# Acidification Effects on Microbial Populations During Initiation of Cucumber Fermentation

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## ABSTRACT

Addition of acetic acid (0.067M) or calcium acetate (0.133 M) to the cover brine (1.94M NaCl) of cucumbers reduced naturally occurring *Enterobacteriaceae*, but not lactic acid bacteria (LAB), during the first 5 days after brining. Naturally occurring LAB were predominantly heterofermentative (≥80%) 1 day after brining and homofermentative (≥90%) on the 5th day. *Enterobacteriaceae* survived longer within cucumbers than in brine, particularly in nonacidified cucumbers. Starter cultures of *Lactobacillus plantarum* or *Pediococcus pentosaceus* decreased 90–99.9% during the first 10 hr after addition and did not increase until about 30 h after brining. Obtaining predominant fermentation of brined cucumbers by added cultures was difficult due to presence of natural microflora on/in the cucumbers and the harsh environment of the brine (high salt, low nutrients).

## INTRODUCTION

MOST COMMERCIAL cucumber fermentations result from the growth of naturally occurring microorganisms and are subject to bloater damage, softening, and other microbially related spoilage problems (Fleming, 1982). Various attempts have been made to control the fermentation of brined cucumbers. Pederson and Albury (1961) added starter cultures of various species of lactic acid bacteria (LAB) to unheated, brined cucumbers. They found that, regardless of LAB species added, *Lactobacillus plantarum* consistently predominated the latter phase of fermentation, apparently because of its greater acid tolerance. Etchells et al. (1964) obtained pure culture fermentation of brined cucumbers with various species of LAB by blanching or irradiating cucumbers before brining to remove natural microflora. The procedure was deemed economically impractical, however, for bulk commercial fermentations. Later, Etchells et al. (1973b) developed a procedure for controlled fermentation of cucumbers that did not require blanching. The procedure was based on washing the cucumbers, acidification (acetic acid) of the cover brine, addition of sodium acetate buffer (to assure complete sugar utilization) after about 1 day. Inoculation with an *L. plantarum* starter culture followed, and purging of the brine with nitrogen to remove $CO_2$. Purging to remove $CO_2$ produced during fermentation prevented bloater formation (Fleming et al., 1973). Acidification of the cover brine resulted in reduction of *Enterobacteriaceae* (Etchells et al., 1975). This controlled fermentation procedure has been tested in open tanks typically used by the pickle industry. Some features of the procedure are in commercial use. Purging (with nitrogen or air) is used widely; some companies acidify cover brines to varying concentrations, and some add a starter culture of LAB. The complete procedure has not been adopted for several reasons. It is rather cumbersome, requiring separate additions of acid, buffer, and culture. Also, the current use of tanks with tops open to the atmosphere exacerbates problems associated with microbial control and use of starter cultures.

A closed tank system was designed and installed for fermentation and storage of brined cucumbers (Fleming et al., 1983). A simplified brining procedure for use in closed tanks was developed (Fleming et al., 1988). The procedure involves washing the cucumbers which are then conveyed into a tank containing a calcium acetate cover brine and LAB starter culture. The procedure resulted in a homolactic fermentation typical of the *L. plantarum* starter culture. The added starter culture predominated only during the first few days of the fermentation, however. Naturally occurring LAB eventually predominated and terminated the fermentation.

In developing procedures for controlled fermentations of cucumbers, conditions must be established to favor growth of the added culture and suppress growth of undesired microorganisms. Also, factors influencing cucumber quality and economy of operations must be considered. The addition of acetic acid (Etchells et al., 1975) or calcium acetate (Fleming et al., 1988) has been shown to suppress growth of *Enterobacteriaceae*, but not naturally occurring LAB in the brine surrounding the cucumbers. In addition to the initial chemical environment of the brine, physical factors may be important in predominance of various microbial groups. Sugars and other nutrients must diffuse from the cucumbers into the brine before fermentation in the surrounding brine occurs. The greatest numbers of naturally occuring microorganisms of fresh cucumbers are located near the skin (Samish et al., 1963, Daeschel and Fleming, 1981). Bacteria of the *Enterobacteriaceae* genus have been reported in the interior of healthy cucumber fruit (Samish et al., 1963; Meneley and Stanghellini, 1975). Once the fermentation starts, LAB may grow within the cucumbers or in the brine surrounding the cucumbers (Daeschel and Fleming, 1981; Daeschel et al., 1985). The LAB apparently gain entry through stomata of the skin (Daeschel et al., 1985).

Objectives of our study were to characterize microbiological and chemical changes associated with three different brining treatments that have been proposed for commercial fermentation of brined cucumbers. Specifically, the effects of acidification and buffering treatments on growth and survival of *Enterobacteriaceae*, LAB (both naturally occurring and added starter cultures), and total aerobes within the cucumbers and in the brine surrounding the cucumbers were studied.

## MATERIALS & METHODS

### Cucumbers

Pickling cucumbers were locally grown, size 3B (3.5–5.1 cm diam), cv. Calypso. All fruit were of good quality and free of serious mechanical damage.

### Brining procedures of uninoculated fermentations

Unwashed cucumbers were brined in 5-gal (19-L) pails with sparging tubes, as described by Fleming et al. (1975). Three brining treatments (in duplicate) were used: (1) no acidification; (2) brine acidified with 0.4% (0.067M) acetic acid to equalize at 0.16%; (3) brine buffered with addition of 0.8% acetic acid and 0.045M calcium hydroxide (= 0.133 M calcium acetate). All brines contained 11.4% (1.94M) NaCl initially, which equalized at 4.5%. Brines 1 and 2 were supple-

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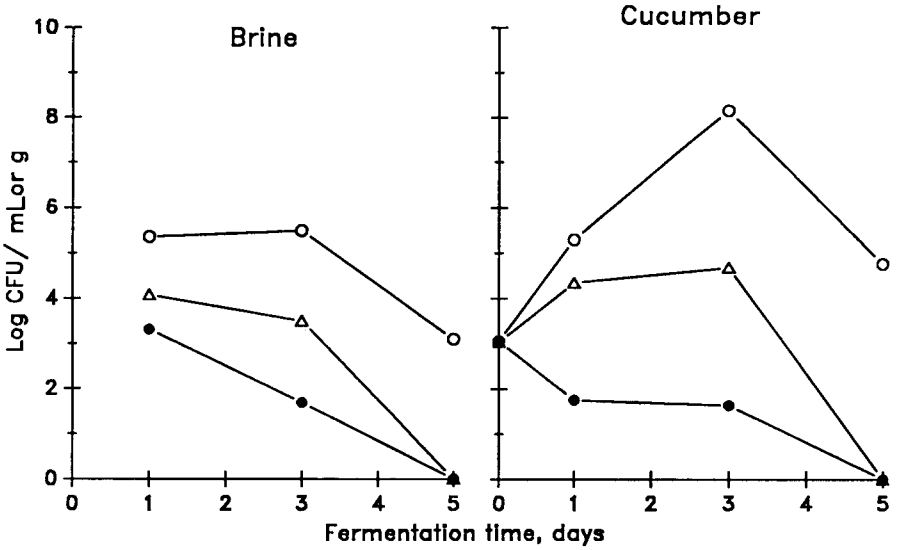


*Fig. 1—Changes in* Enterobacteriaceae *population caused by using nonacidified (○), buffered (△), and acidified (●) brining treatments.*

mented with 0.045M $CaCl_2$. The cucumbers:brine ratio was 60:40, w/v. Brines were purged continuously with $N_2$ at 25 mL/min. In addition, ultraviolet light was placed above the pails to inhibit growth of surface fungi. Starter cultures were not added to those brines.

### Brining procedures of inoculated fermentations

Fermentations (in duplicate) were done in 3.8-L jars using washed cucumbers and brining treatment 3 as described above. Cucumbers, buffered brine, and starter culture were added initially. The jars were closed and purged continuously with $N_2$ at 25 mL/min.

### Sampling

Ten mL of brine were taken from each 19L pail at 0, 1, 3, and 5 days for microbiological and chemical analyses. Three cucumbers were taken from each pail at days 1, 3, and 5 and blended aseptically with 100 mL saline (0.85%) in a Waring Blendor. For day 0, three cucumbers were sampled before brining and blended as described. The cucumber slurry was used for chemical and microbiological analyses. For the inoculated fermentation, 10 mL aliquots of brine were taken at 0, 6, 12, 24, and 48 h for microbiological analysis.

### Microbial cultures and enumeration of microorganisms

Starter cultures were *L. plantarum* WSO MDC$^+$ (a streptomycin-resistant variant), *L. plantarum* WSO MDC$^-$ (a streptomycin-resistant variant unable to decarboxylate malic acid), and a commercial starter culture of *Pediococcus pentosaceus*. Both *L. plantarum* cultures were from the culture collection of the Food Fermentation Laboratory, Raleigh, NC, obtained as previously described (Fleming et al., 1988). The *L. plantarum* WSO MDC$^-$ culture was a mutant of the MDC$^+$ (decarboxylates malic acid) parent culture (Daeschel et al., 1984). All cultures were grown 16 h at 30°C in MRS broth (Difco Laboratories, Detroit, MI). Starter cultures were added to each brine and mixed immediately before addition of cucumbers. The initial population of each starter culture was about $2 \times 10^6$ cells per mL of brined material.

In the inoculated fermentations, the lactobacilli were enumerated on LBSF agar (LBS broth + 1% fructose + 1.5% agar) containing 1 mg/mL streptomycin sulfate. The LBS broth was obtained from BBL Microbiology Systems (Cockeysville, MD). The *P. pentosaceus* culture was enumerated on LBSF agar without streptomycin sulfate, and about 20 colonies were selected at each sampling period and microscopically confirmed as pediococci.

Populations in uninoculated fermentations were enumerated for aerobic plate count using standard methods agar (BBL Microbiology Systems). *Enterobacteriaceae* were enumerated using violet red bile agar (BBL Microbiology Systems) containing 1% glucose (VRBG). LAB were enumerated using MRS broth (Difco) containing 1.5% agar and 0.02% sodium azide. All pour plates were duplicated and incubated at 30°C. For differentiation of heterofermentative and homofermentative LAB, 20–30 colonies from each duplicate plate were inoculated into MRS broth containing Durham tubes as described by Gibson and Abd-El-Malek (1945). Production of gas was a positive test for heterofermentative LAB.

### Chemical analyses

Salt and reducing sugars were analyzed as outlined by Fleming et al. (1984). A Fisher Accumet (model 825 MP) pH meter with a Fisher gel-filled combination electrode was used for pH measurement.

## RESULTS & DISCUSSION

### Uninoculated, brined cucumbers

During the fermentation of uninoculated, brined cucumbers, microbial growth responded to two distinct environments, the cucumber tissue and the surrounding brine (Figs 1 to 3). The effects of acidification treatments on microbial flora are indicated for the cucumbers and the brine. In interpreting growth responses of the microbial groups, the chemical environments of the cucumbers and brine are important as summarized in Table 1. The cucumbers were placed in 11.4% NaCl brine containing acetic acid, calcium acetate buffer, or no acidification treatment. The brine initially was very high in NaCl, high in acid for the acidified treatments, and devoid of nutrients since none had diffused from the cucumbers into the brine. In contrast, the cucumbers initially contained no NaCl, but an abundance of nutrients. Thus, initial conditions for microbial growth were relatively harsh in the brine, but quite favorable inside the cucumbers. The chemical environments of the brine and cucumbers subsequently changed due to microbial activities, and in response to diffusion of nutrients from cucumbers into the brine and salt and acid from brine into the cucumbers.

The effect of acidification treatment on growth of *Enterobacteriaceae* in the brine and in the cucumbers is illustrated in Fig. 1. In the brine of the nonacidified cucumbers, *Enterobacteriaceae* remained nearly constant at $3 \times 10^5$ CFU/mL for 1 and 3 days after brining, but then were reduced to about $10^3$ CFU/mL after 5 days. Inside the cucumbers, however, *Enterbacteriaceae* had increased to $10^8$ CFU/g at 3 days and then decreased to $10^4$ CFU/g at 5 days. The 10,000-fold increase (compared to fresh cucumbers) of *Enterobacteriaceae* within the cucumbers 3 days after brining indicated a favorable environment for growth. The practical significance of this lies in the potential problems that bacterial species within this fam-

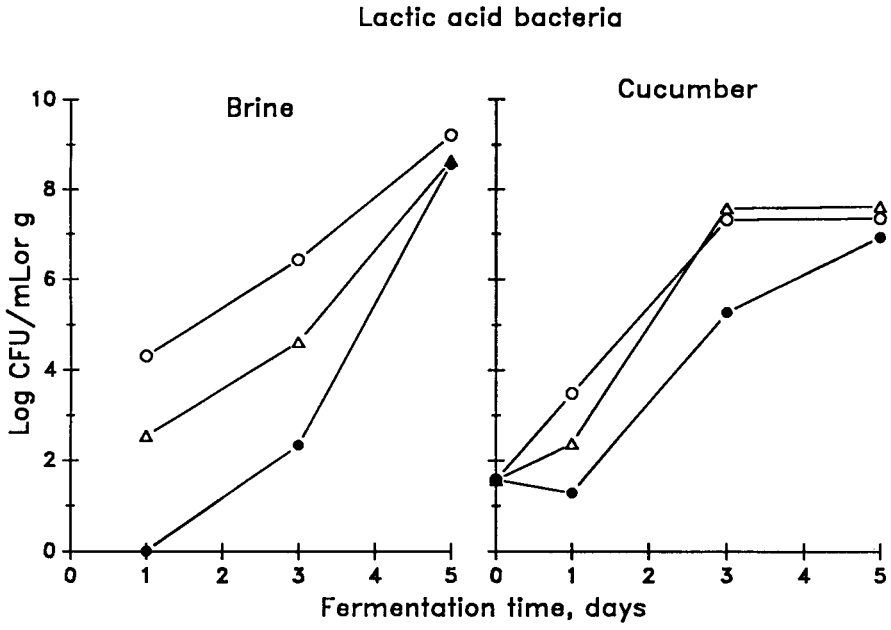


*Fig. 2—Changes in LAB population brought about by using nonacidified (○), buffered (△), and acidified (●) brining treatments.*

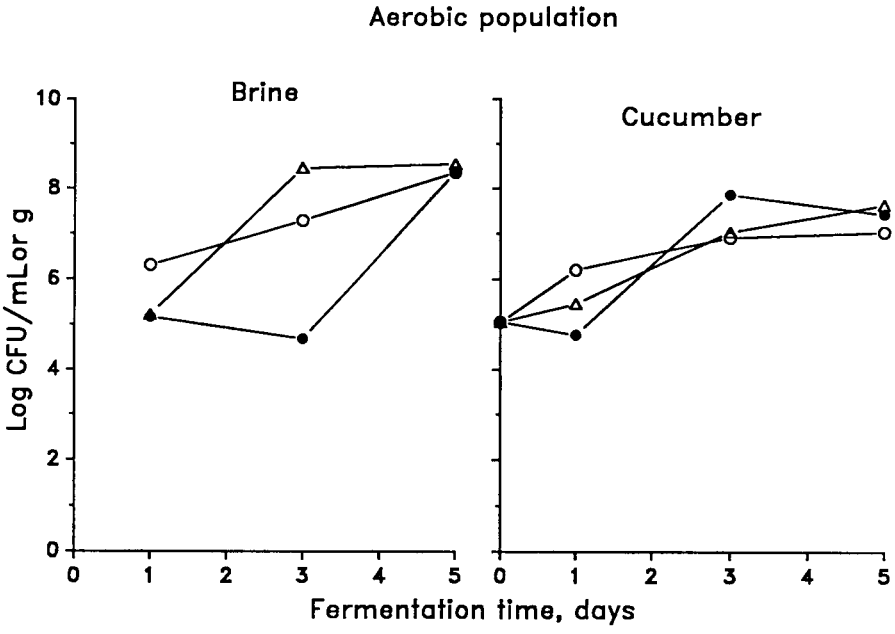


*Fig. 3—Changes in aerobic plant count brought about by using nonacidified (○), buffered (△), and acidified (●) brining treatments.*

*Table 1—Effects of three different brining methods on LAB in brine and in cucumbers*

| Brining treatment | Days after brining | % Heterofermentative LAB[a] | | Brine | | | Cucumber | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Brine | Cucumber | pH | Sugar, % | NaCl, % | pH | Sugar, % | NaCl, % |
| Nonacidified | 0 | ND[b] | 80 | 5.56 | 0.0 | 11.4 | 5.17 | 1.3 | 0.0 |
| | 1 | 90 | 80 | 6.57 | 0.1 | 6.8 | 5.10 | 1.3 | 0.3 |
| | 3 | 50 | 50 | 4.26 | 0.3 | 4.8 | 4.55 | 1.1 | 3.1 |
| | 5 | <1 | <1 | 3.71 | 0.2 | 4.4 | 3.74 | 0.9 | 3.7 |
| Acidified | 0 | ND | 80 | 3.10 | 0.0 | 11.4 | 5.17 | 1.3 | 0.0 |
| | 1 | 80 | 60 | 3.63 | 0.2 | 6.8 | 4.11 | 1.0 | 0.7 |
| | 3 | <1 | <1 | 3.62 | 0.3 | 4.8 | 3.77 | 0.6 | 3.2 |
| | 5 | <1 | <1 | 3.42 | 0.3 | 4.4 | 3.41 | 0.4 | 3.9 |
| Buffered | 0 | ND | 80 | 4.83 | 0.0 | 11.4 | 5.17 | 1.3 | 0 |
| | 1 | 80 | 100 | 4.52 | 0.2 | 7.3 | 4.97 | 1.0 | 0.8 |
| | 3 | <1 | <1 | 4.45 | 0.5 | 4.7 | 4.30 | 0.4 | 3.0 |
| | 5 | <1 | <1 | 3.83 | 0.2 | 3.6 | 3.69 | 0.2 | 3.7 |

[a] Remaining percentages were homofermentative LAB. LAB were enumerated on MRS agar containing 0.02% sodium azide. Heterofermentative LAB were determined by gas production in MRS broth containing Durham tubes.
[b] ND = not determined.

ily can cause with quality of brine-stock cucumbers. Gas-forming bacteria within the *Enterobacteriaceae* family have been implicated as causative agents for bloater damage (Etchells et al., 1945). *Erwinia* species within this family have been re-

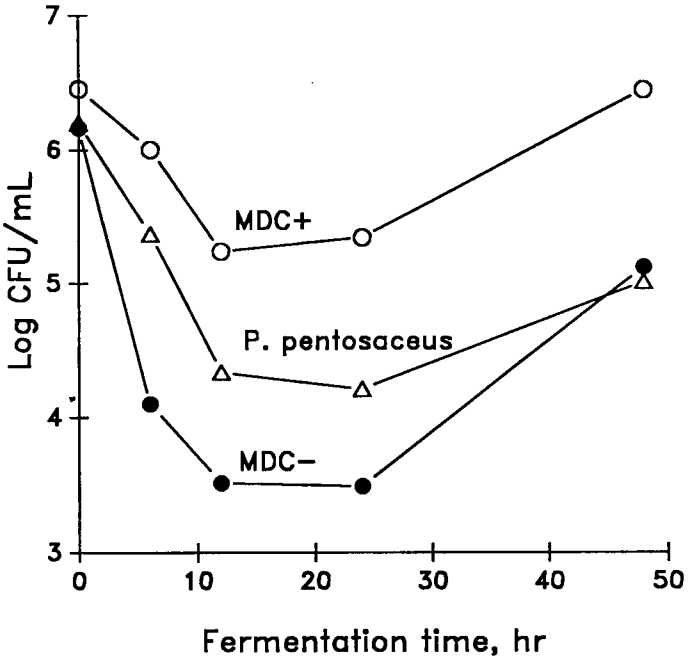


*Fig. 4—Survival and growth of starter cultures of LAB in brines of cucumbers buffered with calcium acetate. The initial salt concentration in the brines was 11.4%, and equilbrated with the cucumbers at a rate similar to that given in Table 1.*

ported in the interior of healthy fresh cucumbers (Meneley and Stanghellini, 1975; Samish et al., 1963). Bloater damage was not observed perhaps because the fermenting brines were purged with $N_2$ to remove $CO_2$. Cucumber softening was not observed.

*Enterobacteriaceae* within the acidified cucumbers steadily declined from $10^3$ in the fresh fruit to <1 CFU/g at 5 d (Fig. 1). The brine reflected a similar decline. A rapid decline in pH within the acidified cucumbers (Table 1) suggested that acetic acid penetration was responsible for reduction of *Enterobacteriaceae*.

We postulated that the buffered treatment might be similar to the acidified treatment regarding inhibition of *Enterobacteriaceae*, since the concentration of protonated acetic acid was about the same in the initial cover brine for both treatments. However, *Enterobacteriaceae* increased about 10- to 15-fold in the buffered cucumbers at 1 and 3 d before declining to <1 CFU/g after 5 d (Fig. 1). Thus, buffering suppressed growth of *Enterobacteriaceae*, but not nearly as much as did acidification. This suggested that suppression of *Enterobacteriaceae* growth may have been partly due to pH effect in addition to the protonated acetic acid effect.

Acidification delayed but did not inhibit growth of naturally occurring LAB within the cucumbers in comparison to nonacidified cucumbers (Fig. 2). The buffering treatment slightly delayed growth of LAB. Heterofermentative species of LAB were predominant (80%) in fresh cucumbers and during the first day after brining (60–100%) for all treatments (Table 1). At 3 d, homofermentative species constituted >99% of the LAB in acidified and buffered cucumbers and brines. Homofermentative species constituted 50% of the LAB after 3 d in nonacidified cucumbers, but at 5 d were >99%.

Total aerobes (APC) within the fresh cucumbers were about $10^5$ CFU/g, about 100-fold greater than could be accounted for by the LAB and the *Enterobacteriaceae* (Fig. 3). Aerobic and anaerobic spore formers, yeasts, and molds are other microbial groups that have been enumerated from fresh cucumbers (Etchells et al., 1973a). At day 3, the APC of the brines in all treatments was higher than either the LAB or the *Enterobacteriaceae* population. Possibly, we were not enumerating for the predominant group of microorganisms or that part of the population of microorganisms was acid-injured. Since we were plating LAB and the *Enterobacteriaceae* on selective media, injured microorganisms may not have been able to grow (Busta, 1976). When $10^6$ CFU/mL of *Enterobacter aerogenes* were inoculated into buffered or acidified cucumber juice, $10^2$ to $10^4$ CFU/mL of injured microorganisms were recovered (data not shown). At 5 d, APC and LAB counts were similar. The APC for cucumbers was similar for all brining treatments, and all treatments reflected a gradual increase in numbers during the 5-d incubation.

### Inoculated, brined cucumbers

LAB cultures added to freshly brined cucumbers decreased in numbers by 10- to 1,000-fold during the first 10 hr (Fig. 4), depending upon the bacterial strain. The chemical environment of the brine into which the cultures were added had a high salt concentration (11.4%) and lacked nutrients (Table 1). The viable counts were similar 10 and 24 hr after brining and then increased as nutrients became available and salt concentration diminished. The *L. plantarum* MDC⁻ culture was most affected during the first 10 hr, while its MDC⁺ parent was least affected. When the microorganisms were inoculated into cucumber juice (high nutrient level) with 11.4% salt, survival of both microorganisms increased. However, *L. plantarum* MDC⁻ remained less competitive than its MDC⁺ parent (unpublished data). Hardiness of the culture obviously was influenced by the mutation procedure, which was expected for mutants obtained from N-nitrosoguanidine exposure.

Data in Fig. 2 and 4 illustrate the dilemma as to when and how to add cultures of LAB to have them predominate in the fermentation. In the brining procedure developed for the anaerobic tank procedure (Fleming et al., 1988), we intended that all additives be incorporated into the tank before closure to simplify commercial operations. We wanted the MDC⁻ culture to predominate since it did not produce $CO_2$ from malic acid. Predominant fermentation by such a bacterium could obviate the need for purging to prevent bloater damage of the cucumbers. In the above brining procedure, however, the starter culture is added to an initial harsh environment, unfavorable for growth. Most naturally occurring LAB are located within the cucumber tissue and may become established during this period.

The *L. plantarum* WSO (MDC⁺) strain has been tested in pilot-scale commercial tests (Fleming et al., 1988). The culture increased nearly 10-fold within 24 hr with an initial salt concentration of 11.4%, and over 100-fold with initial salt concentration of 6.8%. In both instances, however, naturally occurring LAB predominated after 1 to 3 days. Whether the natural flora predominated due to being "protected" within the cucumbers initially, or simply were more competitive in the environment was not clear.

Various options are being considered to resolve the dilemma. These include development of cultures to withstand the initial harsh environment, delay of culture addition, inoculation of the cucumbers prior to brining, addition of nutrients to the cover brine to prevent cell starvation, reduction in the initial salt concentration, and others. Our goal is development of an inoculation procedure that will not compromise the simplicity of commercial operations in closed tanks, nor the conditions favorable for growth and predominance of added cultures.

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