Chapter 8

THE LACTOBACILLI, PEDIOCOCCI, AND LEUCONOSTOCS: VEGETABLE PRODUCTS*

H. P. Fleming, R. F. McFeeters, and M. A. Daeschel

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I. INTRODUCTION

The use of pure cultures of lactic acid bacteria for fermentation of cucumbers, cabbage, olives, and other produce has been explored for several decades with varying degrees of success. However, pure cultures presently are used only on a limited commercial scale for these commodities. The lack of widespread commercial use is a consequence of several factors, including the following.

1. Cucumbers, olives, and cabbage undergo natural fermentation by lactic acid bacteria if the product is properly handled and held at salt concentrations that have been established for each particular product.
2. Brine from one natural fermentation can be used to inoculate other containers.
3. Heat is the only effective and acceptable means known for ridding vegetables of the natural lactic acid bacteria. Heating is expensive, and it changes the flavor and other characteristics of the product.
4. The fermentation vessels and general handling procedures to date are not compatible with pure culture fermentations.
5. No sufficiently unique strains of lactic acid bacteria have been revealed as yet that have mandated their use as starter cultures.

While each of the above factors may be subject to argument, overall they probably account for the low commercial usage of cultures for vegetables. There are indications, however, that increased use of pure cultures of lactic acid bacteria may occur within the foreseeable future. Changes in brining technology, acceptance of anaerobic fermentation tanks that are more compatible for pure culture usage, and selection and/or modification of lactic acid bacteria with unique and valuable properties loom as possible reasons for use of pure cultures in certain vegetable fermentations.

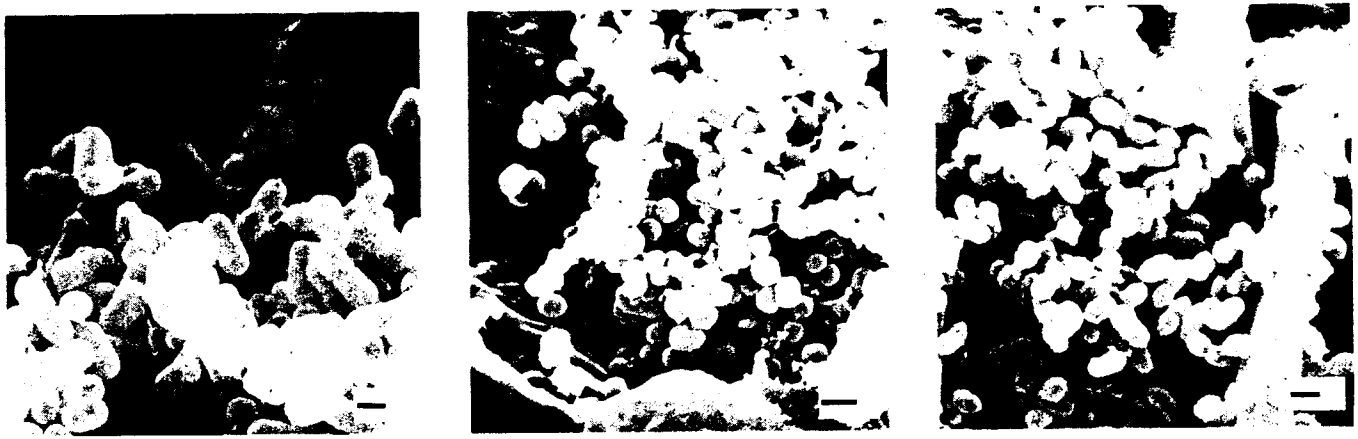
II. SPECIES INVOLVED

Four species of lactic acid bacteria historically have been associated with the natural fermentation of sauerkraut: *Leuconostoc mesenteroides, Pediococcus cerevisiae, Lactobacillus brevis,* and *L. plantarum*[1] (Figure 1). The latter three species are also associated with fermentation of cucumbers[2] and olives.[3] *L. mesenteroides,* which is very important in the initial stages of fermentation of sauerkraut (approximately 2.25% NaCl), has not been considered as playing an important role in fermentation of cucumbers (5 to 7% NaCl) due to its relatively low salt tolerance. Characteristics of these four species related to vegetable fermentations are summarized in Table 1. These characteristics are largely consistent with *Bergey's Manual of Determinative Bacteriology*[4] (8th edition). The exception are the *Pediococcus* species, which have been classified variously over the past 20 years. *P. cerevisiae* is no longer recognized as a valid species name.[5] Plant pediococci referred to as *P. cerevisiae* by Breed et al.[6] are now called *P. pentosaceus.* For a review of the nomenclature problem, see the review by Garvie.[7] In this chapter, the name *P. pentosaceus* will be used in referring to pediococci present during vegetable fermentations.

III. IMPORTANT METABOLIC PATHWAYS FOR VEGETABLE FERMENTATION CULTURES

A. Fermentation of Sugars

The carbohydrates available for lactic acid fermentation of most fruits and vegetables consist almost exclusively of glucose, fructose, and sucrose. *P. dextrinicus*[8] utilizes



A B C

FIGURE 1. Lactic acid bacteria associated with vegetable fermentations. (A) *Lactobacillus plantarum*, (B) *Pediococcus pentosaceus*, (*P. cerevisiae*), (C) *Leuconostoc mesenteroides*. Bars = 1 μm.

Table 1
RELEVANT CHARACTERISTICS OF LACTIC ACID BACTERIA ASSOCIATED WITH VEGETABLE FERMENTATION

Property	Species				Ref.
	L. plantarum	*L. brevis*	*P. pentosaceus*	*L. mesenteroides*	
Morphology	Short to medium rods, usually singly	Short rods, occurring singly or in short chains	Cocci occurring singly, in pairs, and in tetrads	Cocci or coccobacilli, usually in pairs	4, 105
Optimum temp.	30—35	30	35	20—30	6
Growth at 45°C	No	No	Yes	No	7, 103, 104
Growth in 8% NaCl	Yes	No	Yes	No	4, 7, 103
Lactic acid produced from glucose	DL	DL	DL	D	4, 105
Glucose metabolism	Homofermenter	Heterofermenter	Homofermenter	Heterofermenter	4, 105
Final pH (% acidity)* in					
Cabbage juice	3.5 (1.04)	3.9 (1.06)	3.5 (0.90)	3.9 (1.04)	102
Cucumbers	3.2 (0.91)	3.7 (0.54)	3.4 (0.63)	— (0.23)	2, 95
Differentiating biochemical characteristics			Variable		4, 6, 7, 103, 104, 106
Arginine hydrolysis	−	+		−	
Dextran from sucrose	−	−	−	+	
Acid from					
Cellobiose	+	−	+	+	
Sorbitol	+	−	−	−	

* Acidity expressed as lactic acid.

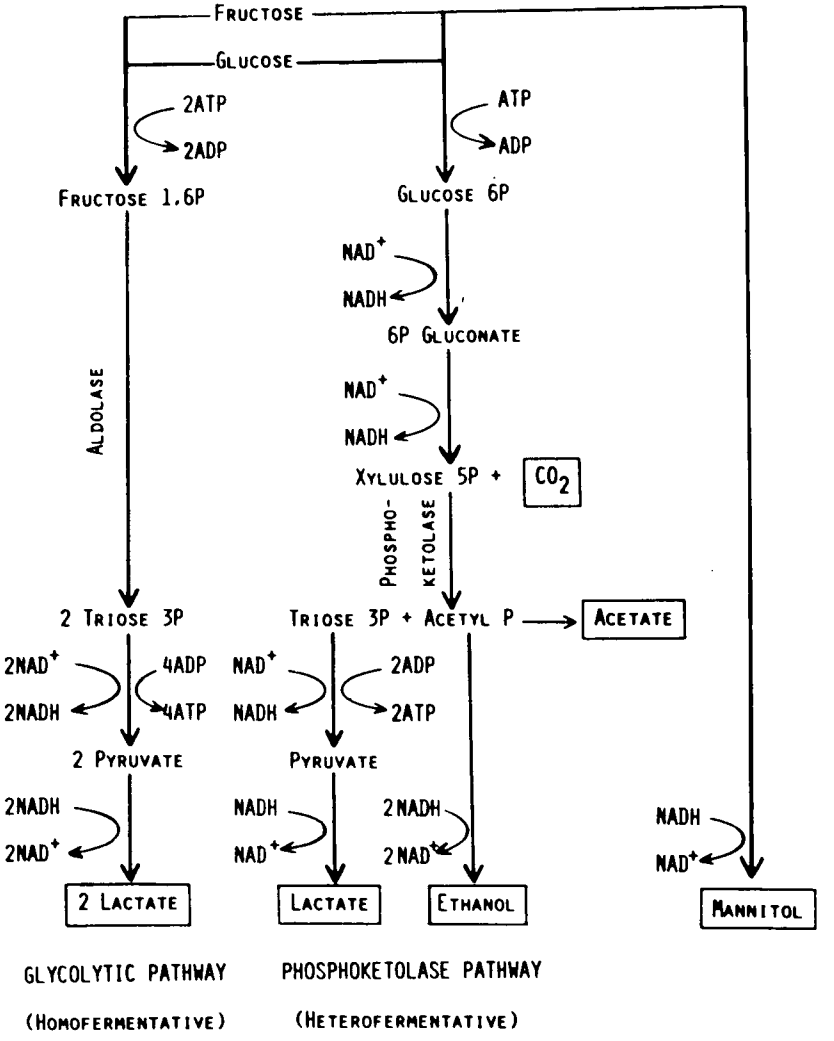


FIGURE 2. Major pathways for sugar fermentation by lactic acid bacteria.

starch, but this bacterium has not been implicated in vegetable fermentations. Free pentoses are not present in vegetables in sufficient amounts to be quantitatively significant in a fermentation. Mannitol may be a significant fermentation substrate in some circumstances. First, it is formed in the heterofermentative phase of sauerkraut fermentation. It then may be a substrate during the homofermentative phase of the fermentation.[1] Second, mannitol is present in substantial amounts in some plants. The most notable case is the common commercial mushroom in which it is the major carbohydrate present.

Lactic acid bacteria involved in vegetable fermentations ferment sugars by two important pathways. Homofermentative lactobacilli, such as *L. plantarum* and pediococci, metabolize hexoses by the glycolytic pathway (Figure 2) to produce primarily lactic acid. A net yield of 2 mol of ATP per mol of hexose fermented is achieved. Electron balance is attained by oxidizing the NADH produced in the transformation of triose phosphate to pyruvate back to NAD by the reduction of pyruvate to lactate. Heterofermentative lactic bacteria (i.e., *L. mesenteroides, L. brevis*) convert glucose

to 1 mol each of lactic acid, acetic acid or ethanol, and CO_2 (Figure 2). A net of only 1 mol of ATP is produced per hexose in the 6-phosphogluconate pathway. This pathway also differs from glycolysis in that 3 mol of NADH are formed per hexose. Only 1 mol of NADH is oxidized by reduction of pyruvate. If alternative electron acceptors are present, acetic acid will be a final product, otherwise acetate will be reduced to ethanol to oxidize the other 2 mol of NADH. Fructose can be fermented to the same products as glucose by the heterofermentative pathway in Figure 2, but it can also function as a preferred electron acceptor to oxidize NADH back to NAD^+. The result of this characteristic is that much of the fructose in a heterolactic acid fermentation is reduced to mannitol.

L. plantarum has been classified as a facultative homofermenter[9] because it is capable of producing glucose-6-P-dehydrogenase, 6-P-gluconate dehydrogenase,[9] and phosphoketolase.[10] It has the ability to ferment pentoses using the phosphoketolase enzyme.

There have been only limited quantitative data obtained on the formation of products in vegetable fermentations by either homo- or heterofermentative organisms. Product distributions, fermentation balances, and sugar utilization have been analyzed for *L. plantarum* and 10 heterofermentative species, including *L. mesenteroides* and *L. brevis*, in sterilized green bean juice.[11] The distribution of products was consistent with the homofermentative pathway for *L. plantarum* in that only lactic acid was obtained as a fermentation product; carbon recovery approximated 100%. *L. mesenteroides* and *L. brevis* strains formed typical heterofermentative products. There were strain differences, but measured carbon recoveries were near 100% for *L. brevis* strains and slightly over 100% for *L. mesenteroides*. Carbon recoveries in 7 different vegetables fermented with pH control varied from 74 to 146%,[12] indicating that some substrates or products were unaccounted for in determining the fermentation balances.

As the use of microbial cultures by vegetable fermentation industries increases, it may be important to consider the different characteristics that can be obtained by using either homo- or heterofermentative organisms. The consequences of using a homofermenter are quite simple. Hexoses are efficiently converted to 2 mol of lactic acid, which may be useful if only limited sugar is available. CO_2 is not produced by the glycolytic pathway. Heterofermentative organisms, on the other hand, allow for more complex fermentation possibilities. First, a large amount of CO_2 will be produced from sugars. Second, a more complex product distribution results since acetic acid, ethanol, and mannitol, in addition to lactic acid, are formed. The acid production from a given amount of sugar in a heterofermentation is generally less than that obtained from the same amount of sugar with a homolactic acid fermentation. If we consider, as an example, a case in which equal amounts of glucose and fructose are fermented, and the fructose is quantitatively reduced to mannitol, product formation will be as shown in Equations 1 and 2.

$$2 \text{ Glucose} \rightarrow 2 \text{ Lactic} + 1 \text{ Acetic} + 1 \text{ Ethanol} + 2\ CO_2 \quad (1)$$

$$2 \text{ Fructose} \rightarrow 2 \text{ Mannitol} \quad (2)$$

From a homolactic acid fermentation, 8 mol of acid are formed from 2 mol of glucose and 2 mol of fructose. Equations 1 and 2 show that only 3/8 as much acid is produced on a molar basis by the heterolactic acid fermentation. A result similar to this example has been observed in the fermentation of green beans by *L. cellobiosus*.[13]

B. CO_2 Production

Table 2 lists compounds that can be degraded to yield CO_2 by lactic acid bacteria.

Table 2
REACTIONS FOR CO_2 PRODUCTION FROM ORGANIC AND AMINO ACIDS BY LACTIC ACID BACTERIA

No.	Reaction	Ref.
4	Malate → lactate + CO_2	15
5	Citrate → acetate + pyruvate + CO_2	20
6	2 Tartarate → lactate + acetate + 3 CO_2	107
7	Histidine → histamine + CO_2	24
8	Tyrosine → tyramine + CO_2	108
9	Arginine → ornithine + NH_3 + CO_2	108
10	Glutamic acid → α-aminobutyric acid + CO_2	24
11	Lysine → cadaverine + CO_2	24

The degradation of malate is the most important of these reactions due to the widespread ability of lactic acid bacteria to degrade it and the common occurrence of substantial amounts of malate in fruits and vegetables.

Korkes et al.[14] were the first to demonstrate an enzyme in lactic acid bacteria that degraded malic acid with the production of CO_2. They proposed that this "malic enzyme" produced CO_2 by the following reaction:

$$\text{Malic acid} + NAD^+ \rightarrow \text{pyruvic acid} + CO_2 + NADH \quad (3)$$

It was later determined that the reaction found by Korkes et al.[14] in *L. plantarum* was actually a conversion of malic acid to lactic acid and CO_2[15] as shown by Reaction 4 (see Table 2). It was suggested that the enzyme in *L. plantarum* be called L-malate:NAD carboxylyase, or as a common name malolactic enzyme[16,17] to distinguish it from malic enzyme that catalyzes Reaction 3 (above). In Reaction 4, lactic acid is formed instead of pyruvic acid as in Reaction 3.

Caspritz and Radler[18] purified the malolactic enzyme from *L. plantarum* to homogeneity and characterized some of its properties. Surveys of malolactic activity by Caspritz and Radler[18] and malic degradation activity[11,19] in lactic acid bacteria indicate that most strains can produce the enzyme. However, one or more strains that lack the malolactic enzyme have been found in each of the four common vegetable fermentation species, except *L. brevis*.

Though not as widespread as the ability to degrade malic acid, a number of lactic acid bacteria are also capable of CO_2 production from citric acid.[20] In contrast to some earlier reports,[21,22] Keddie[23] found that several cultures of *L. plantarum* isolated from silage degraded citric acid. Citrate is converted to acetate and oxalacetate by citrate lyase. The oxalacetate is then decarboxylated to pyruvate and CO_2. The formation of diacetyl and acetoin from citrate has been the subject of many studies in streptococci, but formation of these compounds has not been investigated in vegetable fermentations.

Amino acid decarboxylation can also be a minor source of CO_2 production by lactic acid bacteria, although in vegetable fermentations it is not established. Rodwell[24] surveyed isolates of rumen lactobacilli and 26 named strains of lactobacilli. Among the named strains, one strain of *L. pentoaceticus* decarboxylated arginine and tyrosine and a strain of *L. bifidus* degraded arginine and glutamic acid. Several of the rumen isolates degraded arginine, histidine, lysine, or ornithine. Radler[17] reported that among 100 isolates of lactic acid bacteria from wines, strains of *L. brevis* could break down arginine, glutamic acid, and isoleucine. One strain of *P. pentosaceus* decarboxylated histidine to histamine.

Table 3
REACTIONS INVOLVING OXYGEN SPECIES IN LACTIC ACID BACTERIA

Reaction	Ref.
$O_2^- \cdot + 2\,H^+ + Mn^{2+} \xrightarrow[\text{nonenzymatic superoxide reduction}]{} H_2O_2 + Mn^{2+}$	34, 35
$2\,H_2O_2 \xrightarrow[Mn^{2+}\text{-pseudocatalase}]{} 2\,H_2O + O_2$	38
$\text{Pyruvate} + O_2 + PO_4^{3-} \xrightarrow[\text{pyruvate oxidase}]{} \text{acetyl phosphate} + CO_2 + H_2O_2$	42, 43
$\text{Lactate} + O_2 \xrightarrow[\text{L-lactate oxidase}]{} \text{pyruvate} + H_2O_2$	45
$\text{Lactate} + O_2 \xrightarrow[\text{NAD-independent D-lactate dehydrogenase}]{} \text{pyruvate} + H_2O_2$	45
$NADH + H^+ + O_2 \xrightarrow[\text{NADH oxidase}]{} NAD + H_2O_2$	43
$NADH + H_2O_2 + H^+ \xrightarrow[\text{NADH peroxidase}]{} NAD + 2\,H_2O$	43

C. Lactic Acid Isomers

Lactic acid bacteria can produce either L(+) or D(−) lactate stereoisomers. Garvie,[25] Stetter and Kandler,[26] and Kunath and Kandler[27] published surveys of the lactic acid isomers produced by this group of bacteria. Among the common species found in vegetable fermentations (Table 1), *L. mesenteroides* produces only the D-isomer, while *L. plantarum, L. brevis*, and *P. pentosaceus* form mixtures of the isomers. It is often said that racemic lactic acid is produced, but in fact equal amounts of the D and L isomer are rarely, if ever, formed. Often the proportion of isomers changes markedly during fermentation.[25,26] The unequal production of isomers is caused by the relative activities of specific D and L lactate dehydrogenases that reduce pyruvate to lactate.[28,29] Hiyama et al.[30] found a second mechanism for formation of mixtures of isomers. *L. sake* produced a lactate racemase that would racemize the L(+) lactic acid initially formed by the organism. However, racemase enzymes have only been found in *L. curvatus* and *L. casei* subsp. *pseudoplantarum* in addition to *L. sake*.[26]

D. Oxygen Reactions

Table 3 shows a list of reactions carried out by lactic acid bacteria that involve oxygen. Lactic acid bacteria have a limited ability to metabolize different forms of oxygen. They are unable to synthesize heme, so they lack cytochromes or heme-containing catalase except in cases in which heme is supplied in the medium.[31-33] *L. plantarum* has also been found to lack superoxide dismutase for dismutation or scavenging of superoxide anion.[34,35] Alternative protective mechanisms against oxygen toxicity have evolved. Scavenging of the superoxide radical is performed by manganous ion in several lactic acid bacteria, including *L. plantarum, P. pentosaceus*, and *L. mesenteroides*.[34,36] To preserve cell viability in late log phase cultures, *L. plantarum* produces a Mn-containing pseudocatalase.[37,38]

Lactic acid bacteria carry out a number of oxidative reactions, usually catalyzed by flavin enzymes.[39,40] *L. delbruckii*[41] and *L. plantarum*[42,43] have a flavin-containing pyruvate oxidase that converts pyruvate to acetyl phosphate and CO_2 with the formation of H_2O_2. The acetyl phosphate can then be utilized for production of ATP. Lactate can be oxidized to pyruvate by two mechanisms that are present in lactobacilli.[44,45] *L.*

curvatus, L. sake, L. acidophilus, L. bulgaricus, and *L. lactis* have a lactate oxidase which reduces O_2 to H_2O_2. *L. plantarum, L. casei,* and *L. coryniformis* can use methylene blue or dichlorophenolindophenol as electron acceptors.

Excess electrons can be removed without the production of ATP by a very active NADH oxidase that catalyzes the formation of H_2O_2 and a NADH peroxidase that reduces H_2O_2 to H_2O.[43]

IV. SIGNIFICANCE OF CULTURES IN FERMENTED PRODUCTS

A. General

Unlike liquid foods and beverages, no economically effective methods have been found to completely remove or inactivate the naturally occurring lactic acid bacteria on vegetables prior to fermentation for bulk storage. Heating and other methods have been used on a limited scale, but heating has not been considered practical for large volumes of produce. Other accepted methods such as washing, chlorination, and acidification may reduce numbers, but do not sufficiently attenuate the natural lactic acid bacteria to provide a "pure culture" fermentation. Hence it is important to understand the ecology of natural fermentations in order to appreciate and perhaps develop controlled fermentation methods for vegetables. Considerable research has been done on the lactic acid fermentation of vegetables during this century, including identification of species and characteristics of bacteria involved and environmental factors affecting their predominance during fermentation. Reviews of the natural fermentations of cabbage,[1] cucumbers,[2] and olives,[3,46] are available.

Microbial growth during the natural fermentation of vegetables has been categorized into four sequential stages:[47] *initiation,* which may include growth by many Gram-positive and Gram-negative microorganisms naturally present on the vegetables; *primary fermentation,* which includes growth by lactic acid bacteria with or without growth by fermentative yeasts; *secondary fermentation,* which includes growth by fermentative yeasts after growth by the lactic acid bacteria has been inhibited by low pH, provided that fermentable carbohydrates remain; and *postfermentation,* which occurs after fermentable carbohydrates have been exhausted and is characterized by the absence of microbial growth under anaerobic conditions and by surface growth of oxidative microorganisms only when the brine surface is exposed to the atmosphere. The naturally occurring lactic acid bacteria proliferate during initiation and primary fermentation, depending upon their presence on the raw product and the chemical and environmental conditions under which the brined or salted product is held.

The use of pure cultures of lactic acid bacteria in the fermentation of vegetables and certain fruits in the 20th century has been the subject of numerous studies.[47] Rationale for selection and use of cultures and factors affecting their performance are presented in the remainder of this chapter.

B. Acid Production

Brined or salted vegetables undergo a natural lactic acid fermentation provided the salt concentration does not exceed approximately 8% NaCl and bacterial inhibitors, added intentionally or naturally present in the vegetable, are not present. In natural fermentation of sauerkraut, total acid production and the growth sequence of the four major species of lactic acid bacteria present occur in the approximate order *L. mesenteroides, L. brevis, P. pentosaceus,* and *L. plantarum.*[1] *L. mesenteroides* characteristically initiates the fermentation and *L. plantarum* characteristically terminates the fermentation. Acid production from these bacteria serves to inhibit growth of acid-sensitive bacteria that greatly outnumber the lactic acid bacteria on the raw plant material.

These species also become inhibited in the order of their sensitivity to acid and/or low pH. Perhaps at least partially for this reason Pederson and Albury[48] found that the fermentation of cucumber brines inoculated with pure cultures of any of the four species were eventually predominated by and terminated by *L. plantarum*.

Lactic acid constitutes most of the acid formed in vegetable fermentations. Acetic acid is also produced, depending on growth by heterofermentative lactic acid bacteria and the oxidation/reduction potential that influences the acetate/ethanol ratio. While lactic acid has a lower pK value, resulting in lower pH values, acetic acid has been shown to be more effective in inhibiting molds associated with softening of brined cucumbers.[49] Acid production with resulting lower pH values is thought to largely account for the eventual predomination of lactic acid bacteria during vegetable fermentations. The extent to which other bacteria grow thus depends upon activity of the lactic acid bacteria.

L. mesenteroides produces only D(−) lactic acid, while most strains of *L. brevis, L. plantarum,* and *P. pentosaceus* associated with vegetable fermentations produce both D(−) and L(+) isomers of lactic acid (Table 1). Both L(+) and D(−) lactic acid are normally metabolized by mammals, but the D(−) isomer is metabolized more slowly.[50] Based primarily on concerns about the ability of infants to metabolize the D(−) isomer, the FAO/WHO Expert Committee on Food Additives[51] recommended that D(−) lactic acid be avoided in infant foods and that consumption of the isomer by adults not exceed 100 mg/kg body weight per day. This recommendation was revised in 1974 to retain the recommendation that D(−) or DL-lactic acid not be used in infant foods, but no limit was set for an acceptable adult intake.[52]

This concern about the consumption of D(−) lactic acid has, however, led to efforts to produce sauerkraut that contains only L(+) lactic acid. Stetter and Stetter[53] found a new species, *L. bavaricus,* isolated from sauerkraut that exclusively forms the L(+) isomer. Use of this bacterium has been tested on an industrial scale to produce up to 50-ton batches yielding sauerkraut with only L(+) lactic acid.[54] A German patent has been granted on the process. *P. dextrinicus* produces exclusively L(+) lactic acid, but it is thought to be found very rarely in nature.[8] The significance of this organism in fermented vegetables is unknown.

C. Sugar Utilization

The rate and extent of sugar utilization during bulk fermentation of vegetables is important for various reasons. Lactic acid produced during fermentation is not subject to further metabolism under anaerobic conditions. If sugars are completely converted to lactic acid during fermentation in bulk containers, it is possible for certain products to be preserved in consumer containers without the requirement for heat processing. The Spanish-style green olive is an example of products that are not heat processed. Fleming et al.[12] concluded that five vegetables (cucumbers, red and green bell peppers, tomatoes, and green beans) fermented by *L. plantarum* with pH control were microbiologically stable under anaerobiosis, provided all fermentable carbohydrates were removed and the pH was 3.8 or below. Failure to completely remove fermentable sugars from carrots and red beets, which had relatively high initial sugar concentrations, resulted in secondary fermentation by yeasts after growth by lactic acid bacteria had ceased, though the pH was maintained at 3.8. Factors that limited growth by *L. plantarum* were not established. If sugar remains after termination of lactic acid fermentation, fermentative yeasts are capable of growing at low pH (<3.8) and under anaerobic conditions. Secondary fermentation by yeasts can result in gaseous spoilage, such as bloater formation in cucumbers.[55] Several factors influence the ability of lactic acid bacteria to completely ferment the sugars of vegetables, including initial sugar concen-

tration, pH, salt concentration, temperature, and buffer capacity of the vegetable. Thus, it is desirable to use cultures of lactic acid bacteria that remove as much fermentable sugar during primary fermentation as possible. *L. plantarum* WSO has been found to be particularly useful for this purpose in fermentation of cucumbers[56] and olives.[57] Even with this culture, however, it was necessary to buffer brined cucumbers with sodium acetate to assure complete sugar utilization.[56] Heterofermentative lactic acid bacteria such as *L. cellobiosus* ferment glucose to acids, but fructose is reduced to mannitol. Chen et al.[58] found that *L. cellobiosus* fermented green beans until all fermentable sugars (primarily glucose and fructose) were removed. However, fructose was reduced to mannitol which could be further fermented by *L. plantarum* WSO above pH 3.6. Below this pH mannitol was not fermented, and the beans were not further fermented by *L. plantarum*. Since mannitol reportedly[59] is not fermentable by yeasts, vegetables fermented to complete hexose removal by heterofermentative lactic acid bacteria may be microbiologically stable even with residual mannitol. This assumption warrants confirmation by further investigation.

The rate of fermentation is important when rapid turnover of the product is desired. Rapid fermentation with brined cucumbers is also desirable from the standpoint of minimizing the time required for purging to remove dissolved CO_2 resulting from fermentation. Rapid fermentation may not be desirable with sauerkraut, however. Higher temperatures may favor rapid fermentation and thus, homo- rather than heterofermentative lactic acid bacteria, which can adversely influence flavor and other quality factors of the product.[60]

D. CO_2 Production

Carbon dioxide production is important in the fermentation of vegetables because of gaseous spoilage problems of the vegetable and stress factors on tank heading devices due to buoyancy pressures. Vegetable tissue and fermenting lactic acid bacteria both contribute to CO_2 production.[61] CO_2 is a major end product in fermentation of hexoses by heterofermentative lactic acid bacteria. However, CO_2 production by homofermentative lactic acid bacteria can occur by decarboxylation of organic and amino acids. Malic and citric acids are present in many vegetables and may be decarboxylated. McFeeters et al.[62,63] found the malolactic reaction to account for most of the CO_2 produced by *L. plantarum* in cucumber juice fermentations.

Perhaps the most notable significance of CO_2 production in brined vegetables occurs with cucumbers. If the critical concentration of CO_2 in the brine is exceeded, the cucumbers will form hollow cavities (bloat) and can represent a serious economic loss (Figure 3d). Bloater damage may be caused by yeasts,[53] coliform bacteria,[64] and homo-[65] and heterofermentative lactic acid bacteria.[66] A mechanism for bloater formation has been proposed[67] that suggests that CO_2 concentration is only one of several factors involved. However, bloater damage can be effectively reduced by purging of CO_2 from the brine.[56,68] Nitrogen is recommended as the purging gas because it will reduce bloater formation and will not cause possible problems due to off-flavors and colors and will not encourage growth of aerobic microorganisms, some of which may produce softening enzymes.[68] Air purging has also been found to prevent bloater formation[68,69] but may result in the problems cited above that do not occur as a consequence of nitrogen purging. Air purging does not appear to adversely influence the growth of lactic acid bacteria at the rates normally used in commercial fermentations, but acid production may be reduced at high rates of air.[49]

Efforts are currently underway to develop strains of *L. plantarum* for use in cucumber fermentations that do not produce CO_2 from malic acid. McFeeters et al.[19] recently observed that bloater damage can be prevented in nonpurged cucumber fermentation if the fermentation is carried out by a homolactic bacterium that lacks the

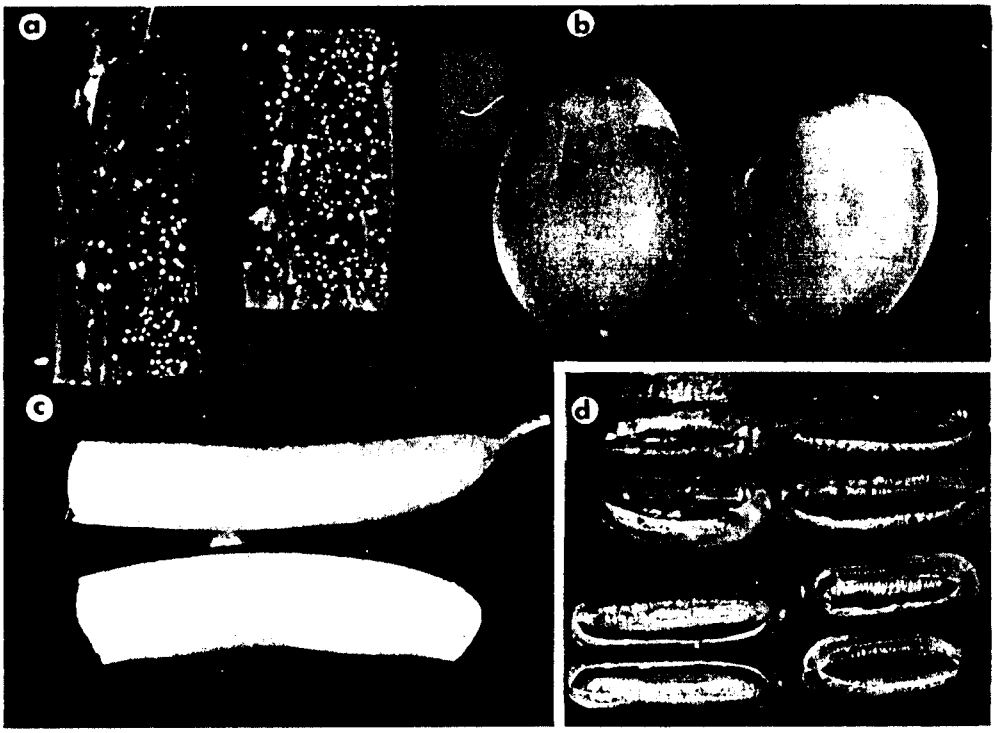


FIGURE 3. Bacterial pustule formation on the surfaces of fermented (a) cucumber, (b) olive, and (c) green bean; (d) gaseous spoilage (bloating) of fermented cucumbers.

ability to degrade malic acid. Predominant growth by such a lactic acid bacterium could obviate the need for purging. Mutants of *L. plantarum* WSO that lack the ability to produce CO_2 from malic acid have been obtained.[70] These and other mutants are being tested and/or further adapted for use in cucumber fermentations.[71]

Gas production, presumably including CO_2, is associated with fisheye spoilage in olives.[72] These lenticular cavities are thought to occur due to spoilage bacteria, but influence due to lactic acid bacteria perhaps should not be excluded.

E. Biogenic Amines

Biogenic amines have been implicated in food poisoning incidents, usually from the consumption of fish. Lactic acid bacteria involved in food fermentations can decarboxylate histidine and certain other amino acids.[73] Mayer et al.[74] investigated histamine formation during sauerkraut production. Commercial sauerkraut samples in the U.S. have been found to contain 0.9 to 13.0 mg/100 g histamine.[75] These concentrations are considered unlikely to cause illness.[75,76] However, it would seem prudent to select cultures that produce minimum amounts of histamine for use in food fermentations.

F. Flavor

Relatively little research has been done on the contribution of lactic acid bacteria to the flavor of fermented vegetables. In the case of fermented Spanish-style green olives, Fleming et al.[77] concluded that the primary contributions of the lactic acid bacteria to flavor were (1) production of a desirable level of acidity and (2) utilization of fermentable sugars to the exclusion of microorganisms that produce end products with undesirable flavor characteristics. They found methyl sulfide to be a major odor component

of fermented as well as unfermented olives through headspace vapor analysis. Acetaldehyde and ethanol varied among fermentations by different species of lactic acid bacteria and were found to contribute secondarily to the odor.

Aurand et al.[78] concluded that the flavor of pickles fermented by four different species of lactic acid bacteria in pure culture was due to a blend of volatile components rather than the presence or absence of a single component. The compounds detected by headspace vapor analysis were formaldehyde, acetaldehyde, propionaldehyde, acetone, butyraldehyde, ethyl alcohol, ethyl butyrate, and isovaleraldehyde. The presence and relative concentrations of those compounds varied among species and strains within species. The degree of acidity produced by the cultures evoked the most commentary from the taste panel. *L. plantarum* fermentations were described as strongly acidic and differed from pasteurized controls chiefly in the degree of acid present.

Perhaps the greatest influence of lactic cultures on the flavor of fermented vegetables has been found in sauerkraut. Good-flavored kraut has been associated with the ratio of volatile to nonvolatile acids.[1] Growth by *L. mesenteroides* during the early stages of fermentation is preferred because it forms relatively large amounts of acetic acid. Thus, sauerkraut is preferably fermented at 65°F or below to favor growth of *L. mesenteroides*. At higher temperatures, *L. plantarum* predominates and results in lower ratios of volatile to nonvolatile acid. Also, the pH may be lower and yet the total acid may be lower because of this lower ratio. Such kraut has a sharp acid flavor as opposed to the milder-flavored kraut produced at lower temperatures.

G. Texture

There are indications that high concentrations of lactic acid may result in softening of fermented vegetables. Bell et al.[79] found that the firmness of fresh-pack pickles became progressively softer at higher concentrations of lactic acid during storage. At similar concentrations, lactic acid was more detrimental than acetic acid to firmness. They proposed that the higher binding affinity of lactic acid as compared to acetic acid for Ca^{++} may be responsible for the effects shown. They suggested that removal of Ca^{++} from the pectic substances that bind the cucumber tissues by lactic acid could result in softening. Etchells et al.[57] found Spanish-type green olives fermented by *L. plantarum* to be less firm than those fermented by *L. mesenteroides*. Those fermented by *P. cerevisiae* were intermediate in firmness. Overall, firmness varied inversely with percentage of titratable acidity calculated as lactic acid. These observations indicate that the type of lactic acid fermentation, particularly as it dictates the amount of total lactic acid formed, may influence firmness of the fermented product. Other factors such as salt concentration, Ca^{++} concentration, and storage temperature probably interact with acid concentration to render an overall influence on firmness.

H. Appearance

Lactic acid bacteria influence the appearance of fermented vegetables in the form of brine turbidity, pustules that form on the surface of certain vegetables such as cucumbers and olives (Figures 3a—c), and in gaseous spoilage (Figure 3d). Although cloudy brine is not considered a problem with certain specialty products such as overnight dill pickles, it is not acceptable in most pickle products and in Spanish-type green olives. In genuine dill pickles, the brine must be clarified before it is used as a cover brine in the final product.

V. FACTORS INFLUENCING OPTIMUM PERFORMANCE OF CULTURES

A. Concentrated Cultures

Concentrated cultures of lactic acid bacteria have been commercially available for fermented vegetables for over 10 years. They have been used experimentally in the

fermentation of cucumbers[56] and olives,[60] and have been used on a limited commercial scale for the bulk fermentation of brined cucumbers for several years. Both *L. plantarum* and *P. pentosaceus* have been marketed commercially for this purpose. The use of these cultures assures a consistent, rapid fermentation of brined cucumbers if the controlled fermentation procedure of Etchells et al.[56] is followed, the temperature is 26 to 29°C, and the salt concentration does not exceed about 7%.

The cultures are grown and stored by commercial culture firms by general methods used for dairy cultures. These methods are described elsewhere in this book and also have been reviewed by Porubcan and Sellars.[61] The cultures may be stored in liquid nitrogen and are shipped on dry ice. They may be held for several weeks on dry ice or in a −40°C freezer. Such cultures are typically packaged in 70 or 360 g containers with instructions for thawing in about 30°C water. The cells are added at the rate of 1 to 4 billion cells per gallon of brined cucumbers. Before liquid nitrogen storage became popular, cultures were lyophilized. Lyophilization came into disfavor when cell recovery and activity of liquid nitrogen cultures were demonstrated to be superior. Recently, however, attempts have been made to produce improved lyophilized cultures;[61] such cultures are available to the pickled vegetable industry. The lyophilized cultures are shipped without refrigeration and can be stored at about 4°C, or for extended storage at −18°C.

B. Treatment of Product Prior to Inoculation

Cabbage, cucumbers, and green olives are variously handled during preparation for brine fermentation and storage (Figure 4). The growth of the natural flora, including endogenous lactic acid bacteria, is greatly influenced by these treatments.

Most cabbage for sauerkraut is shredded or chopped before tanking, although a small quantity is brined whole or cut into chunks. Thus, nutrients from the cabbage are immediately available and microbial growth probably occurs on the coring and shredding equipment.

Green olives are treated with alkali prior to brining to remove the natural bitterness caused by the phenolic glucoside, oleuropein, in the olives. The olives must then be washed to remove the alkali. The olives may be heat shocked (74°C, 3 min) as proposed by Etchells et al.[57] to enhance the fermentability of certain varieties; normally they are not heated in commercial practice. Sugar may be added to the brine if alkali and washing treatments remove too many of the natural sugars. The brines normally undergo a lactic fermentation by the natural flora, although various abnormal fermentations may result.[3] Brines may be inoculated with an actively fermenting brine, as has been done commercially, or with pure cultures of *L. plantarum*, as has been done experimentally.

Cucumbers are size graded and brined whole. Most commercial fermentations are by naturally occurring lactic acid bacteria. Limited commercial use of lactic cultures has begun during the past decade according to modifications of the controlled fermentation procedure outlined by Etchells et al.[56] This procedure is outlined in Figure 4. Acidification (acetic acid or vinegar) serves to virtually eliminate growth of the natural microbial flora that occurs during the initiation stage of natural fermentations. The brines are purged with nitrogen to remove dissolved CO_2 and thereby prevent bloater formation. After about 24 hr, the brine is buffered with sodium acetate,[56] or the acetic acid is neutralized to form acetate as practiced commercially.[62] The buffering serves to eliminate secondary fermentation by yeasts. With the pH at about 4.7, the brine is inoculated with either *L. plantarum* or *P. pentosaceus* or a combination for a total cell count of 1 to 4 billion cells per gallon of brined cucumbers.

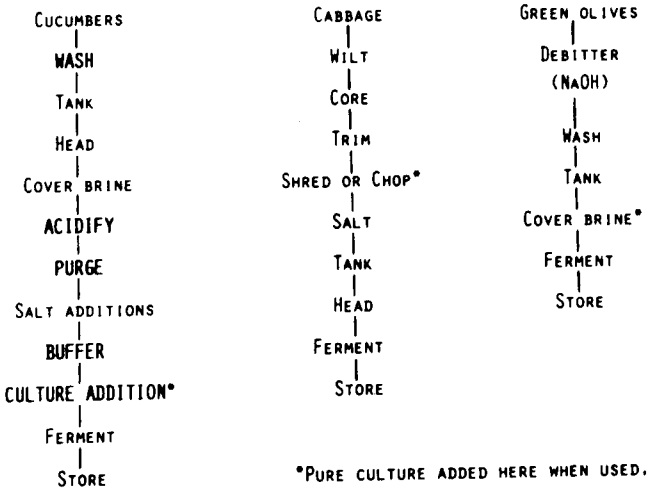


FIGURE 4. Flow charts for the fermentation of produce. For cucumbers, steps that have been added to the natural fermentation procedure are indicated in boldface.[47] For cabbage and olives, steps are those involved in natural fermentation; culture, if used, is added where indicated.

C. Salt Concentration

The use of salt in fermentation serves two primary functions: (1) it helps direct the type and extent of microbial action and (2) it prevents softening of the vegetables. The concentration of salt required to prevent softening determines the minimum that can be used, and varies among products. The concentration for fermentation of cabbage is 2 to 3%; cucumbers, 5 to 8%; and green olives, 4 to 7%. These salt concentrations, in turn, at least partially dictate growth by the four species of lactic acid bacteria. *L. mesenteroides* is active in the primary fermentation of sauerkraut but is not prominent in the fermentation of cucumbers and olives, at least partially because of the higher salt concentrations in the latter products. Currently, there are efforts being made to reduce the concentration of salt in the fermentation of cucumbers to help abate environmental problems and problems related to high salt usage. Addition of calcium acetate[83] or calcium chloride[84] has been shown to result in firmness retention of cucumbers at relatively low concentrations of sodium chloride (1.4 to 4%). It is conceivable that *L. mesenteroides* could be useful in cucumber fermentations at these concentrations of salt. *L. cellobiosus* could also prove to be useful, especially since it was shown to be relatively effective in removing the fermentable sugars of green beans.[13]

D. Temperature

Relatively low fermentation temperatures seem to favor growth of *L. mesenteroides*, while higher temperatures favor growth of *L. plantarum*, which can have important effects on the quality of sauerkraut. Pederson and Albury[60] reported that *L. mesenteroides* predominated an early and extended primary fermentation of sauerkraut at 7.5 and 18°C. At 32 and 37°C, the period of growth by *L. mesenteroides* was shortened and the predominance by *L. plantarum* occurred earlier. They reported that sauerkraut fermented with high salt concentrations or at high temperatures was poor in color, flavor, and texture. Pederson and Albury,[1] based on years of study, concluded that sauerkraut fermented at temperatures of 13 to 18°C will be superior in quality to that fermented at 24°C and above since the heterofermentative lactics exert a great effect at the lower temperatures.

For controlled fermentation of cucumbers, however, Etchells et al.[56] recommended brine temperatures of 26 to 29°C. The homofermentative *L. plantarum* and *P. pentosaceus* cultures ferment rapidly at these temperatures. For controlled fermentation of cucumbers, it has been considered desirable to complete the fermentation as rapidly as possible to reduce purging costs. In the southern and southwestern U.S., brine temperatures during summer months normally exceed 26°C and favor rapid growth of these starter cultures. In cooler regions of the U.S. such as the northwest, and in late fall brine temperatures may be 21°C or lower. For this reason, efforts have been made to obtain cultures capable of growing rapidly at these temperatures. A strain of *P. pentosaceus* has reportedly been developed that is capable of growing more rapidly at the lower temperatures.[85] Since *L. plantarum* is more acid tolerant, however, it is likely to terminate the fermentation. Indeed, this has been our experience.[71]

E. Availability of Nutrients

Fermentable carbohydrates in cabbage and olives include sucrose, fructose, and glucose. Glucose and fructose constitute essentially all of the fermentable sugars in cucumbers and olives. All other essential nutrients for growth of lactic acid bacteria are present in the raw produce, as evidenced by the occurrence of a vigorous natural lactic fermentation in the brined products. Availability of these nutrients for growth of lactic acid bacteria depends upon treatment of the produce before salting or brining. Nutrients are immediately available in cabbage that is shredded or chopped, whereas they must diffuse from cucumbers and olives into the brine before they are available for fermentation in the brine surrounding the produce.

It has been hypothesized that the fermentation of cucumbers occurs solely in the brine.[66] It has been shown, however, that lactic acid bacteria enter and proliferate within brined cucumbers.[86] Entrance into and growth within cucumbers was shown to be influenced by the gas composition of the cucumber before brining. Thus, fermentation evidently occurs within cucumbers and the brine surrounding the cucumbers. Later evidence has indicated that the relative amount of sugar that diffuses into the brine before inoculation influences the relative amount of fermentation that occurs in the cucumbers and in the brine.[87]

While green olives contain 2 to 3% fermentable sugars[57] (which is more than sufficient for adequate acid production), alkali and washing treatments can greatly reduce the amount of sugars available for fermentation. It is assumed that other nutrients would also be removed or altered by these treatments. The degree of alkali penetration can be an important factor in this regard. California processors customarily allow alkali to penetrate to the pit, thereby more completely removing bitterness from the olives. Fermentable sugar is removed to such an extent that glucose is added to assure adequate acid production. In Spain, briners typically allow alkali to penetrate only about three fourths of the flesh distance to the pit. This leaves a slight bitterness that is preferred in these olives, as well as adequate sugar for acid production.

F. Natural Inhibitors

Unidentified substances in cabbage have been reported to inhibit certain undesirable Gram-negative bacteria present on cabbage.[1] They presumably do not inhibit desirable lactic acid bacteria.

Etchells et al.[57] found that heat shocking of lye-treated, green Manzanillo olives greatly increased their brine fermentation by pure cultures of lactic acid bacteria. When the olives were neither lye-treated nor heated, added lactic cultures caused essentially no fermentation; only yeasts grew. They suggested that the heat treatment destroyed a naturally occurring inhibitor of lactic acid bacteria, and that the inhibitor may account for the occurrence of stuck fermentations which are characterized by the absence of

lactic acid bacteria and the presence of yeasts in the brine. Subsequent studies revealed that hydrolysis products of oleuropein, including its aglycone and elenolic acid, are inhibitory to lactic acid bacteria but not to yeasts.[88,90] Fleming et al.[90] found that oleuropein was not greatly inhibitory to lactic acid bacteria, contrary to earlier suggestions by Vaughn[3] and Juven et al.[91] In fact, lactic acid bacteria have been found to utilize oleuropein.[92] Fleming et al.[90] suggested that oleuropein is degraded to its aglycone when unheated olives are brined, perhaps by the natural β-glucosidase that Cruess and Alsberg[93] reported, and by further degradation to elenoic acid. Juven and Henis[94] found that oleuropein became more inhibitory when it was treated with β-glucosidase. Perhaps heat inactivates β-glucosidase in the olives, preventing breakdown of oleuropein to yield the inhibitory aglycone when the olives are brined. Apparently not all varieties of olives are inhibitory to lactic acid bacteria. Balatsouras et al.[80] found no evidence that the Conservolea variety of olives popular in Greece contains compounds in concentrations inhibitory to lactic acid bacteria.

Antagonism among species of lactic acid bacteria has been indicated. Etchells et al.[95] found that *P. pentosaceus* delayed the onset of growth by *L. plantarum* in fermenting cucumbers. Fleming et al.[96] found that certain strains of *P. pentosaceus* were inhibitory to other strains of this species, to other species of lactic acid bacteria, and to certain other Gram-positive bacteria.

G. Bacteriophage

We are not aware of any problems that occur due to bacteriophage in *L. plantarum* or *P. pentosaceus* cultures that are used for pure culture inoculations of vegetables. The presence of bacteriophage in *L. plantarum* and other lactobacilli has been reported,[97] but its significance in cultures for vegetable fermentations is yet to be determined.

VI. CONCLUSIONS

Much research has been done over the past 50 years to characterize the lactic fermentation of vegetables. This work has yet to be sufficiently integrated to encourage wide-scale commercial use of controlled fermentation methods. In this regard, the vegetable fermentation industry contrasts with other food and beverage fermentation industries such as the dairy, brewery, and wine industries. Part of the lag in development and commercial acceptance of such methods is caused by the complexities of fermenting particulate material. The vegetable fermentation industries have been able to produce acceptable products using salt and the mechanisms of nature for fermentation control. A major obstacle for the use of pure cultures and controlled fermentation methods has been the fermentation vessel, especially for brined cucumbers.

Recent efforts to develop anaerobic tanks for fermentation and storage of brined cucumbers[98] are expected to make controlled fermentation an attractive option to picklers. It is conceivable that development of strains of lactic acid bacteria with desirable and unique properties will further encourage acceptance of pure cultures. Development of cultures for use in vegetable fermentations that do not produce CO_2 from malic acid, that exclusively produce L(+) lactic acid, and that have other desirable traits are examples of recent progress that hint of possible breakthroughs for the industry in the near future. Rapid progress is being made in understanding the genetic systems of the lactic acid bacteria that are used in the food industry. Much is already known about the lactic streptococci used in the dairy industry. *L. plantarum* and *P. pentosaceus* are only now being studied with respect to their genetic information transfer systems. Conjugal plasmid transfer has been demonstrated between streptococci and pediococci,[99] and recently, plasmids have been observed in *L. plantarum*.[100,101] Rapid progress is

likely in understanding how these important vegetable fermentation bacteria can be engineered to have desirable traits for use as starter cultures.

Thus, development of improved controlled fermentation methods for vegetables seems possible, but commercial acceptance will depend on development of superior cultures and suitable tanking and handling procedures to make such methods economically attractive.

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Bacterial Starter Cultures for Foods

Editor

Stanley E. Gilliland

Professor
Animal Science Department
Oklahoma State University
Stillwater, Oklahoma

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