Preparation of a *Lactobacillus Plantarum* Starter Culture for Cucumber Fermentations That Can Meet Kosher Guidelines

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Abstract: A method is described for growth of a *Lactobacillus plantarum* starter culture in jars of commercially available pasteurized fresh-pack kosher dill cucumbers so that jars can be used to inoculate commercial scale cucumber fermentation tanks. A procedure is also described to transfer lactic acid bacteria from frozen storage in MRS broth into cucumber juice and commercial jars of kosher dill cucumbers so that a selected strain of lactic acid bacteria can be kosher certified for commercial fermentations in processing plants that operate under kosher certification. The strain of *L. plantarum* used in these experiments grew to maximum cell numbers in 4 d at 20 to 25 °C and then maintained viable cell numbers for 2 wk at $>10^8$ CFU/mL so the culture was suitable for inoculation of fermentation tanks. Refrigeration of jars of culture after they grow to maximum numbers minimizes die-off of cells sufficiently so that a pure culture can be maintained by aseptically transferring brine containing viable bacteria to a new pH-adjusted jar only once every 4 mo.

Keywords: fermentation, lactic acid bacteria, *Lactobacillus*, starter culture

Practical Application: This report describes a method to prepare a lactic acid bacteria starter culture suitable for kosher vegetable fermentations.

Introduction

Most processors that ferment cucumbers for hamburger dill chips and other processed pickle products rely upon the fact that the salt used in brining of cucumbers prevents the growth of spoilage bacteria, but allows growth of homofermentative lactic acid bacteria that are present on fresh cucumbers. However, addition of a starter culture to cucumber fermentations has been used by some processors to obtain more rapid and consistent fermentations. Starter cultures specifically selected for cucumber fermentations are not produced commercially. Lactic acid bacteria starter cultures that are produced commercially are often grown in a whey based-medium, which cannot be used for the manufacture of parve kosher products in production plants with kosher certification. A second and less commonly recognized issue with regard to kosher certification is that lactobacilli are typically isolated and propagated on deMan Rogosa and Sharpe (MRS) medium, which contains non-kosher animal derived ingredients. Moreover, such cultures are often stored long term as frozen stocks containing glycerol from an animal source in addition to MRS broth.

Availability of a starter culture that could meet requirements for kosher certification and that grows rapidly under the low pH, high salt conditions in commercial fermentation tanks would allow more processors to use a starter culture to improve the consistency of fermentations. In addition, the availability of a starter culture suitable for kosher certification will be a necessary component to develop a process to carry out cucumber fermentations in brines that contain no NaCl, but use $CaCl_2$ to maintain cucumber firmness during fermentation and subsequent storage (McFeeters and Pérez-Díaz 2010). Thus, this study was motivated by the need to ferment cucumbers in brines with calcium chloride, but no NaCl (McFeeters and Pérez-Díaz 2010) with a *Lactobacillus plantarum* strain that had been shown to rapidly grow and ferment cucumbers (McDonald and others 1993). This *L. plantarum* strain was originally isolated from a commercial pilot scale tank because it was the dominant microorganism found in a cucumber fermentation that had been inoculated with 10^5 CFU/mL of a naturally occurring streptomycin resistant *L. plantarum* strain, which was the preferred strain for inoculation of cucumber fermentations at that time (Fleming and others 1988).

Relatively large quantities of culture are required for pilot scale fermentations in 240 L barrels as well as for commercial cucumber fermentations in up to 40000 L tanks. The culture must be produced from frozen stocks in MRS broth in a way that would be suitable for kosher certification. This note describes a procedure to grow this culture aseptically from frozen stocks in up to 3.8 L (1 gallon) quantities such that the culture may receive kosher certification. This volume of culture would be sufficient to inoculate a 40000-L (10000 gallon) tank of cucumbers with 10^4 to 10^5 CFU/mL compared to typical numbers of only 10^1 to 10^2 CFU/g naturally occurring lactic acid bacteria on fresh cucumbers. The approach used to grow this strain in quantities sufficient to inoculate tanks of several thousand gallons should be applicable to other lactic acid bacteria. Also, the procedure used to transfer this *L. plantarum* strain from frozen storage in MRS

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broth and glycerol into cucumber juice in a manner that would be suitable for kosher certification should be generally applicable for other lactic acid bacteria.

Materials and Methods

L. plantarum and growth medium

L. plantarum LA0445 was obtained from the culture collection of the U.S. Dept. of Agriculture-Agricultural Research Service, Food Science Research Unit, located in Raleigh, N.C., U.S.A. Standard cultures were prepared on MRS agar (Becton, Dickinson and Co., Sparks, Md., U.S.A.) from stock cultures frozen in MRS broth supplemented with 10% glycerol (Sigma-Aldrich, Saint Louis, Mo., U.S.A.), from which isolated colonies were selected for further propagation. Cultures were propagated twice before used for experimental purposes. Agar plates and broth cultures were incubated for 24 h at 30 °C under aerobic conditions.

Preparation of cucumber juice

Fresh cucumber juice was expressed from two lots of size 1B pickling cucumbers (20 to 25 mm diameter) using an automatic juice extractor (JM400 Juiceman Jr., Black and Decker, Towson, Md., U.S.A.). The extracted juice was frozen until used, thawed and centrifuged for 1 h at 9000 rpm (25 °C) using an Eppendorf Centrifuge 5810 R (West Bury, N.Y., U.S.A.) to remove solids, and then filtered sterilized through a 0.22 μ filter (Millipore, Billerica, Mass., U.S.A.).

Transfer of a starter culture strain for kosher certification

The following procedure for transfer of a bacterial strain from a frozen culture in MRS broth with added glycerol was developed in consultation with the Orthodox Union (Rabbi Menachem Adler and Rabbi Gavriel Price, personal communication). A sterile loop was touched to the surface of a frozen culture of *L. plantarum* LA0445 stored at −80 °C. The cells were gently streaked on the surface of an MRS agar plate, taking care not to disturb the surface. The inoculated plate was then incubated at 30 °C for 48 h to allow individual colonies to grow such that the surface of the bacterial colony was slightly above the surface of the agar. A new sterile loop was used to transfer bacteria at the surface of a colony into filter sterilized cucumber juice. The inoculated cucumber juice was incubated at 30 °C for 48 h until the inoculated juice was very turbid as a result of growth of the inoculated *L. plantarum* cells.

Growth of starter culture in pasteurized kosher dill pickles

Jars of pasteurized kosher dill pickles (spears or whole cucumbers) were purchased from local grocers or obtained from a local processor. A total of 4 different experiments with jar sizes of 480 mL (24 oz), 1360 mL (46 oz), 2400 mL (80 oz), and 3840 mL (1 gallon) were used. These pickles were preserved in a brine that contains salt, vinegar (0.6% to 0.8% acetic acid), sodium benzoate, FD&C yellow nr 5 food coloring and spices with pH in the range of 3.6 to 4.0. The pH of the commercial pickles was aseptically adjusted to 5.1 to 5.4 by addition of 8 N NaOH solution (Sigma-Aldrich Chemical Co.) to allow rapid growth of the starter culture. The amount of NaOH required for pH adjustment was determined by titrating a sample of the cover brine solution to the target pH and then adding the calculated amount of base to a jar. After addition of the NaOH, jars were held for 48 h with occasional mixing at ambient temperature to allow for pH equilibration between the cucumbers and cover brine solution.

Initial inoculation of *L. plantarum* LA0445 into pH-adjusted kosher dill pickle jars was done by transferring cells from either a cucumber juice culture or from an MRS broth culture after incubation for 24 h at 30 °C. The bacteria were removed from either growth media by centrifugation at 3824× *g*. The cell pellet was washed twice with saline solution and centrifuged. The final cell pellet was resuspended into an equal volume of saline solution. Jars were inoculated with the washed cells to an initial population of 10^5 CFU/mL.

After the initial inoculation and growth of culture in pH-adjusted kosher dill pickles, cultures were maintained at 4 °C or used as a source of inoculum for new jars. Cucumber jars in which the inoculum had reached high cell numbers were shaken to resuspend the bacteria, and aseptically handled to transfer 1 mL of brine to a new pH-adjusted jar of cucumbers for each liter of volume of the new jar. For example, a 3840-mL pH-adjusted jar of cucumbers was inoculated with 3.8 mL of brine containing the *L. plantarum* cells. In this way, the culture can be maintained and multiplied provided the jar to jar inoculations are done aseptically. Triplicate jars from 3 different production lots of kosher dill pickles were inoculated for each trial. Inoculated jars were incubated at ambient temperature for 3 to 4 d until maximum cell densities were reached and then refrigerated (4 °C).

Monitoring microbial growth and survival

Jars inoculated with *L. plantarum* were visually monitored for the development of turbidity in the cover brine solution. After turbidity developed in a container, brine samples were taken using aseptic techniques for microbiological analysis. Samples were plated on MRS agar plates and incubated at 30 °C for 48 h. Decline in pH, which reflected the extent of growth due to lactic acid production, was monitored by measurement of the pH of samples with an Accumet pH meter (Cole-Palmer, Vernon Hills, Ill., U.S.A.) with a combination electrode. The progression of the fermentation was also monitored by high-performance liquid chromatography (HPLC) analysis (McFeeters and Barish 2003) to determine the decline in the concentrations of glucose, fructose, and malic acid, the main fermentation substrates, and the formation of lactic acid.

Results and Discussion

Pasteurized fresh-pack dill pickles were selected as a readily available food grade growth medium for a *L. plantarum* strain to be used as a starter culture in cucumber fermentations. These dill pickles are commercially sterile, acidified products with a usual pH in the range of 3.5 to 4. Pickles of this type have up to a 2-y shelf life at ambient temperature. Therefore, at a processing plant it is not necessary to have the capability to sterilize media to maintain a starter culture without contamination. Since the cucumbers are not fermented, there is sufficient sugar to serve as the primary fermentation substrate and other required nutrients for lactic acid bacteria to grow to high numbers. If the starter culture is to be used to ferment cucumbers or other vegetables with kosher certification, use of kosher certified pickles as the growth medium will assure that the culture will be produced using the appropriate ingredients. Provided the starter culture grows to 10^8 to 10^9 CFU/mL, a 10000-fold volume of product can be inoculated to start a commercial fermentation with one volume of culture.

The procedure for transfer of a *L. plantarum* strain, that had previously been found to be a desirable fermentation organism for cucumber fermentations (McDonald and others 1993) from frozen

storage to a cucumber based medium, was done in consultation with representatives of the Orthodox Union (New York, N.Y., U.S.A.) such that when done under appropriate supervision a starter culture may receive kosher certification. This procedure maintains normal microbiological standards to assure the strain does not become contaminated with other microorganisms.

When jars of commercial dill pickles were inoculated with *L. plantarum* culture without pH adjustment (initial pH 4 ± 0.1), cell counts increased to a maximum of 5×10^8 CFU/mL, pH was reduced by approximately 0.3 units, and less than 100 mM lactic acid was produced 8 d after inoculation of the jars. The viable cell numbers then decreased by 5 logs in the following week (Figure 1, Panel A, open circles). Adjustment of the initial pH to 5.2 with NaOH resulted in maximum cell numbers near 10^9 CFU/mL, a decrease in pH from 5.2 to 4.2, and more than 100 mM lactic acid produced 5 d after inoculation (Figure 1). Cell numbers declined much more slowly so viable cell counts were still near 10^8 two weeks after inoculation. A total of 1 L of starter culture would provide an inoculum at 10^4 CFU/mL for 10000 L of brined cucumbers.

The pH of jars decreased from 5.2 at the time of inoculation to 4.1 ± 0.2 when cell numbers increased to the maximum levels. The effect of raising the pH back to 5.2 prior to refrigeration to improve survival of the *L. plantarum* during refrigerated storage was investigated. Figure 2 shows that raising the pH did not improve cell survival in the refrigerated dill pickle jars. Regardless of the pH, die-off of the culture began to accelerate after 7 mo. This result indicated that it would not be beneficial to raise the pH after growth of a culture.

The effect of temperature on the growth of starter culture in dill pickles adjusted to pH 5.2 is shown in Table 1. Between 20 and 30 °C, the maximum cell numbers were slightly above 5×10^8 CFU/mL. However, viable cell counts decline more rapidly as temperature increased. An incubation temperature of 20 to 25 °C will result in a starter culture with greater than 10^8 CFU/mL from 3 to 10 d after inoculation of cucumbers adjusted to pH 5.2.

Continued production of a starter culture during a brining season in an industrial setting will require that the culture be aseptically transferred and re-grown in new jars of pH-adjusted dill pickles so that a culture is available as tanks are filled with cucumbers. Figure 3 shows that this can be done. Panel A shows

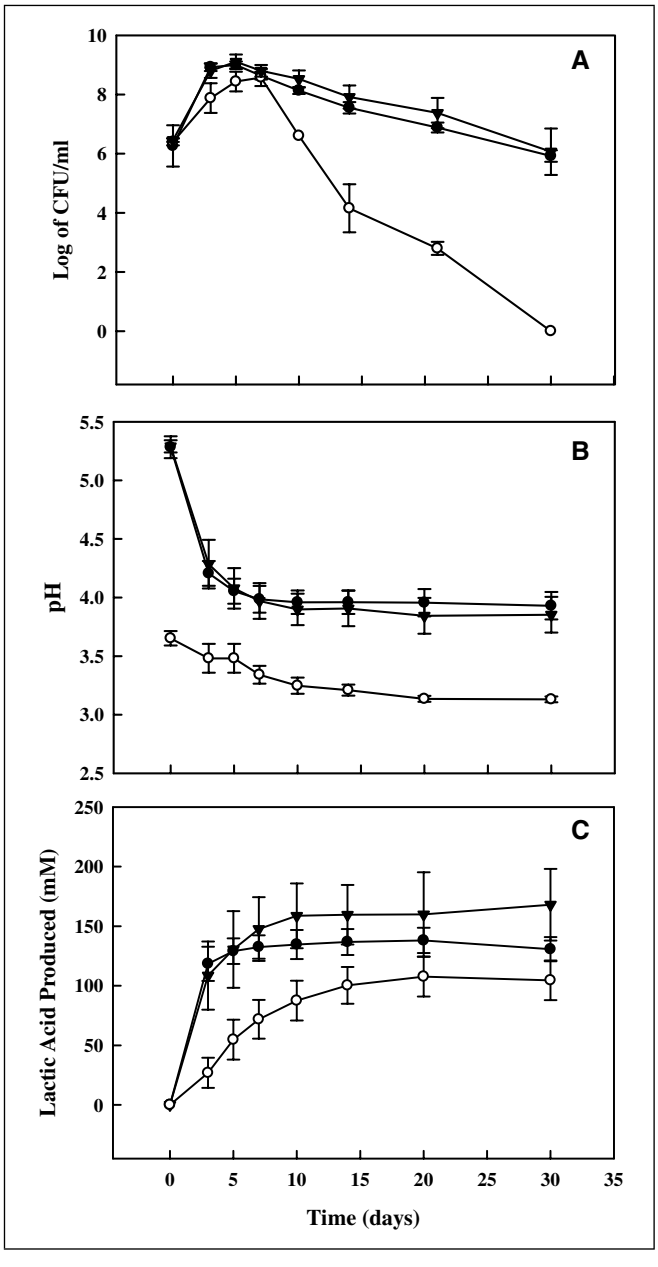

Figure 1–Changes in viable cell numbers, pH, and lactic acid production of a *L. plantarum* LA0445 culture inoculated at 10^5 CFU/mL in pasteurized kosher dill pickles from companies A (●, ○) and B (▲) in which the initial pH was adjusted to 5.2 (filled symbols) or inoculated into jars without pH adjustment at pH 4 ± 0.1 (open circles). The average and range of duplicate jars are shown at each sampling time.

Table 1–Effect of incubation temperature on the growth of *L. plantarum* LA0445 in jars of kosher dill spears.

Incubation temperature (°C)	Cell counts (log of CFU/mL)		
	Day 3	Day 6	Day 10
30	8.57 ± 0.12	8.09 ± 0.23	7.09 ± 0.39
25	8.69 ± 0.11	8.77 ± 0.06	8.16 ± 0.05
20	8.42 ± 0.13	8.75 ± 0.12	8.79 ± 0.16

Standard deviations shown were calculated considering the log of CFU per milliliter for 3 independent jars.

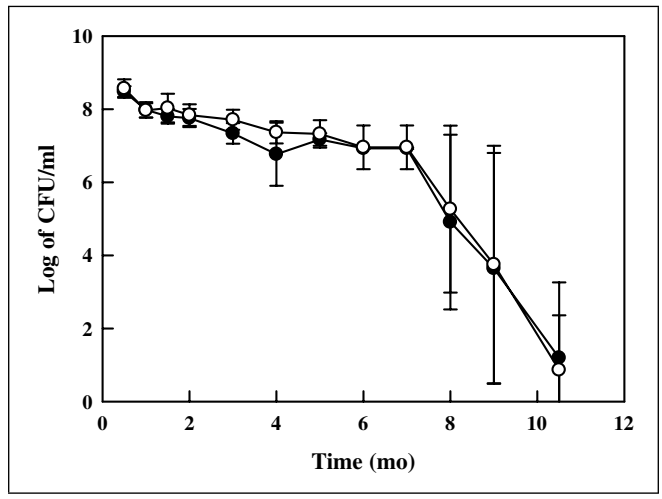

Figure 2–*L. plantarum* LA0445 survival under refrigeration. Survival of a *L. plantarum* starter culture in kosher dill spear jars in which initial pH was adjusted to 5.2 and the final post-fermentation pH was maintained at 3.8 ± 0.2 (○) or re-adjusted to 5.2 ± 0.1(●). The averages of independent triplicates are presented.

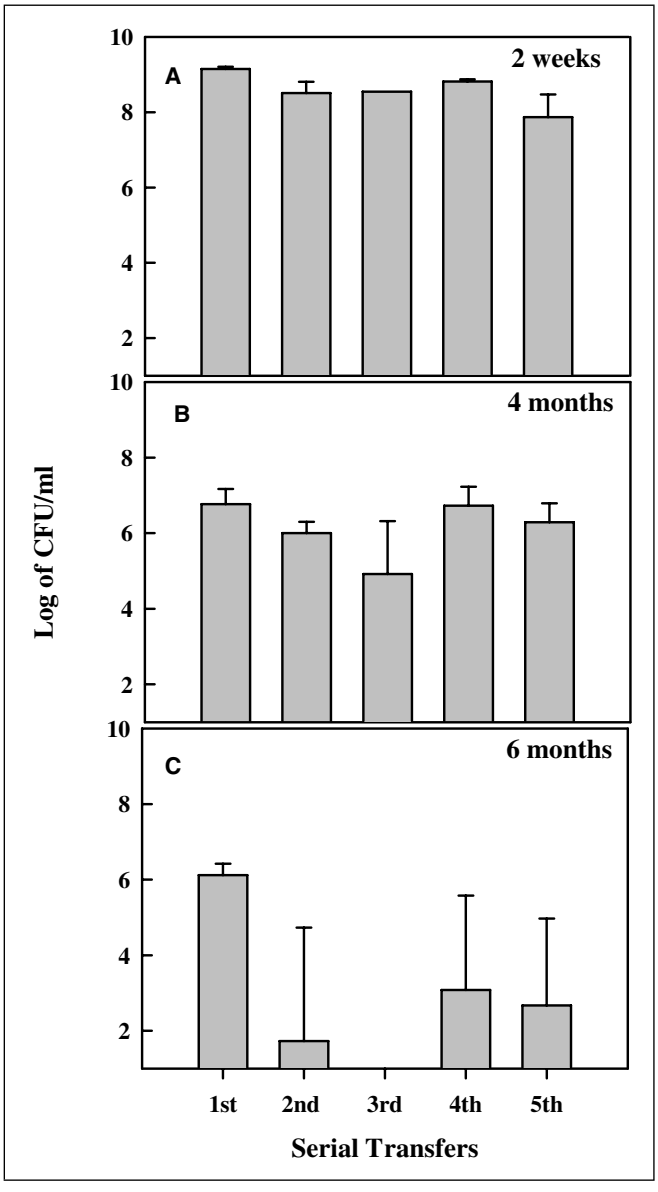

Figure 3–*L. plantarum* survival under refrigeration upon sequential inoculation of commercial kosher dill spear jars. *L. plantarum* was sequentially transferred 5 times to independent triplicate jars. There was a 2-wk refrigeration period between inoculations. Jars were maintained at ambient temperature (25 °C/77 °F) for 4 d immediately after inoculation and then refrigerated for 11 d prior to the initial sampling. The A, B, and C panels show the survival of *L. plantarum* during refrigeration at 2 wk, 4 mo, and 6 mo after inoculation, respectively.

the serial transfer of culture to new jars every 2 wk through 5 transfer cycles. After inoculation jars were incubated for 4 d at ambient temperature (approximately 25 °C), they were then refrigerated for 10 d. After 14 d viable cell counts were at or above 10^8 CFU/mL. These jars would allow inoculation of 10000 L of brined cucumbers with a liter of culture to give an initial inoculation of 10^4 CFU/mL in a fermentation tank.

The extent of *L. plantarum* survival in refrigerated kosher dill pickle jars was determined, to minimize the number of transfers required for a possible long-term maintenance routine, so that the opportunities for contamination of a culture with foreign microorganisms is reduced. Panel B of Figure 3 shows that viable cells remained at numbers in the range of 10^5 to 10^7 after refrigeration for 4 mo. These numbers would not be sufficiently high to inoculate tanks, but would be adequate for the inoculation of new jars of pH-adjusted cucumbers with 10 to 20 mL of brine from the refrigerated jars. In this way, a starter culture could be maintained with only 3 transfers per year by allowing the cells to grow to high numbers and then holding them at 4 °C. Panel C of Figure 3 shows that after 6 mo of refrigerated storage, cells died off to such low numbers that it might not be possible to maintain a culture.

The procedure used to transfer this *L. plantarum* strain from frozen storage in MRS broth and glycerol into cucumber juice in a manner that would be suitable for kosher certification should be generally applicable for other lactic acid bacteria. For instance, the type strain of *Leuconostoc mesenteroides* ATCC8293 has been found to grow to numbers $>10^9$ CFU/mL in 4 d at a temperature of 22 °C in pH-adjusted kosher dill pickles (data not shown).

Guidelines for the preparation of a *L. plantarum* starter culture for vegetable fermentation are available online at: http://www.ars.usda.gov/SP2UserFiles/Place/66451000/GuidelinesforthePreparationofaStarterCultureforCucumberFermentationTanks.pdf.

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