be used in fermentation brines and in finished pickle products to reduce softening and improve product texture. It contributes to the ionic strength of fermentation brines, which may influence LAB. A convenient analysis of calcium in fermentation brine and fermented products utilizes ethylenediaminetetra-acetic acid (EDTA) complexometric titration, as described by the AOAC Official Method 968.31.[158] Sufficient hydroxynaphthol blue is mixed into samples that have been adjusted to pH 12–13 by potassium hydroxide to produce a pink color. The pink hydroxynaphthol–calcium complex is then immediately titrated with standard EDTA solution (0.01 N) to a deep blue endpoint color, which is the color of the indicator free of calcium.[102] The amount of calcium in the solution, based on the amount of titrant used, is determined from a standard curve or calculated using the equivalent weight of calcium, as 1:1 complexes occur with EDTA.[158] Usually, these methods are sufficient for NaCl and calcium determinations in both fermentation brines and vegetable tissue. However, precise quantification can be achieved by atomic absorption spectroscopy[124] or inductively coupled plasma mass spectroscopy, methods commonly used for soil analysis.

Potassium sorbate is effectively used to inhibit the growth of yeasts and molds in fermentation brines and some processed products. Benzoate is an important preservative used in certain fermented and fresh-pack products to inhibit bacterial growth, especially LAB. Organic solvent extraction and spectrophotometry provide a simple method for estimating the amounts of both of these preservatives in samples of brine.[207] A 5 µL sample is acidified with concentrated phosphoric acid (5 µL) and then extracted by isooctane (5 mL) for 30 minutes. The decanted organic solvent containing the preservatives is scanned against the control solution from 215 to 265 nm. Benzoic acid and sorbic acid will have distinct maximum absorbance at 225 nm and 255 nm, respectively. Amounts of the preservatives can be determined from standard calibrations developed from the known amounts. Certain chemicals from spices used to flavor finished products interfere with this analysis. The HPLC method described by the AOAC Official Method 994.11[158] for the separation and detection of preservatives has reduced interference problems and improved accuracy. Although sample analysis times are long (60 minutes), sorbate and benzoate can also be quantified simultaneously with the other components important in fermented and acidified vegetables using the HPLC method described above.

Sulfites are added to some processed fruits and vegetables to prevent discoloration caused by oxidation reactions, especially when exposed to light. Sulfites may also be used to inhibit microbial spoilage in certain products although it is rarely used for this purpose by manufacturers of vegetable products in the USA. A common procedure for estimating free sulfur dioxide (SO_2) content is the Ripper method, which is an iodometric titration;[156,197] however, the presence of iodine-reducing substances in many foods yields unreliable results. The modified and optimized Monier–Williams methods (AOAC Official Methods 975.32 and 990.28) have been the most widely accepted procedures for analysis of total sulfites, although sulfur-containing substances in *Allium* and *Crucifer* species were measured as sulfites.[158] Interferences caused by naturally occurring substances may be avoided by using ion exclusion chromatography, with electrochemical detection of SO_2 released from foods by alkali according to AOAC Official Method 990.31. This is currently the preferred procedure for sulfite analysis.[158] An alternative HPLC procedure for the analysis of sulfites in acidified vegetables has been developed that employs a ultraviolet detector and chromatographic conditions suitable for the simultaneous separation and quantification of the major acids and sugars observed in fermented and acidified vegetables.[137]

Polygalacturonase (PG, EC 3.3.1.15) activity in cucumber fermentation brines is a major concern as it can cause softening of processed vegetables. Even very low PG activity in brines can reduce the firmness of the vegetables, which are often stored in their fermentation brines for several months before processing. To reduce the risk of texture loss due to PG, brine cover solutions used during storage or to be reused for fermentations may be analyzed for such enzymatic activity. PG activity is determined from changes in viscosity,[14] reducing sugar concentrations,[104] or by the diffusion plate assay, which requires the staining of polygalacturonate (PGA)[146] after incubation with a sample of brine or tissue extract. Cover brine solutions or tissue samples require dialysis to reduce NaCl and remove $CaCl_2$ prior to analysis by viscosity or reducing sugar procedures. Also, these procedures typically utilize pH 5–7 buffer solution to avoid precipitation of the PGA substrate. The diffusion plate assay for PG activity involves the addition of samples to wells in plates of agarose gel containing PGA, incubation at 38°C for 48 hours, then staining with ruthenium red and destaining in water. The unstained area of the gel is caused by hydrolysis of PGA by PG and represents its activity. This procedure allows the screening of a large number of samples for PG activity at pH 4 rather than pH 5 or higher, and usually without sample preparation. Although the diffusion plate assay is widely used for identifying PG activity in industrial brines, more rapid and accurate procedures are needed. Fermentation brine cover solutions, which test positive with this test, and are to

be reused, can be treated with PG-absorbing clay.[24] Fermented vegetables stored in brines which are positive for PG activity should be processed as soon as possible to minimize the risk of damage.

51.8 INTERPRETATION OF DATA

Keeping proper records of salting procedures and chemical and microbiological data can greatly aid the commercial briner in assessing the causes of 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 record-keeping system for brined cucumbers has been published.[65]

For fermented vegetables, it is important that lactic acid fermentation becomes established early to preclude the growth of spoilage bacteria. Acidity and pH data provide an indication of this information. Expected values for total acidity and pH for a selection of fermented and acidified vegetable products are shown in Tables 51.1 and 51.2. Combined acidity plays a more significant role in Spanish-style green table olive fermentations, with typical values around 125 mEq/L in the fermented product and around 25 mEq/L in the finished products. Higher combined acidity values may prevent proper pH values and the prevention of spoilage being achieved. If necessary, partial substitution of fermentation brine, or washing prior to packing, may be convenient to adjust the combined acidity to adequate levels.[97] 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.

Table 51.3 shows typical microbial counts for fermented and fresh cucumbers. In cucumber and olive fermentations *L. plantarum* and *L. pentosus* should reach maximum counts of 8–9 $\log_{10}$ CFU/mL by day 7, but this depends on a considerable number of variables, such as temperature, raw material, and initial brine conditions, among others. In sauerkraut fermentation, heterofermentative LAB may reach 9 $\log_{10}$ CFU/mL during the first week of fermentation and then decline in numbers as they are replaced by the more acid-tolerant homofermentative microorganisms. This biphasic pattern of growth and death can be seen by plating total LAB using MRS agar, with anaerobic growth at 30°C.[82,112] Mathematical models can describe the growth/decay parameters of microbial populations during vegetable fermentations.[48,152].

If the dissolved CO_2 concentration in the brine of fermenting cucumbers is allowed to exceed about 50% saturation (=54 mg/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.[87] 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.[37] Since brines must be purged for 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 (PG) enzyme activity in brine cover solutions may indicate the cause of soft brine-stock vegetables, especially if they are held at relatively low brine strengths (5–8% NaCl). Higher salt concentrations added after primary fermentation will prevent softening by these enzymes,[11] but high salt levels present disposal problems. Studies have indicated that calcium chloride, *ca*. 0.2–0.4%, and other calcium salts may inhibit the action of softening enzymes.[26,140,141] Calcium chloride is now being added to commercial cucumber brines. The extent of protection against enzymatic softening offered by calcium has not been fully assessed, although it has been reported that elevated calcium in fermentation brines greatly assists in maintaining product crispness.[25] The absence of softening enzyme activity in older brine-stock pickles does not necessarily mean that such activity did not contribute to 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 the natural PG in overly mature cucumbers,[178] rather than microbial activity.

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, whereas recovery of >100% suggests that unknown substrates have been fermented. Examples of fermentation balances have been published.[31,65,81,83,86] Expected concentrations for acetic and lactic acids on a number of fermented and acidified vegetable products are described in Tables 51.1 and 51.2. The identification of fermentation end-products can be useful to determine the microorganisms responsible for spoilage of fermented cucumbers.

Properly acidified, packaged, pasteurized, and/or fermented vegetable products are typically not subject to microbial spoilage. When spoilage occurs, it is usually due to incomplete pasteurization or fermentation. Some commercial packers minimize heat processing in order to maintain greater product quality, but minimal processing is done at the risk of spoilage. LAB and yeasts are usually present in this type of spoilage. Improper acidification and/or closure can also be a source of spoilage, with potential public health significance. Improper closure allowing oxygen into containers can result in the growth of aerobic microorganisms on the surface of the brine and a reduction in acidity. Improper acidification even with an appropriate closure may result in some or all of the following: increased pH, reduced lactic acid, increased gas pressure and white scum on the surface of the brine, and the development of brine turbidity. No known public health problem exists in pasteurized processed vegetable products that have been properly acidified or fermented.

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