

Pure Culture Fermentation of Brined Cucumbers¹

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ABSTRACT

ETHELLES, J. L. (North Carolina Agricultural Experiment Station, Raleigh), R. N. COSTILOW, T. E. ANDERSON, AND T. A. BELL. Pure culture fermentation of brined cucumbers. *Appl. Microbiol.* 12:523-535. 1964.—The relative abilities of *Pediococcus cerevisiae*, *Lactobacillus plantarum*, *L. brevis*, and several other species of lactic acid bacteria to grow and produce acid in brined cucumbers were evaluated in pure culture fermentations. Such fermentations were made possible by the use of two techniques, gamma radiation (0.83 to 1.00 Mrad) and hot-water blanching (66 to 80 C for 5 min), designed first to rid the cucumbers of naturally occurring, interfering, and competitive microbial groups prior to brining, followed by inoculation with the desired lactic acid bacteria. Of the nine species tested, strains of the three common to cucumber fermentations, *P. cerevisiae*, *L. plantarum*, and *L. brevis*, grew to the highest populations, and produced the highest levels of brine acidity and the lowest pH values in fermentations at 5.4 to 5.6% NaCl by weight; also, their sequence of active development in fermentations, with the use of a three-species mixture for inoculation, was in the species order just named. This sequence of occurrence was similar to that estimated by others for natural fermentations. The rates of growth and acid production in fermentations with a mixture of *P. cerevisiae*, *L. plantarum*, and *L. brevis* increased as the incubation temperature was increased from 21 to 27 to 32 C; however, the maximal populations and acidities attained were essentially the same for fermentations at each temperature. Further, these same three species were found to be the most salt tolerant of those tested; their upper limit for appreciable growth and measurable acid production was about 8% salt, whereas thermophilic species such as *L. thermophilus*, *L. lactis*, *L. helveticus*, *L. fermenti*, and *L. delbrueckii* exhibited a much lower salt tolerance, ranging from about 2.5 to 4.0%. However, certain strains of *L. delbrueckii* grew very rapidly in cucumbers brined at 2.5 to 3.0% salt, and produced sufficient acid in about 30 hr at 48 C to reduce the brine pH from above 7.0 to below 4.0. An inexpensive, pure culture fermentor which was suitable for gamma radiation, resistant to salt and acid, and which permitted repeated aseptic sampling of the fermenting brine, is illustrated and the specifications are given.

be quite complex and highly variable. The lactic acid fermentation usually involves at least three species of acid-forming bacteria, namely, *Pediococcus cerevisiae*, *Lactobacillus plantarum*, and *L. brevis* (Pederson and Albury, 1950; Pederson and Ward, 1949; Borg, Etchells, and Bell, 1955; Costilow et al., 1956). The active fermentation is further complicated by the frequent participation of *Aerobacter* (Etchells, Fabian, and Jones, 1945) and of both fermentative and oxidative yeasts (Etchells and Bell, 1950a, b; Etchells, Costilow, and Bell, 1952). Such heterogeneous microbiological activity leads to much variation between fermentations. Gaseous fermentations by yeasts, coliform bacteria, and gas-forming lactic acid bacteria result in the formation of "bloaters" (hollow cucumbers; Jones et al., 1940; Etchells et al., 1945; Etchells and Bell, 1950a; Borg, Etchells, and Bell, 1956; Costilow, 1957); and oxidative yeasts growing on the surface of brines may reduce the lactic acid levels sufficiently to allow the growth of other types of spoilage organisms.

There have been recent attempts to control the lactic fermentation of brined cucumbers by inoculation of natural fermentations with various species of lactic acid bacteria (Pederson and Albury, 1956, 1961), and some effects were evident on the flora present. However, there is no way in such a heterogeneous natural microflora to determine with reasonable certainty the interactions of the various microbial groups and species and to ascertain whether effective control of the fermentation proper was actually obtained as the direct result of the inoculated organism. No previous attempts to study pure culture fermentations of brined cucumbers in the absence of undesirable and competitive microorganisms have come to our attention. However, a process for pure culture fermentation of shredded cabbage for sauerkraut was patented (Engelland, 1962).

Herein are reported the results of extensive studies of (i) the efficiency of a number of species of lactic acid bacteria to ferment irradiated cucumbers covered with sterile brine; (ii) interactions occurring between species of lactic acid bacteria in the same pure culture fermentation; (iii) the effects of temperature and salt concentration on the pure culture lactic acid fermentation; (iv) the role of lactic acid in controlling the growth of coliform bacteria in brined cucumbers; and (v) the effectiveness of hot-water blanching treatments for cucumbers to control interfering microbial groups in pure culture fermentations.

Natural fermentations of brined cucumbers have been studied extensively, and their microbiology found to

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MATERIALS AND METHODS

Immature cucumber fruit, consisting of no. 1 size Model variety [diameter, 0.75 to 0.87 in. (1.9 to 2.2 cm)], was obtained in 30- to 40-lb (13.6 to 18.1 kg) amounts as needed during the 1960 and 1961 growing seasons from a cucumber receiving and grading station located at Zebulon, N.C., about 20 miles from the laboratory. This material was used for all fermentation studies where irradiated or heat-blanced cucumbers were used; quart jars of pasteurized no. 1 size SR-6 variety of cucumbers were used for the salt-tolerance tests. The details of the three treatments used to prepare the cucumbers for pure culture fermentation, together with the brining, inoculation, and sampling procedures, are given below.

Irradiated series. When gamma radiation was used to rid the cucumbers of undesirable and competitive microbial groups prior to pure culture fermentation, the cucumbers were packed into specially made polyethylene fermentors (Fig. 1) and were irradiated in a Gammacell-220 (Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada). All received 0.83 Mrad of gamma radiation unless otherwise indicated. Previous data (Etchells et al., 1961) demonstrated that this exposure would destroy essentially all microorganisms on cucumbers except bacterial endospores; these organisms would not be considered competitive in acid fermentations even at low brine strengths. Sterilized brine (11.2% NaCl for most experiments) was inoculated with the desired culture(s) and was added to the fermentors aseptically. The ratio of brine to cucumber was such that salt concentration usually equalized at about 5.5%. The brined cucumbers were usually incubated at 32 C, except that 48 C was used for thermophilic fermentations. The fermentors were rotated back and forth between the hands 20 times before sampling to ensure suspension of the microbial cells in the brine. Samples were removed at frequent intervals during the fermentation and storage period by use of a sterile syringe and needle through the rubber serum stopper located in the side of the fermentor (Fig. 1). In a few instances, fermentors were inoculated in the same manner.

Blanched series. Hot-water blanching was also investigated as a means of controlling competitive microorganisms occurring naturally on cucumbers. This was accomplished by placing 4.5 to 5 lb (2 to 2.3 kg) of washed cucumbers in a deep-fry wire basket [diameter, 11 in. (27.9 cm); depth, 5 in. (12.7 cm)], fitted with a hardware cloth top, and immersing them for 5 min in about 50 gal (189.2 liters) of water maintained at the desired temperature in a steam-jacketed kettle. To ensure even heating of the cucumbers, the basket was kept in constant motion during the exposure time, and the water was circulated. Next, the cucumbers were packed hot into previously steamed (30 min) and cooled 1-qt (0.946 liter) glass jars by use of sterile tongs. Boiled and cooled brine containing sufficient amounts of salt to equalize at the

desired concentration was then inoculated with the culture desired and was poured over the packed cucumbers with the use of aseptic precautions. For acidified brines, 2 ml of 85% lactic acid were added per liter. The packed, brined, and inoculated jars were then sealed with four-lug, "Twist-Off" metal closures (diameter, 70 mm; White Cap Co., Chicago, Ill.), which were steamed 10 min before use. All jars were incubated at 32 C and were sampled at the time intervals indicated in Tables 2 and 3 by use of sterile 5-ml pipettes.

Pasteurized series. Pasteurized cucumbers used for studying the effect of eight different salt levels on the growth of lactic acid bacteria were prepared as follows: First, 12 to 14 cucumbers were packed into a series of 1-qt jars containing sufficient amounts of dry salt to equalize at the approximate brine strengths desired (i.e., 0, 4, 6,

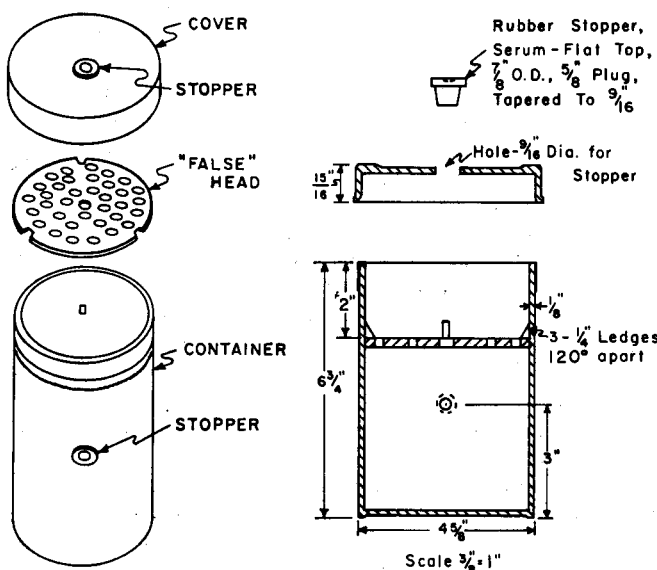


FIG. 1. Pure culture fermentor for brined cucumbers. The container was fabricated from a 2-qt (1.9 liter) size polyethylene jar (Nalge Co., Inc., Rochester, N.Y.) to the specifications shown, and had a snug-fitting, friction-type cover of the same material, which was further sealed in place during fermentation with a 1-in. (2.54 cm) wide rubber band. A perforated, circular "false" head was fashioned from sheet polyethylene, and was shaped so as to fit inside the container and hold the cucumbers beneath the brine. The head was slotted at three points equidistant around the edge, and thus could be keyed in place by pressing down and turning it below three corresponding triangular polyethylene ledges which were heat-sealed to the side of the container. The fermentor was supplied with two tight-fitting rubber serum stoppers; one was located in the center of the cover to permit the escape of fermentation gases through an inserted sterile, 20-gauge hypodermic needle plugged with cotton, and the other was located in the side of the container, and was used for sampling the fermenting brine by means of a sterile 10-ml syringe fitted with either a 20- or 22-gauge needle. The fermentor, suitable for radiation and resistant to salt and acid, conveniently held 550 to 600 g of no. 1 size [diameter, 0.75 to 0.87 in. (1.9 to 2.2 cm)] cucumbers, and 530 to 550 ml of brine brought the liquid level approximately 0.5 in. (1.27 cm) over the head. For use, the containers were filled, irradiated, brined, and inoculated with pure cultures as described in Materials and Methods.

8, 10, 12, 16, and 20% NaCl); next, lactic acid (85%) was added to each container in an amount calculated to equalize at 0.40%. The jars were then filled with water, sealed (as described for the blanched series), shaken to dissolve the salt, and pasteurized in a commercial unit which gave an internal product temperature of 74 C. The jars were promptly cooled in water to less than 38 C. Prior to use, sufficient sterile NaOH was added to each jar to reduce the acidity to about 0.25%, and was allowed to equalize overnight before inoculation. All jars were incubated at 32 C and were sampled by use of sterile 5-ml pipettes at eight intervals during the active fermentation and storage period (2, 4, 6, 9, 11, 13, 20, and 25 days). The brines were examined for total acidity and populations of acid-forming bacteria at each sampling interval for all salt levels, but only data from samplings pertinent to establishing the relative salt-tolerance of the species tested are reported.

Cultures used. Unless otherwise specified (see Table 1) the following cultures of lactic acid bacteria isolated from cucumber fermentations (Costilow et al., 1956) were used for the pure culture fermentations: *P. cerevisiae* (FBB-61), *L. plantarum* (FBB-67), and *L. brevis* (FBB-70); all strains denoted as FBB were from this source. Those labeled AF were isolated from cucumber fruit or natural fermentations in the U.S. Food Fermentation Laboratory, Raleigh, N.C. [These cultures were all gram-positive, elongated cocci or very short rods, microaerophilic, catalase-negative, and produced acid from simple sugars. Thus, they were classed as lactic acid bacteria, but could not be identified with any of the species described in *Bergey's*

Manual (Breed, Murray, and Smith, 1957). The individual cultures were representative of different groups, varying primarily in carbohydrates fermented.] The cultures with L numbers were isolated by Borg et al. (1955) from commercial cucumber fermentations. The NRRL-designated cultures were obtained from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill. Stock cultures were maintained in stabs of Trypticase Sugar Agar (TSA; BBL); for inoculation of brined cucumbers, 24- to 36-hr cultures were grown in a liquid medium of the same composition, except that the agar was omitted and 2 to 6% salt was added (TS broth). Cultures of mesophilic species were incubated at 32 C, and thermophilic species, at 48 C.

Enumeration and species separation of lactic acid bacteria. Total population estimates of lactic acid bacteria in most of the pure culture and control fermentations were determined by plating decimal dilutions of the brine on TSA plus 0.5% yeast extract and 0.01% bromocresol green dye; the medium was adjusted to pH 5.6 to 5.7 at the time of preparation. For fermentations with blanched cucumbers (Table 2 and 3), LBS agar (BBL) was used, but with the modification we found necessary to prevent marked inhibition of certain species of lactic acid bacteria (Costilow, Etchells, and Anderson, 1964). The cited modification consisted essentially of careful adjustment of the pH to 5.6 ± 0.05 , plus the addition of 0.0075% bromocresol green dye to aid in colony counting and detection of acid production. In its modified form, LBS agar was employed successfully for many pure culture and natural fermentations during the 2-year study as a companion

TABLE 1. Summarized results for 34 pure culture fermentations of brined cucumbers^a conducted with various species of lactic acid bacteria

Species	Strains tested	No. of fermentations	Fermentation temp	Equalized brine strength ^b	Range of		
					Maximal populations ^c	Maximal acidities ^d	Final pH
<i>Pediococcus cerevisiae</i>	FBB-61, 39, NRRL B-1325, 1326	8	32	5.4-5.6	182-590	0.46-0.63	3.4-3.6
<i>Lactobacillus plantarum</i>	FBB-12, 67, L-346, 442	5	32	5.4-5.6	520-920	0.66-0.91	3.2-3.5
<i>L. brevis</i>	FBB-50, 70, L-106, 336	6	32	5.4-5.6	61-285	0.25-0.54	3.7-4.4
Unidentified lactics from cucumber fruit	AF-18, 139, 177	3	32	5.4-5.6	43-109	0.27-0.31	4.3-4.6
Unidentified lactics from natural fermentations	AF-287, 362	2	32	5.1-5.6	197-308	0.36-0.49	4.1-4.3
<i>L. delbrueckii</i>	NRRL B-763, 445, 1658, 1933, 1934	5	48	2.5-2.9 ^e	14-290	0.45-0.70	3.7-4.4
<i>L. thermophilus</i>	NRRL B-1952	2	48	2.5 ^f	1-32	0.31-0.51	4.1
<i>L. lactis</i>	NRRL B-736	1	48	2.8	14	0.41	4.0
<i>L. helveticus</i>	NRRL B-1842	1	48	2.8	20	0.54	3.9
<i>L. fermenti</i>	NRRL B-585	1	48	2.9	108	0.47	4.1
<i>L. bulgaricus</i>	NRRL B-734	1	48	3.0	No growth	No growth	6.4

^a Cucumbers received 0.83 Mrad of gamma radiation in polyethylene fermentors (Fig. 1) prior to brining and inoculation.

^b Expressed as per cent NaCl.

^c Expressed as number per milliliter $\times 10^{-6}$.

^d Expressed as per cent lactic acid.

^e *L. delbrueckii* failed to grow in brines of 3.8% NaCl and higher.

^f *L. thermophilus* grew very poorly in brines of 3.8% NaCl.

medium to check the population estimates obtained with TSA.

When mixed inocula of lactic acid bacteria were used for pure culture fermentations, differential colony counts of the individual species present were made by using variations in TSA with respect to composition of ingredients. Actual selectivity was based on certain biochemical properties of the species to be detected. For example, of the three species used in the same fermentation, *L. plantarum* was the only one which would ferment lactose; therefore, it was estimated by plating on a medium of the same composition as TSA, except that lactose (1%) was the only fermentable sugar present. Polymyxin-B (15 ppm) in TSA adjusted to pH 5.4 to 5.5 completely inhibited the growth of *L. brevis* without effect on *L. plantarum* and *P. cerevisiae*; thus, the difference between counts on regular TSA and TSA plus polymyxin-B was used as the estimate of the numbers of *L. brevis*. Finally, the difference between the sum of the estimated numbers obtained for *L. plantarum* and *L. brevis* and the total number obtained on TSA was the estimate of the *P. cerevisiae* population. Tests of this method with known populations of mixtures of cells of the three species named indicated that it gave reasonable estimates of the numbers of the individual species present.

Control measures for irradiated series. The inoculated containers and controls were sampled at frequent intervals during the fermentation and storage period. In a number of experiments, the brines were examined for microbial groups other than the introduced pure cultures, namely, total aerobes, coliform bacteria, yeasts, and molds. Measurements for optical density, acidity, pH, and salt content were made on brines from all experiments with irradiated cucumbers. In addition, for certain experiments in the irradiated series, separate samples of cucumber fruit, representative of that in the containers, were checked for the presence of the same microbial groups plus lactic acid bacteria and the number of surviving aerobic and anaerobic spores. The same examination procedure was usually followed for irradiated, noninoculated brined controls.

Thus, in this manner a check was maintained on the effectiveness of the cucumber irradiation treatment to rid the fruit of competitive microorganisms, as well as on the technique of brining, inoculating, and sampling of the pure culture fermentations to detect the presence of any contaminants that may have gained entrance to the containers during these operations. As a further precaution to establish whether full control of the fermentations had been obtained by pure culture inoculations, slide mounts of the brine were usually made at sampling interval for subsequent Gram staining and microscopic examination as to cellular morphology and staining reaction.

Bacteriological control tests. The procedures used for estimating the numbers of the different microbial groups

mentioned in the foregoing statement, other than for lactic acid bacteria described above, were those used by Etchells et al. (1961).

Brine analyses. Total acidity was measured by the use of 2-ml samples of the brine and by titration with 0.111 N NaOH with phenolphthalein as the indicator; the acidity is expressed as grams of lactic acid per 100 ml of sample. Brine pH was measured with a Beckman pH meter (Zero-matic). Optical density of the brines was taken with a Lumetron colorimeter by use of a 650-m μ filter and 13-mm diameter test tubes. The salt content of the brines was determined by titrating a 1-ml sample in 15 to 20 ml of distilled water with 0.171 N silver nitrate solution, 3 to 5 drops of 0.5% dichlorofluorescein as the indicator; the results were recorded as grams of NaCl per 100 ml of sample, but are reported here on a per cent by weight basis.

RESULTS

Irradiated series. During the 2-year study, 82 pure culture fermentations of irradiated cucumbers with controls were observed and, with very few exceptions, there was no bacteriological evidence obtained to indicate that microbial groups other than those introduced as pure cultures grew in the fermenting brines. In three separate instances, a film-forming yeast contamination was noted after 10 to 14 days; these organisms probably were introduced during the brining and inoculation operation.

The irradiated uninoculated controls, in some instances, developed latent microbial growth originating from the few aerobic spores on the cucumbers that survived the gamma radiation treatment. This microbial activity was first evident after about 7 to 14 days of incubation, and continued to develop very slowly throughout the observation period (3 to 4 weeks). However, such growth by aerobic sporeformers was fully inhibited in irradiated, inoculated lots by the acid fermentation resulting from rapid growth by the pure cultures of lactic acid bacteria. In the nonirradiated, natural controls, growth by aerobic sporeforms was similarly inhibited by the acid fermentation resulting from the naturally occurring lactic acid bacteria. In addition to the latter group, coliform bacteria and both fermentative and film-forming yeasts were usually found in high populations in the natural controls.

Fermentations by pure cultures of lactic acid bacteria. The initial experiments were conducted with the three species of lactic acid bacteria most common to cucumber fermentations, namely, *P. cerevisiae*, *L. plantarum*, and *L. brevis*. Each of these species initiated rapid growth in cucumber brines when inoculated singly, reaching maximal populations within 2 days (Fig. 2A). *P. cerevisiae* attained peak populations the earliest, followed closely by *L. plantarum*; *L. brevis* was slowest, and failed to attain populations as large as the other two species.

When all three species were inoculated into the same brine (Fig. 2B), the total counts were similar to those

noted with the individual species. However, the differential counts indicated an interesting phenomenon; the growth of *L. plantarum* in the mixed culture was greatly delayed, and *P. cerevisiae* completely predominated in this fermentation through 2 days. *L. brevis* initiated early rapid growth, but attained a relatively low population before the numbers leveled off. After a lag phase of over 1 day, *L. plantarum* multiplied rapidly, and predominated in the fermentation from 4 days through the remainder of the sampling period.

The prompt initiation of rapid growth of *P. cerevisiae* was reflected in the rate of acid production in a pure culture fermentation (Fig. 3A). However, after the initial lag, *L. plantarum* produced acid more rapidly and to a greater extent than did the *Pediococcus* species. *L. brevis* produced the least amount of acid of the three cultures tested. Acid production by all possible combinations of these three species in fermentations is shown in Fig. 3B. The presence of *P. cerevisiae* in the culture combination

eliminated the lag phase, and the presence of *L. plantarum* assured a reasonably high level of lactic acid production.

Differential counts were made on fermentations inoculated with various combinations of the three species to determine the effect of each of the other two on *L. plantarum*. *P. cerevisiae* was the organism responsible for the great increase in the lag phase of growth of *L. plantarum* (Fig. 4); it also greatly reduced the maximal population attained. The initial count of *L. plantarum* was exceptionally low for some unknown reason when *L. brevis* was added to the brine; the maximal population was considerably reduced, but the growth rate of *L. plantarum* was not affected by the presence of *L. brevis*.

Comparisons were made of pure culture fermentations by use of a mixture of the three species of lactic acid bacteria, with natural fermentations of nonirradiated cucumbers of the same lot (Fig. 5). Maximal cell populations were attained about 1 day earlier in the pure culture fermentations than in the natural system, but the total

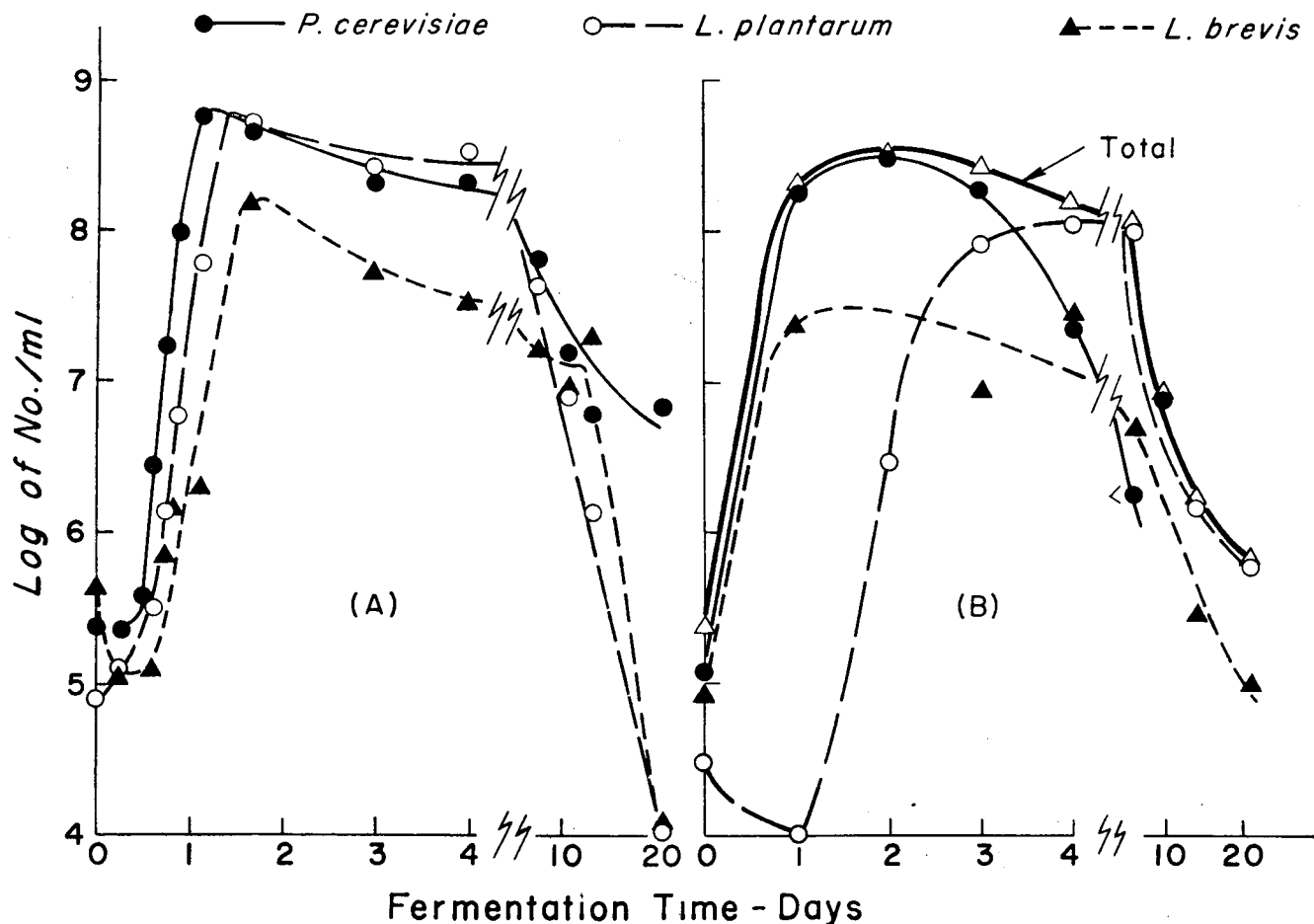


FIG. 2. Growth of *Pediococcus cerevisiae*, *Lactobacillus plantarum*, and *L. brevis* during pure culture fermentation of brined cucumbers. Part A reflects their growth in individual fermentations, and part B, their growth in a single fermentation inoculated with all three species. The cucumbers were prepared for fermentation by exposure to 0.83 Mrad of gamma radiation. Sterilized brine containing sufficient NaCl to equalize at 5.4 to 5.6% was inoculated with the desired culture(s) and was aseptically poured over the cucumbers. Incubation was at 32 C.

maximal populations of lactic acid bacteria were usually higher in the latter. The rate of acid production correlated with growth. Although the extent of acid production was lower in the natural fermentation, this is not a fair comparison, because high numbers of film-forming yeasts capable of acid oxidation were present on the surface of these brines within 4 to 6 days.

The incubation temperature greatly affected bacterial growth and acid production in pure culture fermentations (Fig. 6). However, the greatest effect was on the time required for maximal levels of both growth and acid production to be attained, rather than on the maximal levels reached. Thus, a temperature decrease from 32 to 27 C or from 27 to 21 C extended the time required to attain maximal cell populations by about 1 day, and had a similar delaying effect on acid production. Differential counts run on these fermentations demonstrated that the temperature affected each of the three species of lactic acid bacteria similarly, and did not result in a selection of one species over another.

The pure culture technique was further used to test the fermentation efficiency of a number of strains of the three lactic species common to brined cucumber fermentations, as well as strains of unidentified species of lactic acid bacteria isolated from cucumber fruit and from natural fermentations. Also included were several species of lactic acid bacteria having relatively high optimal temperatures for growth. These data are summarized in Table 1. Only the three species common to brines (*P. cerevisiae*, *L. plantarum*, and *L. brevis*) and the five unidentified cultures grew well and produced significant amounts of acid in brines at salt levels of 5.4 to 5.6%. *L. delbrueckii* grew rapidly in fermentations brined to equalize at 2.5 to 2.9% salt; furthermore, some strains of this species produced sufficient acid to reduce the brine pH below 4.0 in 30 hr (Fig. 7). All of the other high-temperature lactic acid species tested grew to a limited extent in 2.8 to 3.0% salt brine, except *L. bulgaricus* which failed to grow.

L. plantarum consistently produced the highest brine acidities and the lowest pH levels of the cultures tested.

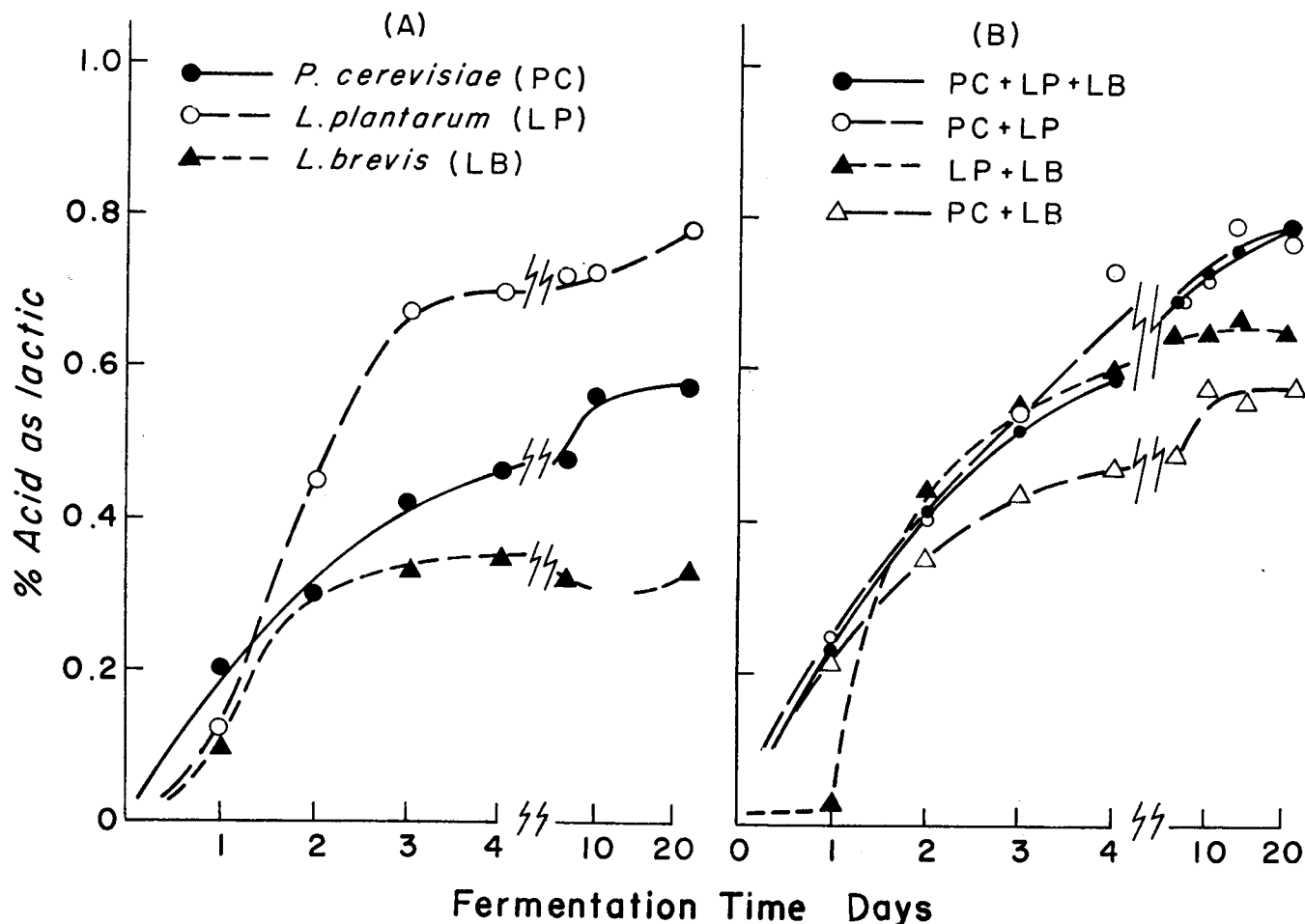


FIG. 3. Acid production by *Pediococcus cerevisiae*, *Lactobacillus plantarum*, and *L. brevis* during the pure culture fermentation of brined cucumbers. Part A reflects acid production in individual fermentations and part B, acid production in fermentations containing the mixtures of species indicated. See Fig. 2 for procedure.

P. cerevisiae was the only other species wherein all strains tested produced sufficient acid to insure that the pH of the brine would be below 4.0.

Effect of lactic acid on the growth of coliform bacteria. The coliform bacteria constitute the most numerous single microbial group found on cucumbers capable of interfering with brine fermentations (Etchells et al., 1961). These organisms appear to be relatively insensitive to salt, but do not compete well in low salt brines where acid is developed rapidly (Etchells et al., 1945). Therefore, the initial acidification of the brine should prevent their growth.

To obtain more exact knowledge of the amount of lactic acid necessary to prevent the growth of coliform bacteria in cucumber fermentations, irradiated cucumbers were covered with sterile brine containing various levels of lactic acid, were allowed to equalize overnight, and were inoculated with a coliform culture isolated from fresh cucumbers. A control fermentation containing 0.24% lactic acid was inoculated with cultures of *P. cerevisiae*

and *L. brevis* to insure that their growth was not appreciably affected by added acid. The rate and extent of growth of the coliform in nonacidified brine was comparable to that of the lactic acid bacteria, although the death rate was more precipitous (Fig. 8). The use of 0.18% lactic acid only partially inhibited coliform growth, but no growth was evident in fermentations with 0.24 and 0.38% lactic acid. In fact, very few viable coliform bacteria could be isolated from these brines after 2 days of incubation. Complete inhibition and loss of viability of these organisms were also noted in brines acidified initially with 0.30% lactic acid.

Control of cucumber microflora by hot-water blanching. In addition to gamma radiation, a second method, hot water blanching, was investigated as a means of ridding the cucumbers of undesirable and interfering surface microorganisms capable of competing with pure cultures of lactic acid bacteria in brine fermentations. First results on the fermentation of blanched cucumbers are presented in Table 2. Early, vigorous acid fermentations took place in all inoculated lots of cucumbers blanched at water tempera-

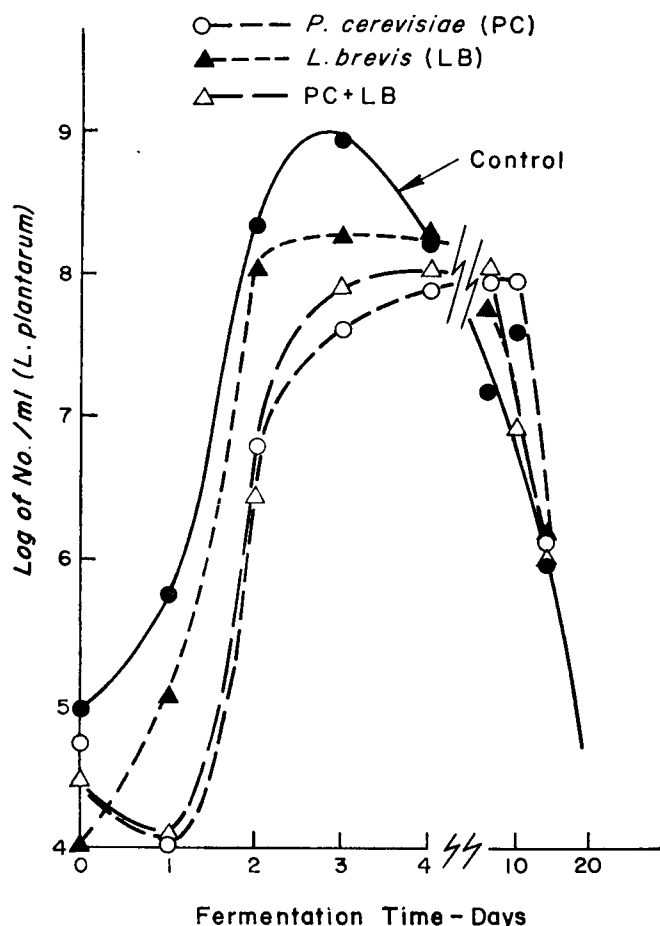


FIG. 4. Effect of the presence of *Pediococcus cerevisiae*, *Lactobacillus brevis*, and a mixture of the two on the growth of *L. plantarum* in pure culture cucumber fermentations. See Fig. 2 for procedure. The differential counts were made as outlined in Materials and Methods. The counts of *L. plantarum* are the only ones shown.

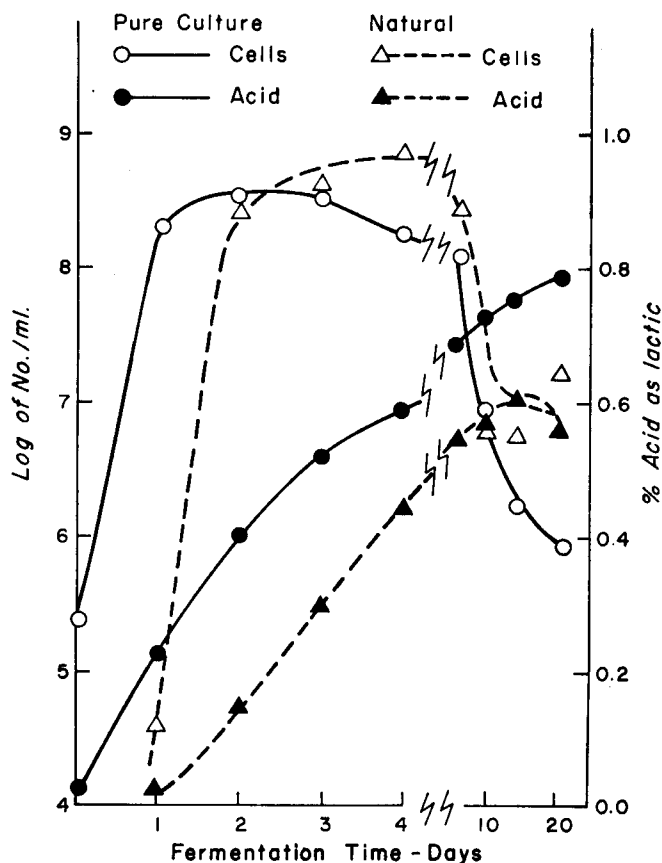


FIG. 5. Comparison of a pure culture fermentation with a natural fermentation. The fermentations were set up as outlined in Fig. 2, but the cucumbers for the natural fermentation were not irradiated and no inoculum was added. A mixture of *Pediococcus cerevisiae*, *Lactobacillus plantarum*, and *L. brevis* was used for inoculating the pure culture fermentation.

tures ranging from 66 to 82 C. These fermenting brines were characterized by rapid acid development with a corresponding drop in brine pH, together with increased optical densities and high plate counts for acid-forming bacteria. In contrast, the brines of the uninoculated lots of blanched material, after 44 hr of incubation, gave no evidence typical of fermentative activity by the usual competitive organisms, including naturally occurring acid-forming bacteria. The latter group of organisms were, with one exception, not found in the uninoculated brines after 5 days of incubation. In the first 82 C treatment listed in Table 2, an acid fermentation developed in the uninoculated lot after about 4 days; the responsible organism probably was introduced during the packing and brining operation.

Although the water temperatures employed (66 to 82 C) were apparently sufficiently high to effectively control the usual organisms associated with cucumber fermentation, such heat treatments would not be expected to affect

materially the survival of aerobic and anaerobic spores known to be present on cucumbers (Etchells et al., 1961). Of the two groups, the anaerobic spores might be expected to develop in the blanched, uninoculated lots at the brine strengths used and in the absence of an acid fermentation. Such was indeed the case and, with time, a gaseous, malodorous fermentation typical of that resulting from butyric acid-producing anaerobes took place in most of the uninoculated jars of blanched cucumbers. Furthermore, this butyric fermentation was found to occur, under the conditions described, over a wide range of incubation temperatures (25 to 50 C).

Of the two natural control fermentations shown in Table 2, the inoculated lot developed acid at a more rapid rate and showed higher acid-former counts during the first 44 hr than did the uninoculated fermentation. However, the total acidities after 5 days of incubation were comparable for both fermentations.

In a second experiment on the fermentation of blanched

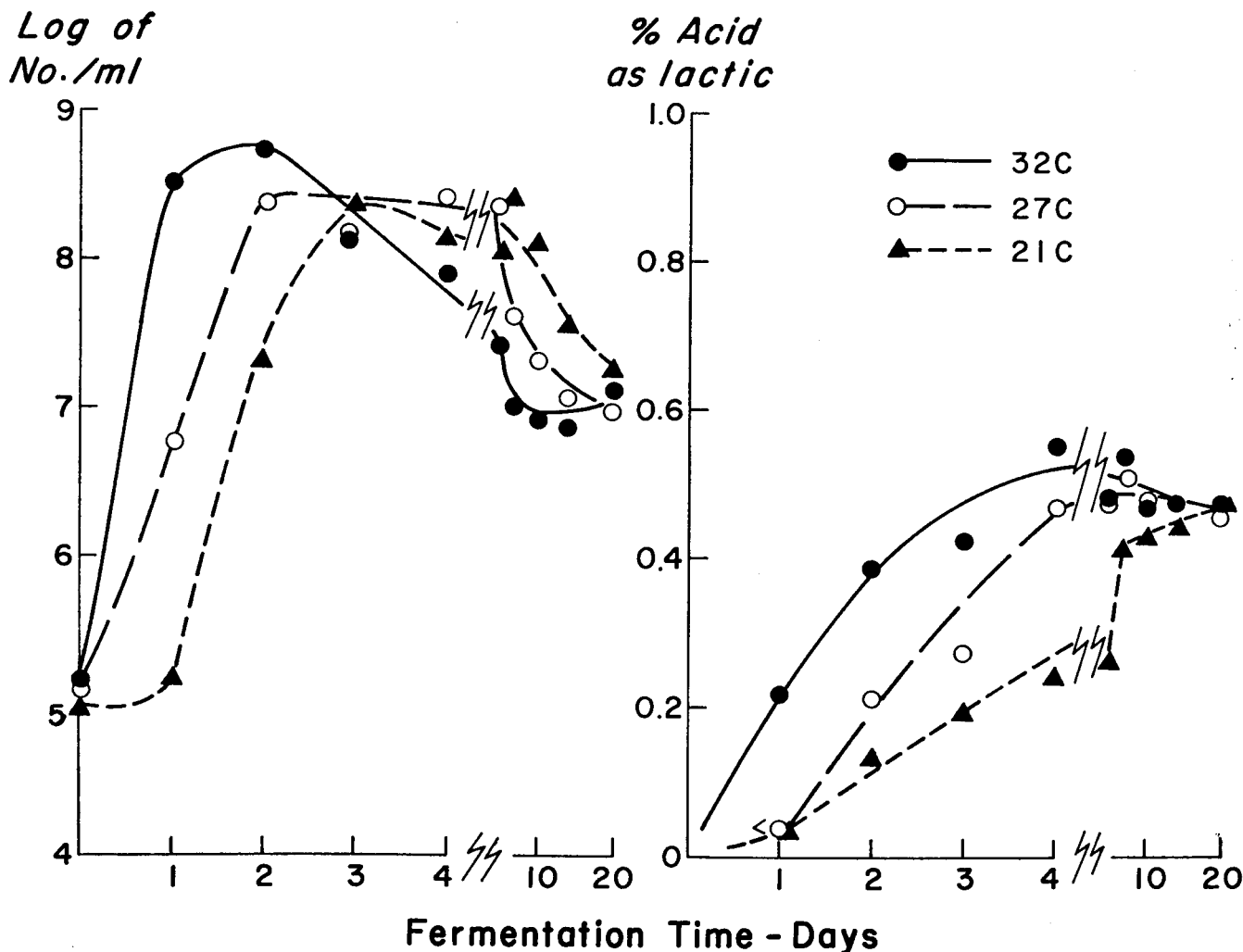


FIG. 6. Effect of incubation temperature on growth and acid production by a mixture of *Pediococcus cerevisiae*, *Lactobacillus plantarum*, and *L. brevis* in a pure culture fermentation. See Fig. 2 for procedure used.

cucumbers, water temperatures ranging from 54 to 82 C were used, and acidification of the cover brine as a means of controlling development of the butyric fermentation in blanched, uninoculated lots was investigated (Table 3). In general, the findings for the inoculated, nonacidified series blanched at 66 C or higher followed the same fermentation trends as those discussed earlier (Table 2). However, the two blanching treatments below 66 C (i.e., 60 and 54 C; Table 3) were not sufficient to preclude slow development of lactic acid bacteria in the uninoculated jars of this series; thus, control of pure culture fermentations by the use of such cucumber blanching temperatures would not be assured. Further, as indicated by the optical densities, other organisms did develop in the nonacidified, uninoculated lots blanched at 66 C or higher. These fermentations were of the same malodorous, butyric acid type discussed earlier. No such butyric acid fermentations developed in inoculated series of the nonacidified brines.

Acidification of the cover brine with sufficient acid to equalize at about 0.10% was of much value in controlling the growth of residual sporeforming bacteria, particularly the anaerobic types, in the uninoculated lots. There was no growth indicated within 5 days in any of the uninoculated, acidified brines when the cucumbers had been blanched at 60 C or higher.

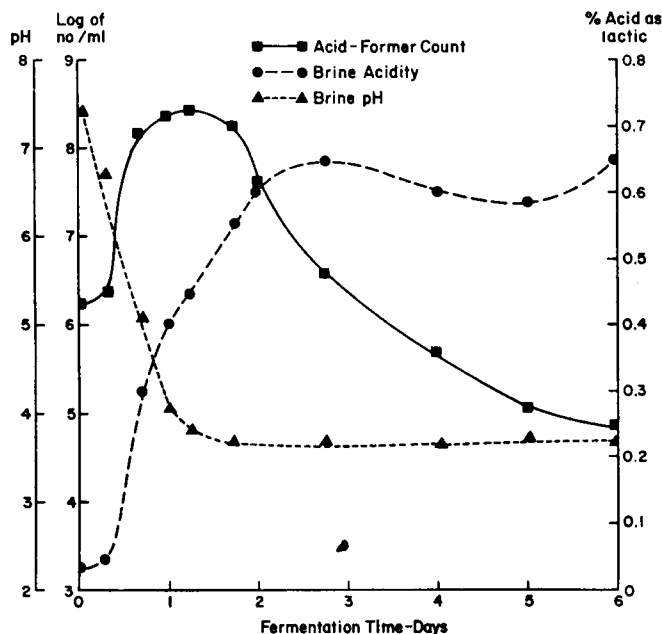


FIG. 7. Growth and acid production by *Lactobacillus delbrueckii* during the pure culture fermentation of brined cucumbers incubated at 48 C. The sterilized cover brine (5.5% NaCl) was aseptically poured over the irradiated (0.83 Mrad) cucumbers and was allowed to stand 6 hr prior to inoculation with 2 ml of an 8-hr TS broth culture through the side stopper of each fermentor by a sterile syringe; the brine strengths at inoculation were 2.9 to 3.2% NaCl, and after equalization, 2.5 to 2.9%. The curves shown represent averaged values for three strains: NRRL B-1658, 1954, and 445.

In two instances (71 and 77 C treatments), a distinct loss of viability of the cells of *P. cerevisiae* was encountered when acidified brines were used. This condition was apparently the result of the combined action of the acid and salt levels of the cover brine before equalization, plus the fact that the blanched cucumbers were not sufficiently cooled before the cover brine-culture mixture was added. It should be noted that *P. cerevisiae* is more sensitive to acid than are other species of lactics commonly found in cucumber fermentations, namely, *L. plantarum* and *L. brevis*. In subsequent studies, involving several hundred successful pure culture fermentations conducted at pickling plants, this problem was solved by adding the inoculum

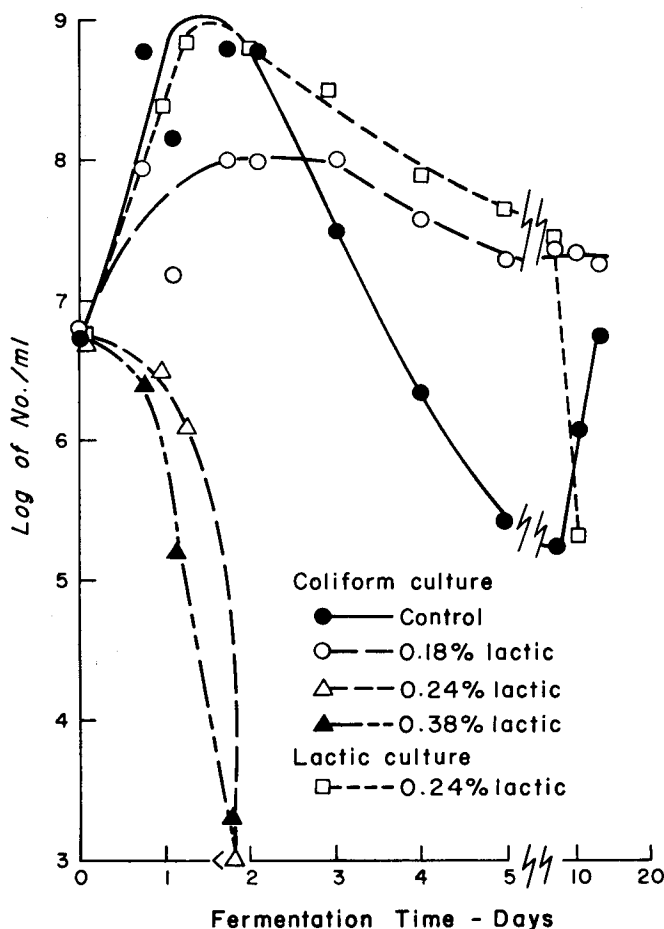


FIG. 8. Effect of acidification of cover brines on the development of coliform and lactic acid bacteria in cucumber fermentations. Fermentations were set up as outlined in Fig. 2, except that the cucumbers were exposed to 1.0 Mrad of gamma radiation, and the brine strength was equalized at 3.0 to 3.2% NaCl. Sufficient lactic acid was added to individual cover brines to equalize at the three levels indicated. A pure culture of a coliform-type bacterium was inoculated into all brines after equalization except the one indicated for lactic acid bacteria. A mixture of *Pediococcus cerevisiae* and *Lactobacillus brevis* cultures was inoculated into this brine. All inoculations were made by sterile syringes through the rubber serum stopper located in the side of each fermentor.

separately, after the packed cucumbers were acidified and covered with a cooled brine (ca. 10 C).

Effect of salt concentration on the pure culture fermentation of cucumbers. Of the three lactic species tested, *P. cerevisiae* was least affected by increasing salt levels insofar as growth was concerned, but it was affected to the greatest extent insofar as acid production was concerned (Table 4). This species grew well in 8.1% salt brine, but produced only a negligible amount of acid. In contrast, *L. plantarum* and *L. brevis* grew poorly in 8.3% salt brines, but produced significant amounts of acid. One strain of *L. brevis* (L-544) produced essentially the same amounts of total acid in the presence of 4.3, 6.1, and 8.3% salt brines as in the absence of salt. With one possible exception, none of the three species tested grew appreciably or produced acid to a significant extent in brines of about 10% salt strength or higher during 25 days of incubation. A latent fermentation by *L. plantarum* was observed at the 10.3% brine strength after about 3 weeks, with counts of 6×10^6 and 3.5×10^6 per ml of brine at 20 and 25 days, respectively.

DISCUSSION

For the first time, the relative abilities of *P. cerevisiae*, *L. plantarum*, *L. brevis*, and several other species of lactic acid bacteria to grow and produce acid in brined cucumbers were evaluated in pure culture fermentations.

The acid production data correspond reasonably well with those obtained in laboratory media (Costilow,

Ferguson, and Ray, 1955), with *L. plantarum* producing consistently higher levels of total acid than the other two species. However, our results are not in agreement with those of Pederson and Albury (1956, 1961). These workers inoculated natural fermentations with individual species of bacteria in an attempt to gain control of the fermentations, and their data indicate that this was possible with *L. plantarum*. However, fermentations inoculated with this species frequently had a lower level of total acid than did those inoculated with *L. brevis*. It is obvious that there were other unknown effects of the inoculation in the experiments of Pederson and Albury (1956, 1961); also, the variability of the natural fermentations attributable to competitive microbial groups was so great that the differences noted were not significant.

In the present study, when all three species were inoculated into the same pure culture fermentation, the growth of the individual species resulted in a fermentation sequence very similar to the one estimated by Costilow et al. (1956) for natural fermentations on the basis of frequency of isolation. Thus, *P. cerevisiae* was dominant during the first few days of fermentation, and then essentially disappeared from the brine flora. This was followed by the predominance of *L. plantarum*. *L. brevis* was present throughout the sampling period but failed to predominate. Pederson and Albury (1956) also noted that *P. cerevisiae* may predominate early in natural fermentations and then may be replaced by *L. plantarum* and *L. brevis*.

TABLE 2. Effect of hot-water blanching of cucumbers on the control of competitive microorganisms in pure culture fermentations with *Pediococcus cerevisiae*

Temp		Inoculum added ^c	Examination of brine for								
			Total acidity as lactic			pH		Optical density		Acid-former plate count ^d	
			18 hr	44 hr	5 days	18 hr	5 days	18 hr	44 hr	44 hr	5 days
Blanch water ^a	Cucumber ^b		%	%	%						
C	C										
66	43	+	0.26	0.37	0.48	3.9	3.7	0.48	0.72	560	
		—	0.04	0.05	0.12	5.9	5.2	0.02	0.04	0	0
71	57	+	0.24	0.37	0.48	3.9	3.7	0.45	0.70	540	
		—	0.09	0.04	0.10	5.6	5.9	0.02	0.08	0	0
77	63	+	0.15	0.36	0.48	4.0	3.7	0.41	0.76	910	
		—	0.08	0.06	0.08	5.7	5.7	0.02	0.04	0	0
82	68	+	0.15	0.27	0.38	4.3	3.8	0.19	0.50	710	70
		—	0.06	0.02	0.40	5.8	3.7	0.02	0.18	0	370
82 ^a	49	+	0.16	0.31	0.48	4.0	3.7	0.34	0.60	920	
		—	0.05	0.02	0.08	5.9	5.7	0.02	0.08	0	0
Unheated, natural control ^c	24	+	0.20	0.43	0.64	4.0	3.6	0.48	0.80	560	
		—	0.06	0.12	0.70	6.9	3.7	0.01	0.16	66	

^a Cucumbers were completely immersed in water at temperatures listed for 5 min, except for the second 82 C treatment shown, which was for 2 min.

^b Internal cucumber temperature at end of the blanch period.

^c A 2-ml portion of a 24-hr culture of *P. cerevisiae* FBB-61 per liter of cover brine or about 0.8 ml per quart jar of cucumbers; the culture was grown in Trypticase Sugar (TS broth) + 6% NaCl. The cover brine for all lots equalized within the range of 4.4 to 5.4% NaCl. All jars were incubated at 32 C.

^d Plate counts made with LBS agar (BBL), pH 5.6 $\pm$ 0.05, as modified by Costilow, Etchells, and Anderson (1964) for use in detecting species of lactic acid bacteria associated with plant material and brine fermentation of cucumbers and other vegetables. Results expressed as number per milliliter $\times 10^{-6}$.

^e Uninoculated, unblanched, washed cucumbers allowed to ferment naturally at 32 C.

The effect of *P. cerevisiae* on the initiation of growth by *L. plantarum* is of considerable interest, and may explain the basis of the sequence of species noted in nature. The rapid acid production by the *Pediococcus* species is the only possible reason evident from our data for this initial inhibition of *L. plantarum* growth. However, *L. plantarum* is so acid-tolerant that it does not appear likely that this is the sole reason for the inhibition, and there may be other factors more important.

The pure culture fermentations were made possible by use of two techniques, gamma radiation and hot-water blanching, designed to rid the cucumbers of naturally occurring, interfering, and competitive microbial groups prior to brining and inoculation with the desired cultures of lactic acid bacteria. The radiation technique provided an ideal means of preparing the cucumbers for fermentation; the 0.83-Mrad dose, predicted from earlier studies (Etchells et al., 1961), appears adequate to insure complete fermentation control by pure culture inocula.

The fermentation experiments in the irradiated series,

TABLE 3. Effect of several hot-water blanching treatments and acidification of the cover brine on the control of competitive microorganisms in pure culture fermentations with *Pediococcus cerevisiae*

Temp		Inoculum added ^c	Microbial growth in			
Blanch water ^a	Cucumber ^b		Nonacidified brines ^d		Acidified brines ^{d,e}	
			Optical density	Acid-formers	Optical density	Acid-formers
C	C					
54	38	+	0.58	510	0.55	850
		—	0.19	>10	0.21	>10
60	46	+	0.70	800	0.53	1,000
		—	0.29	>10	0.04	0
66	48	+	0.66	1,030	0.50	1,320
		—	0.20	0	0.03	0
71	51	+	0.67	930	0.50 ^f	21 ^f
		—	0.16	0	0.03	0
77	60	+	0.50	920	—	540 ^g
		—	0.16	0	0.02	0
82	71	+	0.45	1,080	0.45	850
		—	0.12	0	0.02	0
Unheated, natural control	26	+	0.71	900	0.38	450
		—	0.29	100	0.43	750

^a Cucumbers were completely immersed in water at temperatures listed for 5 min.

^b Internal cucumber temperature at end of blanch period.

^c See Table 2 for details.

^d Optical density and acid-former plate counts on LBS agar were run on the inoculated series after 44 hr of incubation at 32 C, and on the uninoculated series after 5 days. Results expressed as number per milliliter $\times 10^{-6}$.

^e All brines equalized in the range of 4.7 to 5.3% NaCl. Acidified brines contained 2 ml of 85% lactic acid per liter calculated to equalize at 0.10%.

^f Fermentation was delayed, but developed normally with time.

^g Plate count shown is for 48 hr after reinoculation; optical density test was not run at this sampling interval, but acid-former populations and a 5+ brine turbidity indicated that a vigorous acid fermentation was underway.

with the use of a small-scale fermentor, laid the ground work for future studies to evaluate, under controlled conditions, the influence of such variables as microbial species, brining treatments, brine additives, and many other factors, not only on the fermentation of cucumbers, but also on other fruit and vegetable material. However, based on our experience with cucumbers, the gamma radiation treatment has certain limitations for practical studies, and is best suited for experimentation on the more basic aspects of pure culture brine fermentations, such as suggested above and reported herein. The necessary dose of 0.83 Mrad of radiation would reduce cucumber firmness approximately 30% (Etchells et al., 1961), and certain other undesirable changes in flavor, odor, and internal color could also be expected (*unpublished data*).

Nonetheless, the findings from the pure culture fermentations with irradiated cucumbers provided a sound basis for the development of the prefermentation, hot-water blanching treatment for cucumbers that not only eliminated the undesirable changes described for gamma irradiated cucumbers but also gave indication of promising potential from a practical standpoint for the pickle industry. During the past 3 years, over 800 successful pure culture cucumber fermentations, both with and without dill, garlic, and spice, have been prepared at pickling plants located in two production areas of the country. These pure culture packs of pickles were evaluated at several storage intervals for various quality characteristics [odor, flavor, external and internal color, firmness, bloater (hollow cucumber) formation, acidity, pH, salt content, and overall acceptability for commercial use].

The findings from the evaluation study will be the subject of another report; however, together with the findings of the extensive studies reported herein, they provided a basis for a proposed patent application in the area of pure culture pickle fermentations (Etchells, Bell, and Costilow, 1963).

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TABLE 4. Growth and acid production by three species of lactic acid bacteria in pasteurized cucumbers brined at various salt concentrations^a

Species	Per cent NaCl	Acid-former plate count after ^b						Per cent acid produced after ^c			
		2 days	4 days	6 days	11 days	20 days	25 days	2 days	4 days	11 days	25 days
<i>Pediococcus cerevisiae</i> , FBB-61	0	1,420.0	1,070.0	1,140.0	306.0	129.0	25.0	0.17	0.30	0.38	0.44
	4.2	1,430.0	1,420.0	160.0	54.0	7.8	2.8	0.08	0.15	0.16	0.21
	6.3	930.0	112.0	120.0	28.0	5.8	1.1	0.05	0.10	0.20	0.16
	8.1	2.1	620.0	180.0	62.0	10.2	1.2	0.00	0.01	0.04	0.04
	10.2	0.20	0.40	0.40	0.16	0.004	0.001	0.01	0.00	0.02	0.00
<i>Lactobacillus plantarum</i> , FBB-67	0	1,900.0	2,200.0	2,180.0	400.0	34.0	6.7	0.46	0.62	0.82	0.84
	4.2	580.0	910.0	715.0	640.0	40.0	8.6	0.25	0.45	0.80	0.72
	7.2	440.0	600.0	580.0	240.0	16.0	0.35	0.09	0.28	0.50	0.56
	8.3	0.4	80.0	44.0	64.0	48.0	8.2	0.02	0.07	0.22	0.30
	10.3	0.003	0.001	<0.001	0.001	6.0	3.5	0.00	0.01	0.01	0.05
<i>L. brevis</i> , FBB-70	0	250.0	1,350.0	500.0	57.0	4.5	4.1	0.10	0.24	0.31	0.26
	4.2	440.0	20.0	22.0	2.5	0.25	3.7	0.19	0.34	0.35	0.30
	ca. 6.2	420.0	—	9.0	2.2	—	—	0.05	0.16	0.35	—
	8.3	0.20	3.2	53.0	7.0	0.25	0.08	0.04	0.00	0.13	0.12
	10.3	0.003	0.008	0.004	<0.001	<0.001	<0.001	0.03	0.02	0.02	0.00
<i>L. brevis</i> , L-544	0	550.0	304.0	46.0	2.9	5.0	3.7	0.24	0.22	0.27	0.22
	4.3	500.0	450.0	55.0	5.1	4.9	6.4	0.18	0.32	0.31	0.32
	6.1	0.30	88.0	165.0	8.1	1.7	0.30	0.03	0.23	—	0.27
	8.3	0.10	0.50	5.8	2.2	3.5	0.80	0.02	0.02	0.25	0.22
	11.8	0.10	0.10	0.10	0.01	<0.001	<0.001	0.01	0.03	0.04	0.02

^a Cucumbers were packed in 1-qt jars, brined, and pasteurized at a commercial plant as outlined under Materials and Methods. The initial acidity (ca. 0.40%) was adjusted to about 0.25% by the addition of sterile NaOH prior to inoculation; 1 ml of a 36-hr TS broth culture of each species was used to inoculate each quart jar in each salt series. This inoculum provided an initial count in the jars, after shaking to suspend the cells, of approximately 10^8 cells per ml of brine for all four cultures used.

^b Plate counts made at three higher salt levels (ca. 12, 16, and 20%) were less than 10^3 per ml throughout the sampling period, except for *P. cerevisiae* at ca. 12% salt, which gave counts of 1.5×10^3 to 3×10^4 during 4 to 20 days of observation. Uninoculated controls (2% NaCl) were plated at each sampling interval for each culture-salt series; all were negative. Results expressed as count per milliliter $\times 10^{-6}$.

^c Calculated as lactic; these data were corrected for the initial acidity (0.25%).

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LITERATURE CITED

- BORG, A. F., J. L. ETCHELLS, AND T. A. BELL. 1955. The influence of sorbic acid on microbial activity in commercial cucumber fermentations. *Bacteriol. Proc.*, p. 19.
- BORG, A. F., J. L. ETCHELLS, AND T. A. BELL. 1956. Bloat formation by gas-forming lactic acid bacteria in cucumber fermentations. *Bacteriol. Proc.*, p. 28.
- BREED, R. S., E. G. D. MURRAY, AND N. R. SMITH. 1957. *Bergey's manual of determinative bacteriology*, 7th ed. The Williams & Wilkins Co., Baltimore.
- COSTILOW, R. N. 1957. Sorbic acid as a selective agent for cucumber fermentations. III. Evaluation of salt stock from sorbic acid treated cucumber fermentations. *Food Technol.* 11:591-595.
- COSTILOW, R. N., F. M. COUGHLIN, D. L. ROBACH, AND H. S. RAGHEB. 1956. A study of the acid-forming bacteria from cucumber fermentations in Michigan. *Food Res.* 21:27-33.
- COSTILOW, R. N., J. L. ETCHELLS, AND T. E. ANDERSON. 1964. Medium for producing cells of lactic acid bacteria. *Appl. Microbiol.* 12:539-540.
- COSTILOW, R. N., W. E. FERGUSON, AND S. RAY. 1955. Sorbic acid as a selective agent in cucumber fermentations. I. Effect of sorbic acid on microorganisms associated with cucumber fermentations. *Appl. Microbiol.* 3:341-345.
- ENGELLAND, G. C. 1962. Food processing. U.S. Patent 3,024,116.
- ETCHELLS, J. L., AND T. A. BELL. 1950a. Classification of yeasts from the fermentation of commercially brined cucumbers. *Farlowia* 4:87-112.
- ETCHELLS, J. L., AND T. A. BELL. 1950b. Film yeasts on commercial cucumber brines. *Food Technol.* 4:77-83.
- ETCHELLS, J. L., T. A. BELL, AND R. N. COSTILOW. 1963. Pure culture fermentation process for cucumber pickles. U.S. Patent Application Serial No. 324,898.
- ETCHELLS, J. L., R. N. COSTILOW, AND T. A. BELL. 1952. Identification of yeasts from commercial cucumber fermentations in northern brining areas. *Farlowia* 4:249-264.
- ETCHELLS, J. L., R. N. COSTILOW, T. A. BELL, AND H. A. RUTHERFORD. 1961. Influence of gamma radiation on the microflora of cucumber fruit and blossoms. *Appl. Microbiol.* 9:145-149.
- ETCHELLS, J. L., F. W. FABIAN, AND I. D. JONES. 1945. The *Aerobacter* fermentation of cucumbers during salting. *Mich. Agr. Expt. Sta. Tech. Bull.* 200.
- JONES, I. D., J. L. ETCHELLS, O. VEERHOFF, AND M. K. VELDHUIS. 1941. Observations on bloater formation in cucumber fermentations. *Fruit Products J.* 20:202-206, 219-220.

PEDERSON, C. S., AND M. N. ALBURY. 1950. Effect of temperature upon bacteriological and chemical changes in fermenting cucumbers. N.Y. State Agr. Expt. Sta. Bull. 744.

PEDERSON, C. S., AND M. N. ALBURY. 1956. Variations in bacterial flora of low salt cucumber brines. Appl. Microbiol. 4: 259-263.

PEDERSON, C. S., AND M. N. ALBURY. 1961. The effect of pure-culture inoculation on fermentation of cucumbers. Food Technol. 15:351-354.

PEDERSON, C. S., AND L. WARD. 1949. The effect of salt upon the bacteriological and chemical changes in fermenting cucumbers. N.Y. State Agr. Expt. Sta. Tech. Bull. 288.

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