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# 14 Safety of Minimally Processed, Acidified, and Fermented Vegetable Products

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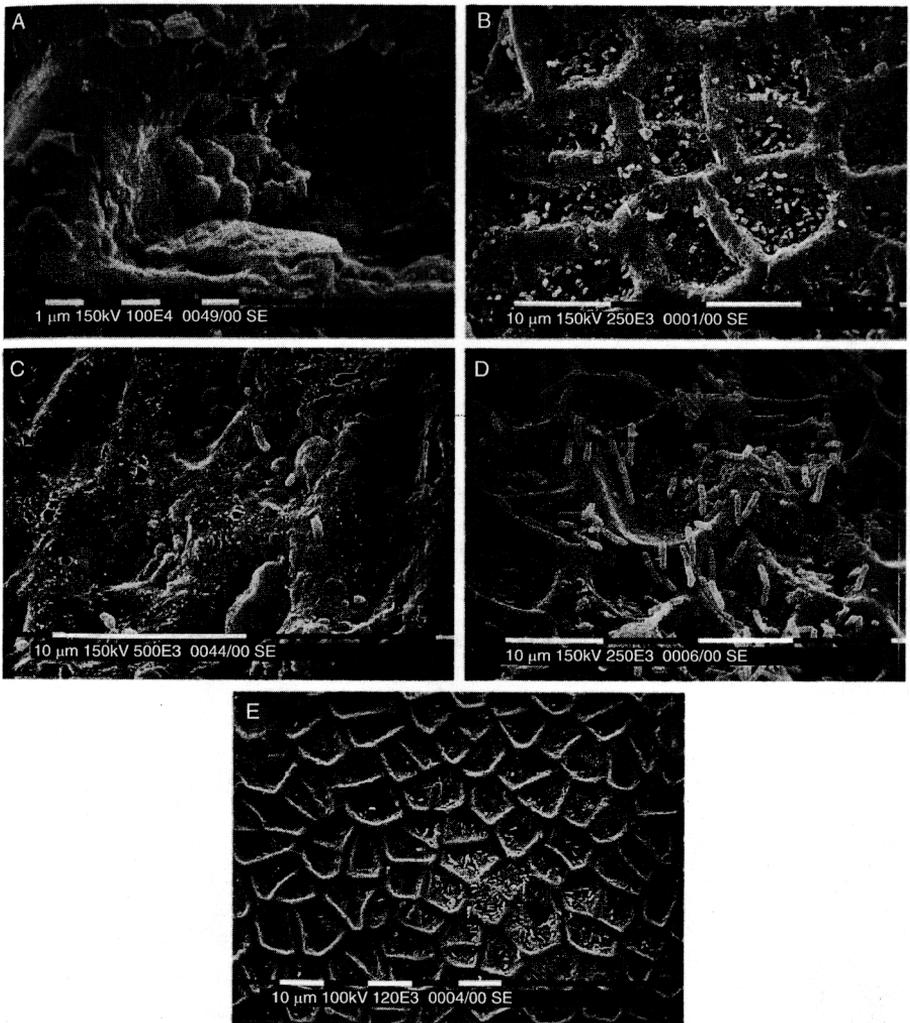
## 14.1 INTRODUCTION

Food fermentation technology likely originated sometime between 8,000 to 12,000 years ago as plants and animals were being domesticated in the Middle East, Africa, and Asia [1–3]. The development of primitive pottery technology likely led to early fermentation experiments, either planned or unplanned. Cheese, bread, and alcoholic beverages may have resulted from the fermentation of milk, grains, fruits, and vegetables stored in ceramic jars or pots. If these “spoiled” or fermented products were found to have desirable sensory properties, they may have been developed as the first processed or fermented foods [2]. An important characteristic of fermentation was the increase in the storage lifetime during which foods could be safely eaten. The microbial nature of food fermentation or foodborne illnesses was not understood, however, until the advent of the science of microbiology in the late 19th century. The fermentation of vegetables by lactic acid bacteria (LAB) is now well understood as an effective means of preserving and ensuring the safety of foods [4,5]. LAB are being considered for use in nonfermented vegetable products as a means of ensuring safety and preventing spoilage [6–8]. Fermented and acidified vegetable products, such as sauerkraut, kimchi, olives, and cucumber pickles, not only have desirable sensory qualities, but also have an excellent safety record with no known reported cases of foodborne illness.

## 14.2 VEGETABLE MICROFLORA

The microflora on fresh fruits, grains, and vegetables can range from as low as  $10^2$  to  $10^9$  colony forming units (CFU) per gram [9,10]. On pickling cucumbers, for example, the aerobic microflora is typically between  $10^4$  to  $10^6$  CFU/ml for fresh fruit, with LAB less than  $10^1$  CFU/g [11]. In the absence of processing, degradative aerobic spoilage of plant material by mesophylic microorganisms occurs, with *Pseudomonas* spp., *Enterobacter* spp., and *Erwinia* spp. initiating the process [10]. A variety of pathogens, including *Salmonella* spp., *Shigella* spp., *Aeromonas hydrophilia*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Campylobacter*, *Listeria monocytogenes*, *Escherichia coli*, and others, may be present on fresh vegetable products [12–15]. Pathogens on fruits and vegetables may also include enteric, hepatitis, or polio viruses [16]. A variety of sources may contribute to the occurrence of pathogenic bacteria on fruit and vegetable crops, including exposure of plants to untreated manure or contaminated water, the presence of insects or birds, personal hygiene practices of farm workers, postharvest washing or hydrocooling water, and conditions of storage during distribution [12,14]. A study comparing the use of organic fertilizer (composted manure) and inorganic fertilizer from farms in Minnesota showed significantly higher coliform counts on the organically grown vegetables [17]. However, in this and related studies [18,19], pathogens, including *E. coli* O157:H7, were not detected.

Removal of pathogenic and spoilage bacteria from fruits and vegetables has proved difficult. Surface adherence of bacteria (Figure 14.1) may serve



**FIGURE 14.1** Attachment of pathogenic bacteria to cucumber fruit. Adhesion of bacteria to the surfaces of pickling cucumbers (Calypso variety) with wax: (A) *Staphylococcus aureus*; (B) *Lactobacillus plantarum*; (C) *Listeria monocytogenes*; (D) *Salmonella typhimurium*; (E) *Enterobacter aerogenes* ATCC 13048. Bar 5, 10mm. (From Reina, L.D., Fleming, H.P., and Breidt, F., *J. Food Prot.*, 65, 1881–1887, 2002.)

to enhance survival of bacteria during washing or sanitizing treatments. Bacterial cell surface charge and hydrophobicity measurements have been found to correlate with the attachment of cells to surfaces of cantaloupes and cucumbers [20,21]. Dewaxing cucumber fruit led to increased adhesion of *L. monocytogenes* and decreased adhesion for other bacteria with higher relative surface hydrophobicity, including salmonella, lactobacilli, and

staphylococci [20]. Biofilms of bacteria may be more resistant to sanitizing agents and organic acid treatments than free or planktonic cells [22–24]. It is likely that the vast majority of microorganisms in food processing environments occur in multispecies or multistrain biofilms on food or equipment surfaces [25,26].

#### 14.2.1 WASHING PROCEDURES

Washing procedures with water or chemical sanitizers typically result in only a 1 to 2 log<sub>10</sub> decrease in bacterial cell numbers [24]. Hydrocooling procedures used for some fruits immediately after harvest may even serve to increase internalization of bacteria due to the vacuum created as internal gases in fruits and vegetables contract with the reduction in temperature [27,28]. Bacteria may be protected in inaccessible locations on fruits and vegetables, such as the cores and calyx of apples [29]. Attachment to wounded regions or entry into the interior of fruits and vegetables through wounded regions or stomata, pores, or channels may occur [20,30–32].

The packaging and storage conditions for minimally processed vegetable products, including the use of modified atmosphere packaging, may significantly alter microbial ecology. The extended shelf life of some minimally processed vegetable products may result in an undesirable “safety index,” a concept developed to define the risks associated with modified atmosphere packaged foods [33]. This safety index is defined as the ratio of spoilage to pathogenic bacteria in foods, measured as the relative cell concentrations of these organisms. It has been argued, however, that the primary effect of modified atmosphere packaging in extending the sensory quality of vegetable products may be to decrease the metabolic activity of the vegetable material [34]. In a model system, it was found that growth rates for *L. monocytogenes*, *A. hydrophilia*, and *Bacillus cereus* may be reduced by modified atmosphere conditions, but final cell density was not affected [35]. One major source of concern is that *Clostridium botulinum* spores have been isolated from a variety of vegetables, and this organism may, under the right conditions of temperature, pH, and atmosphere, grow and produce toxin in minimally processed vegetable products if the O<sub>2</sub> concentrations drop to 1% or lower [10].

#### 14.2.2 BIOCONTROL IN MINIMALLY PROCESSED VEGETABLE PRODUCTS

The survival and growth of bacteria on vegetable products can depend on the competitive microflora present and the environmental conditions and processing treatments [15,36]. The use of competitive microflora to enhance the safety of minimally processed foods, including vegetable products, has been proposed by a number of authors [5,37–39]. LAB have been nominated for this role, partly because of their GRAS (generally regarded as safe) status and their common usage in food fermentations. Application of this approach for minimally processed fruit and vegetable products has led to mixed results. Vescovo

and co-workers isolated LAB from salad vegetables and, subsequently, re-inoculated the vegetables with both the biocontrol cultures and selected food pathogens, including aeromonas, salmonella, staphylococcus, and listeria species [6,40]. The added LAB cultures were found to reduce or prevent the growth of microbial pathogens. Conversely, a *Lactobacillus delbruckii lactis* strain, known to inhibit *E. coli* on chicken skin due to the production of hydrogen peroxide, did not alter the survival of *E. coli* O157:H7 on fresh-cut vegetables, possibly due to the presence of catalase on the plant surfaces [8].

Competition from aerobic microflora isolated from fresh vegetables, other than LAB, including yeasts, *Bacillus* spp. and *Pseudomonas* spp., can influence the survival and growth of microbial food pathogens. *Pseudomonas* spp. have been shown to enhance [41], inhibit [42–44], or have no effect [45] on the growth of *L. monocytogenes* in fruits and vegetables. A variety of pseudomonas and aeromonas isolates from fresh vegetables were found to confer inhibitory activity against *E. coli*, salmonella, listeria, and staphylococcus strains using an agar diffusion assay [46]. Competition studies have shown iron sequestration by siderophores may influence the competition between pseudomonads and *L. monocytogenes* [42,47], although some *Listeria* spp. may be able to use exogenous siderophores as an iron source [48]. Buchanan and Bagi [49] demonstrated that the effects of salt and temperature can control the outcome of competitive growth of a *L. monocytogenes* Scott A and a *Pseudomonas fluorescens* culture that was screened for the inability to produce siderophores or bacteriocins. In a study by Del Campo *et al.* [45], competition for nutrients between a Scott A strain of *L. monocytogenes* and saprophytic bacteria from green endive was investigated. Enterobacteriaceae and pseudomonas were grown in competition with *L. monocytogenes* in minimal media and media supplemented with yeast extract. In this case, enterobacteriaceae but not pseudomonads species were effective in reducing the growth of the *L. monocytogenes* culture. Because culture filtrates from enterobacteriaceae were found to have no inhibitory effects in broth supplemented with yeast extract, the data indicated that competition for nutrients (not end product inhibition) was responsible for the inhibitory effect [45].

These studies illustrate the complexity of microbial interactions in and on fruit and vegetable products. Varying environmental conditions may include changes in the availability of nutrients, salt concentration, temperature, atmosphere, pH, and others. While further research is clearly needed, the use of protective cultures should only be considered as a supplement to good manufacturing practice, not as a substitute for the proper handling and packaging of vegetable products [5]. The use of biocontrol cultures may, therefore, be considered to enhance existing hurdle technology to prevent the growth of pathogens in foods. The hurdle concept [50] advocates the use of multiple preservative factors to prevent the growth of pathogens. In fresh fruit and vegetable products, the main factors affecting the growth of the indigenous bacterial populations are sanitation, modified atmosphere packaging, and refrigeration, as well as the competitive interactions of bacteria.

Bacteria cultures selected for use in biocontrol applications should ideally be isolated from the products for which they are intended to be used [39]. Development of successful biocontrol strategies for fresh fruit and vegetable products may include the following steps: (1) isolation of potential biocontrol LAB from the product for which they are intended to be used; (2) reduction of the total microflora in and on the vegetable product by one of a variety of procedures, including heat, washing using chemical sanitizers, irradiation, or others; (3) addition of the biocontrol culture to achieve an appropriate initial population, as determined experimentally; (4) storage of the product under refrigeration temperatures [39]. The shelf life of the product would then be dictated by the growth of the biocontrol culture, but, to be successful, the growth rates of a biocontrol culture presumably should be faster than that of the target pathogens. While rapid growth and production of inhibitory metabolites may be desirable from a safety standpoint, this may be a liability as far as the quality of the product is concerned. Breidt and Fleming [7] investigated the kinetics of acid production and inhibition of *L. monocytogenes* by *L. lactis* using a mathematical modeling approach [7]. It was observed that the growth and death of the *L. monocytogenes* culture could only be accurately predicted by the model if pH was assumed to be the limiting variable, rather than acid concentration, with cessation of growth around pH 4.6. Further studies to characterize the kinetics of bacterial competition are needed to aid in the development of biocontrol strategies.

### 14.3 FERMENTED VEGETABLES

Under the anaerobic conditions found with brined vegetables, rapid fermentation by LAB and yeasts occurs, resulting in the destruction of most other microflora, usually within a few days of the onset of fermentation [51]. In the U.S., cucumber pickles and sauerkraut represent the majority of fermented vegetable products. For pickles, fermentation was the primary means of preservation until the 1940s, when direct acidification and pasteurization of cucumber pickles was introduced (reviewed by Fleming *et al.* [51]). Currently, fermented cucumbers represent roughly 30% of commercial production of pickles, mostly for institutional markets (hamburger dill slices), with the majority of the retail market being nonfermented acidified pickles which are pasteurized to destroy vegetative microflora.

Vegetable fermentations typically begin with heterofermentative LAB, such as *Leuconostoc mesenteroides* and end with the most acid-resistant homofermentative LAB, usually *Lactobacillus plantarum* [1,52,53]. *Lactobacillus plantarum* is able to tolerate a lower internal pH than other LAB, and this feature may allow it to predominate in the terminal stages of most vegetable fermentations [54]. During the fermentation of cucumbers and cabbage, hexose sugars, including glucose and fructose, are typically converted to lactic acid by homofermentative LAB via the Embden–Myerhof–Parnas pathway, while the heterofermentative LAB will produce a combination of

lactic acid and acetic acid or ethanol, along with CO<sub>2</sub> via the phosphoketolase pathway [55]. When fructose is present, LAB can use this sugar as an electron acceptor, producing mannitol, which subsequently can be converted anaerobically to lactic acid with an appropriate electron acceptor [56]. In cucumber fermentation where malate is present, *L. plantarum* and other LAB have been found to carry out a decarboxylation of malate to produce lactic acid and CO<sub>2</sub> [57]. This one-step reaction occurs via malolactic enzyme, and is analogous to the amino acid decarboxylation reactions described below [119]. During the reaction, a proton is taken up from the surrounding medium, which helps to buffer cellular pH and causes the pH in the surrounding medium to rise.

#### 14.3.1 FERMENTATION CHEMISTRY

In the U.S., commercial cucumber fermentations are typically carried out with 5 to 6% NaCl, while cabbage fermentations are carried out with 2 to 3% NaCl [51]. During the growth of LAB in vegetable fermentations, a variety of antimicrobial metabolic end products are produced, including organic acids, peroxides, amines, thiols, bacteriocins, and other enzymes and compounds [1,4,5,58–61]. These inhibitory compounds begin to accumulate in the initial stages of fermentation. A combination of several factors, including organic acids from the fermentation (up to 2 to 3% organic acids may be produced), complete fermentation of available sugar, terminal pH values around 3 to 3.5, and salt, can serve to destroy most vegetative bacterial cells, including human pathogens. Desirable textural and nutritional properties of the fermented vegetables may be maintained during storage in the fermentation brine for extended periods of time (a year or more) without refrigeration.

### 14.4 ACIDIFIED VEGETABLES

For nonfermented, acidified vegetable products, acetic acid is commonly used as an acidulant. At a concentration of 3.6% or greater, acetic acid-acidified foods can be preserved without the addition of other antimicrobial agents or use of heat treatments [62,63]. For pickled pepper products, acidification with 2% acetic acid to pH values around 3.2 was found to prevent microbial growth for 6 months or more [64]. In general, preservation by organic acids alone results in products that can only be consumed in small amounts, as condiments, or as ingredients in other foods. Many acidified vegetable products contain between 0.5 and 2% acetic acid and are pasteurized to prevent spoilage, as well as to ensure safety. For nonfermented pickled vegetables, the combination of heat treatments, acid, and sugar concentration (for sweet pickles) serves to prevent microbial growth. Fresh-pack cucumber pickle products typically contain between 0.5 and 1% acetic acid. A recommended pasteurization procedure consists of heating to an internal temperature to 74°C for 15 minutes [65].

Both acidified and fermented vegetable products have enjoyed an excellent safety record with few or no reported cases of foodborne disease resulting from consumption of these products. Recently, however, there have been reports of disease outbreaks in juice products with pH values below 4.0, in the same range as many fermented and acidified vegetable products. *Escherichia coli* O157:H7 and salmonella serotypes have caused serious illness and death from the consumption of apple cider and orange juice [66,67]. These disease outbreaks have raised questions about the safety of acidified and fermented vegetable products. While pathogenic microorganisms have not been found to grow in these products due to the low pH (typically below 4.0), these microorganisms may adapt to acid conditions and survive for extended periods [68]. Acid types and concentrations vary considerably for acidified foods. Factors affecting acid inhibition of microbial pathogens include the pH of the product, as well as specific effects of the acid or acid anion on cellular enzymes or membranes, and the ability of bacteria to transport protons and organic acids out of the cell interior [69–72].

#### 14.4.1 DEFINITIONS AND REGULATIONS FOR ACID AND ACIDIFIED FOODS

Acid foods are defined in the U.S. Code of Federal Regulations (21 CFR part 114) as foods that have a natural pH value at or below 4.6. These foods include fermented vegetables; vegetable fermentation is considered a “field process” and typically results in a product with a final pH below 4.6. A pH value of 4.6 is used in the definition of acid foods because this is a limiting pH at or below which *C. botulinum* spore outgrowth and neurotoxin production is prevented [73]. Foods with pH values above 4.6 are defined as low-acid foods, and, when packaged in hermetically sealed containers, must be made commercially sterile as defined in 21 CFR part 113. Acidified foods are defined in 21 CFR part 114 as foods to which acid or acid food ingredients have been added that have a water activity ( $a_w$ ) greater than 0.85 and have a finished equilibrium pH value at or below 4.6. The regulation requires producers of acidified foods to verify that the final equilibrium pH is maintained at or below 4.6 to ensure safety. This regulation governing acidified foods in the U.S. was promulgated by the U.S. Food and Drug Administration (FDA) in 1979. At that time, vegetative pathogenic microorganisms were not considered to be a significant risk for acidified or fermented food products. Included in the regulation, however, is the requirement for a heat process “to the extent that is sufficient” to destroy vegetative cells of microorganisms of public health significance or those of nonhealth significance capable of reproducing in the product. The regulations governing acidified foods are, therefore, based primarily on the pH needed to prevent botulism, and do not include any specification about the type or concentration of acid needed to meet the pH requirement.

In a study of beef carcass wash water, a treatment with 0.2% (33.3 mM) acetic acid and a pH of approximately 3.7 showed that an *E. coli* O157:H7

strain survived for up to 14 days at 15°C, while cell numbers dropped about 4 log cycles [74]. In that study, competitive microflora were also present and could have influenced the survival of the *E. coli* strains. A statistical analysis of several published studies showed that, under typical storage conditions for apple cider (which typically has a pH value less than 4.0 and contains malic acid), the acid conditions alone were not sufficient to ensure a 5 log reduction in the cell numbers of *E. coli* [75]. From these and other studies [68,75–79], it is clear that the potential for *E. coli* to survive for extended periods in acidified vegetable products with a pH below 4 clearly exists, and pasteurization for some acidified food products may be needed to ensure safety.

#### 14.4.2 PATHOGENIC BACTERIA

After recent outbreaks of *E. coli* O157:H7 in apple cider and salmonella in orange juice [66,67], the FDA in 2001 proposed that all new process filings (which are required for the production of acidified foods) should include a heating or pasteurization step. Of primary concern was *E. coli* O157:H7 because of its low infectious dose and lethal sequelae which can result from infection [80,81]. *Escherichia coli* and other food pathogens have been shown to have inducible acid resistance mechanisms [76,82–85]. If only pH is considered, acid-resistant pathogens might, therefore, pose a potential threat to acidified foods. It is likely that the organic acids present in these products have contributed to their excellent safety record because some acidified products have been produced safely for many years without heat treatments [84], although quantitative measurements of the independent effects of organic acids and pH on the killing of pathogens in these products are lacking. In response to the pathogen outbreaks in juice products, 21 CFR part 120 was promulgated in 2001. This regulation mandated a HACCP (hazard analysis critical control point) system with a processing step designed to deliver the equivalent of a 5 log reduction in target pathogen populations in juices. Typically, a heat pasteurization process is used, based on thermal destruction time data for inactivation of *E. coli* O157, which was found to be the most heat- and acid-resistant pathogen in fruit juices [86]. In recent experiments (Breidt, unpublished data), the thermal resistance of *E. coli* O157:H7 and *L. monocytogenes* was found to be identical under the conditions typical of acidified pickle products, and salmonella strains were significantly less heat-resistant. Similarly, salmonella was found to be less heat resistant than *L. monocytogenes* or *E. coli* O157:H7 in fruit juices [86]. For the variety of acidified vegetable products currently available, the time and temperature needed to ensure a 5 log or greater reduction (although a 5 log reduction is not currently mandated by existing federal regulations) in numbers of microbial pathogens will depend on the type and concentration of organic acid present, the composition of the brine or suspending medium during heating, heat resistance of the microorganisms, and other factors.

Some pickled pepper products with high concentrations of acetic acid (greater than 2% acetic acid) and pH values around 3.1 to 3.3 may not need

a heat treatment to ensure the destruction of acid-resistant pathogens because sufficient acid is present. In a study of firmness retention with unpasteurized pickled peppers, which typically have pH values around 3.1 to 3.3, and cucumbers, using 2 to 5% acetic acid, microbiological stability was achieved for a 6-month period [64] for all products tested. A heat process is typically not used for these pickled peppers because sliced peppers are susceptible to softening during pasteurization. Historically, pasteurization treatments were designed to prevent spoilage by LAB in brined vegetables and inactivate softening enzymes. Currently, most commercial acidified vegetable products with pH values between 3.3 and 4.1 are produced using a pasteurization process to prevent spoilage. In addition, low water activity and preservatives can reduce the amount of acetic acid needed for preservation. A preservation prediction chart showing the effects of acid and sugar in preventing the growth of spoilage yeasts in sweet pickles was developed in the 1950s [62]. The acid concentrations that will ensure the death of microbial pathogens for many acidified foods remain to be determined.

#### 14.5 ORGANIC ACIDS AND DESTRUCTION OF PATHOGENS

Organic acid preservatives have widespread application for preventing food spoilage and contribute to the manufacture of safe food products [87-89]. The survival or death of pathogenic bacteria in acid and acidified foods has been investigated in a variety of products, including apple cider [68,90-93], mayonnaise, dressings and condiments [76,84,94,95], and fermented meats [96-98]. The mechanism of action of organic acids is commonly attributed to acidification of the cytoplasm of target cells, but also to intracellular accumulation of anions [99]. The protonated form of weak acid preservatives may diffuse across microbial cell membranes and then dissociate in the cell cytoplasm, releasing protons and anions because the intracellular pH must be maintained at a higher value than the external environment. Internal acid anion concentrations may correlate with the cessation of growth. Goncalves *et al.* [100] found that the specific growth rate of *L. rhamnosus* approached zero at approximately 4 molar lactate (anion), with pH values between 5.0 and 6.8. In vegetable fermentations, *L. plantarum* was found to tolerate a lower internal pH than other LAB and, therefore, would have lower acid anion concentrations.

Data on the relative effects of various organic acids and preservatives on the inhibition of microbial pathogens are often conflicting in the scientific literature. For example, Young and Foegeding [101] showed that with equal initial pH values in brain-heart infusion broth ranging from 4.7 to 6.0 and on an equimolar basis, the order of effectiveness in inhibiting the growth of *L. monocytogenes* for three weak organic acids was acetic > lactic > citric. However, when based on initial undissociated acid concentrations, the order

was reversed. Ostling and Lindgren [102] determined MIC values for the inhibition of *L. monocytogenes* by lactic, acetic, and formic acids. They found lactic acid was the most inhibitory over a range of pH values from 4.2 to 5.4, with an MIC value of less than 4 mM (protonated acid) for aerobic growth and less than 1 mM for anaerobic growth. They used cells grown in glucose-containing nutrient broth and reported MIC values for the protonated acid as no growth for 5 days. Similar MIC values for the inhibition of growth of *Listeria innocua* were reported as 217 mM sodium lactate at pH 5.5, corresponding to about 5 mM protonated lactic acid [103], and 4.7 mM protonated lactic acid in another study [7]. Buchanan and Edelson [104] looked at the effects of a variety of organic acids on *E. coli* O157:H7 at a fixed concentration of 0.5% and pH 3.0. They examined the effects of citric, malic, lactic, and acetic acids on the viability of this organism; variables included growth phase and the presence or absence of glucose in the growth medium. The ability of the cells to survive when held in an acid solution varied in a strain-dependent manner. For nine strains, lactic acid was the most effective at reducing the viable cell population, and HCl was the least effective [104]. This study clearly demonstrated that strain-to-strain variability, as well as growth conditions (induction of acid resistance by growth in the presence of glucose), must be considered in studies of the effects of weak acids and low pH on *E. coli*.

The effect of acetate on *E. coli* O157:H7 was investigated by Diez-Gonzalez and Russell [70,105]. They investigated intracellular pH, acetate anion accumulation, glucose consumption rates, and intracellular potassium concentrations. They showed that *E. coli* O157:H7 cells could divide in the presence of about twice as much intracellular acetate anion (80 vs. 160 mM) as *E. coli* K12. In cells grown at a constant pH of 5.9, *E. coli* O157:H7 lowered its internal pH to close to 6.0 and accumulated significantly less anion when compared to *E. coli* K12, which kept a constant internal pH of 7. To test the theory that acetate acted as an uncoupler (i.e., ferrying protons across the *E. coli* cell membrane), Diez-Gonzales and Russell [105] compared the effects of acetate and the uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (CCCP). They found that the effects of acetate and CCCP differed, specifically in reference to intracellular ATP concentrations of *E. coli* O157:H7. Acetate had very little or no effect on intracellular ATP, even at concentrations greater than 200 mM, while about 10 mM CCCP reduced intracellular ATP concentrations by about 50%. These and similar experiments showed that acetate was having effects other than simply acting as an uncoupler on *E. coli* O157:H7. It was also apparent from these studies that *E. coli* O157:H7 and *E. coli* K12 regulate internal pH differently.

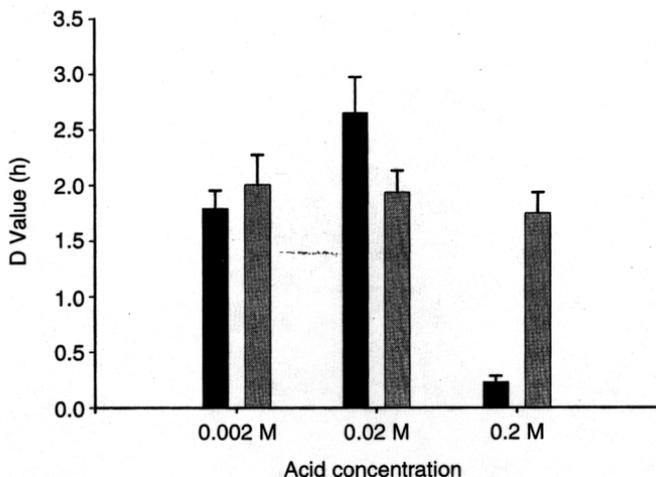
#### 14.5.1 SPECIFIC EFFECTS OF ACIDS

A complicating factor in the study of acid inhibition of microorganisms is that protonated acids and pH (which are interdependent variables linked by the Henderson-Hasselbalch equation for common conditions) may both

independently inhibit growth [106], or they may interact. Tienungoon *et al.* [107] modeled the probability of growth of *L. monocytogenes* using a logistic regression procedure with a function relating specific growth rate to temperature, water activity, pH, lactic acid, and lactate ion concentrations. They found that their equation accurately predicted conditions allowing growth using their own laboratory data, as well as examples from the literature [107]. They presented no data, however, on the growth/no growth interface for *L. monocytogenes*, based on protonated acid and pH; they cited a lack of independent data sets available in the literature.

To address the safety concerns of the FDA and the acidified foods industry, Breidt *et al.* [79] investigated the specific effects of organic acids independent of pH. This study was made possible by using gluconic acid as a noninhibitory low pH buffer. While gluconic acid has been investigated for use as an antimicrobial agent in meats [108,109], it has not proven to be as effective as acetic or lactic acid. The antimicrobial effects of gluconic acid solutions were found to be primarily due to pH rather than to specific effects of the acid itself [79]. No change in the log reduction time (*D* value) was observed over a 100-fold range of gluconic acid concentrations (Figure 14.2).

By using gluconic acid as a noninhibitory buffer, the inhibitory effects of pH alone were compared with the combined effects of pH and acetic acid, while holding ionic strength, temperature, and other variables constant [79]. As expected, survival of *E. coli* O157:H7 was reduced with the addition of acetic acid at concentrations typically found in acidified foods, and with



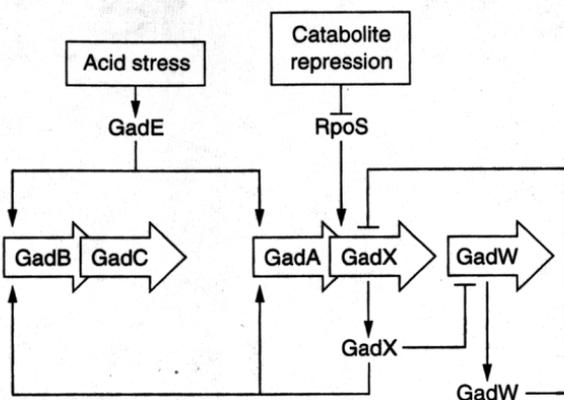
**FIGURE 14.2** Effects of acetic and gluconic acid on the destruction of *Escherichia coli* O157:H7 (cocktail of strains). The log reduction times (*D* values) for acetic (black bars) and gluconic (gray bars) acids at 0.002, 0.02, and 0.2 M concentrations in water at 25°C and pH 3.1. The error bars indicate the upper 95% confidence intervals. No statistically significant difference was detected for the gluconic acid *D* values ( $p > 0.05$ ). (From Breidt, F., Hayes, J.S., and McFeeters, R.F., *J. Food Prot.*, 67, 12–18, 2004.)

increasing temperature for a given pH and ionic strength. Gluconic acid may have wider application as a noninhibitory buffer for similar experiments with other organic acids.

In addition to the proposed mechanisms for the effects of weak acids on microorganisms mentioned above (acidification of the cytoplasm and intracellular accumulation of anion), the effectiveness of these compounds may be modulated by additional factors. Examples of these include: specific effects of the acid or acid anion on cellular enzymes or membranes, the internal buffering capacity of cells, proton pumping at the expense of cellular ATP, and facilitated transport of acid molecules, among others. To investigate the relative importance of these effects for the inhibition of yeasts with sorbic acid, Stratford and Anslow [71] compared the effects of acids with similar pK values (acetic acid, pK = 4.76; sorbic acid, pK = 4.74) and used structural analogs of sorbic acid that have similar lipophilic properties. Interestingly, a variety of structural analogs, including aldehydes and alcohols, had similar MIC values to sorbic acid (which was 3 mM) for the inhibition of a *Saccharomyces cerevisiae* strain, and a survey of yeast strains showed sorbate resistance correlated with ethanol tolerance [71]; they proposed that sorbic acid acted specifically on yeast membranes. Krebs *et al.* [69] examined glycolysis intermediates in yeast cells treated with benzoate and showed an increase in the intracellular concentrations of glucose 6-phosphate and fructose 6-phosphate, while fructose 1,6-bisphosphate and triose phosphate concentrations were reduced. The specific inhibition of phosphofructokinase, however, could be attributed to a lack of ATP required for the function of this enzyme [69]. Alakomi *et al.* [72] showed that lactic acid had a specific membrane effect on Gram-negative bacteria. They found that lactic acid could sensitize *E. coli* O157:H7, pseudomonas, and salmonella to lytic agents such as detergents and lysozyme, presumably by disrupting the outer membrane. Lactic acid (5 mM, pH 3.5) was found to have a greater ability to liberate lipopolysaccharides from the outer membrane of *Salmonella* serovar Typhimurium than a 1 mM EDTA solution under similar conditions [72]. The effect of sorbate on the germination of *C. botulinum* spores was investigated [110]. This study indicated that sorbate inhibited spore outgrowth by disrupting the cell membrane after the start of germination. In addition to membrane effects, organic acids may have a variety of other possibly minor effects on the inhibition of microorganisms. A review by Shelef [88] cites additional effects of lactate salts on the inhibition of microorganisms. These effects include lowering water activity, chelating iron, and the inhibition of lactate dehydrogenase.

#### 14.5.2 GENETIC REGULATION OF ACID RESISTANCE

Induction of acid resistance genes in *E. coli* can be accomplished by growing cells statically to stationary phase in media containing an excess of glucose, resulting in a pH of about 5.5 [104]. The acid resistance systems in *E. coli* and other pathogenic bacteria are also subject to crosstalk, or regulation by



**FIGURE 14.3** Regulatory network governing *gadA/BC* expression and glutamate-dependent acid resistance. (Adapted from Ma, Z., Gong, S., Richard, H., Tucker, D.L., Conway, T., and Foster, J.W., *Mol. Microbiol.*, 49, 1309–1320, 2003. With permission.)

stresses other than acid [111–113]. A clear example of this crosstalk is exhibited by *E. coli* O157:H7 in which acid resistance is induced in response to heat stress [114], and heat tolerance is induced in response to acid stress [115]. Crosstalk can be mediated by two-component (sensor–effector) regulatory systems used by bacteria, where sensor kinases phosphorylate noncognate regulatory proteins [116,117]. The precise nature of the signal(s) recognized by the cells for controlling acid resistance remains unclear, although considerable research has been carried out investigating genes induced by exposure to acid and other stresses.

*Escherichia coli* has several known inducible acid resistance systems that allow the organism to respond to the presence of organic acids and low pH in the environment [118,119]. The most well studied system uses decarboxylation of glutamic acid as a means for modulating internal pH [120]. The system consists of two inducible proteins, glutamate decarboxylase (GadA and an isozyme GadB), and an antiport transporter (GadC) for glutamate and the decarboxylated product of glutamate, gamma-aminobutyric acid. The genetic regulation of this system has been found to be quite complex (Figure 14.3). RpoS, a sigma factor produced in response to stress, mediates expression of two regulatory proteins, GadW and GadX, that control expression of the decarboxylase and transport proteins [121]. In addition, there is a two-component regulatory system that responds to (unidentified) external acid signals and can cause expression of the proteins of the glutamate decarboxylase system through the action of another regulatory protein, GadE [118,122]. The other acid resistance systems include arginine and lysine decarboxylase systems [119] similar to the glutamic acid system and a glucose-repressed, acid-induced system also controlled by RpoS which does not require external amino acids [83]. Inducible acid resistance mechanisms have been observed in a variety of other food pathogens, including *Salmonella*

spp., *L. monocytogenes*, *Shigella flexneri*, *B. cereus*, and others [111,123–126]. As the details of gene regulation of acid resistance of microbial food pathogens become clearer, strategies may be devised to help prevent the survival of these pathogens in acidified foods.

## 14.6 CONCLUSIONS

Preservation of vegetables by fermentation is one of the earliest and most widespread technologies developed by humans. Fermented and acidified vegetable products are produced and consumed in every culture and society around the world, usually based on traditional processing methods. This is because the products produced are safe even in the absence of refrigerated storage, due to the inhibitory metabolites, primarily organic acids produced by lactic acid bacteria. The lactic acid bacteria may also be used to control spoilage of fresh vegetable products. The factors influencing microbial competition during fermentation or spoilage of fresh vegetable products have proved to be difficult to understand, but biocontrol strategies have the potential to ensure the safety and control the microbial ecology of food spoilage for many types of nonfermented foods. Significant challenges remain, however, in understanding the mode of action of organic acids in killing bacterial pathogens, and how those pathogens respond and adapt to acid challenge.

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**MICROBIOLOGY OF**

# **Fruits and Vegetables**

Edited by

**Gerald M. Sapers**

**James R. Gorny**

**Ahmed E. Yousef**



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