

Pure Culture Fermentation of Green Olives¹

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ABSTRACT

ETHELLES, J. L. (U.S. Food Fermentation Laboratory, Raleigh, N.C.), A. F. BORG, I. D. KITTEL, T. A. BELL, AND H. P. FLEMING. Pure culture fermentation of green olives. *Appl. Microbiol.* 14: 1027-1041. 1966.—The method previously developed by us for the pure-culture fermentation of brined cucumbers and other vegetables has been applied successfully to Manzanillo variety olives. Field-run grade fruit was processed first by conventional procedures to remove most of the bitterness. Then the relative abilities of *Lactobacillus plantarum*, *L. brevis*, *Pediococcus cerevisiae*, and *Leuconostoc mesenteroides* to become established and produce acid in both heat-shocked (74 C for 3 min) and unheated olives, brined at 4.7 to 5.9% NaCl (w/v basis), were evaluated. The heat-shock treatment not only proved effective in ridding the fruit of naturally occurring, interfering, and competitive microbial groups prior to brining and inoculation, but also made the olives highly fermentable with respect to growth and acid production by the introduced culture, particularly *L. plantarum*. Of the four species used as inocula, *L. plantarum* was by far the most vigorous in fermentation ability. It consistently produced the highest levels of brine acidity (1.0 to 1.2% calculated as lactic acid) and the lowest pH values (3.8 to 3.9) during the fermentation of heat-shocked olives. Also, *L. plantarum* completely dominated fermentations when used in two-species (with *P. cerevisiae*) and three-species (with *P. cerevisiae* and *L. brevis*) combinations as inocula. In contrast, when *L. plantarum* was inoculated into the brines of unheated olives it failed to become properly established; the same was true for the other species tested, but even to a more pronounced degree. *L. brevis* was the only species used that failed to develop in brines of both heat-shocked and unheated olives. Modification of the curing brine by the addition of lactic acid at the outset, either with or without dextrose, led to a much earlier onset of fermentation with accompanying acid development, as compared to treatments with dextrose alone or nonadditive controls. Reasons for the marked improvement of the fermentability of Manzanillo olives receiving the prebrining heat-shock treatment are discussed.

Vaughn (28) called attention to the similarity of the predominating microbial changes occurring during the brine fermentation of the Sevillano variety of green olives to those of cucumbers fermented in brine with a low salt concentration (ca. 5% by weight). Species of bacteria in three genera—*Aerobacter*, *Leuconostoc*, and *Lactobacillus*—were considered common to both types

of fermentations, and their sequence of occurrence in the brines was in the order named. The sub-surface yeast flora in 20 (low salt concentration) cucumber fermentations was shown to be almost completely dominated by two species of *Candida*—*C. krusei* and *C. tropicalis* (19). These species accounted for 202 and 50 isolations, respectively, of the 287 isolates obtained. Of particular significance here is the fact that the most commonly isolated species, *C. krusei*, was considered by Mrak et al. (25) to be the principal yeast present in fermenting olive brines maintained at 6.5% salt. The relatively low salt concentration of the cucumber and olive brines is suggested

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as the ecological factor mainly responsible for this species dominating the yeast flora and outgrowing species of *Brettanomyces*, *Hansenula*, *Torulopsis*, and *Saccharomyces*, usually found in abundance at higher brine strengths (16, 17, 20).

The microbially induced spoilage problems of brined olives are, with one notable exception, similar to those encountered with brined cucumbers. The most common types of spoilage of brined olives listed by Vaughn (28) were softening, gassy deterioration, and butyric acid fermentation; the last named is not a problem with brined cucumbers. For olives, these problems have been dealt with in detail by Vaughn, Douglas, and Gililand (29), who studied production of Spanish-type green olives in California. Cucumbers spoiled by gassy deterioration are referred to in the pickle industry as "bloaters." They are caused by gaseous fermentations resulting from the growth of yeasts, coliform bacteria, and gas-forming lactic acid bacteria (10, 16, 20, 22, 23; Borg, Etchells, and Bell, *Bacteriol. Proc.*, p. 19, 1955; Borg, Etchells, and Bell, *Bacteriol. Proc.*, p. 28, 1956). Filamentous fungi definitely were shown to be the causative agent responsible for the softening-spoilage of cucumbers brined under commercial conditions. The softening-enzyme systems were introduced into the curing brines by way of the fungus-laden flowers that remained attached to the cucumbers (1-4, 18).

Although an organized research program on the technology of brined cucumbers in this country (24) began about 10 years earlier than that for brined olives (12), workers associated with the latter commodity were first to become concerned with the need for control of the complex and heterogeneous microbiological activity of the fermentation. Cruess (12) in 1930 first recommended the use of a normally fermenting brine as a starter for newly brined olives. He was first to suggest (1937) the use of pure cultures of lactic acid bacteria for starters; *L. pentosus* (syn. *L. plantarum*) was considered to be worthy of commercial brining tests (13). Vaughn et al. (29) greatly expanded the pioneering studies of Cruess and inquired further into the matter of pure culture starters for olive brines. This was in connection with an investigation of green-olive fermentations, mentioned earlier.

The California researchers (29) reported successful results with pure-culture inoculations of olives, both in the laboratory and on an industrial scale. Throughout, they recommended use of one species of the genus *Lactobacillus*, namely, *L. plantarum* (the same strain originally used in 1937 by Cruess, who obtained it from E. B. Fred, University of Wisconsin). The best results were reported for accelerating fermentations when the

Sevillano olive variety was brined. The Manzanillo variety, which is considered more difficult to ferment properly, gave less-consistent results, and usually a supplementary source of fermentable carbohydrate was needed in addition to the starter culture. However, the examples of Sevillano olive fermentations chosen by these authors to illustrate the advantages of inoculation show at best only a very modest degree of acceleration of acid formation, compared to the control brines. Further, the final acidities (calculated as lactic acid) of the inoculated lots in two separate experiments (involving 15 barrels of olives) were less than 0.10% higher than the controls (actually, 0.074 and 0.081%). Their four examples of inoculated lots of Manzanillo olives, particularly those with corn sugar added, show rather favorable results when compared with uninoculated controls; inoculation led to an increase in the total amount of brine acid formed and an accelerated rate of acid formation. In their tests with Manzanillo olives, the prebrining treatment with lye to remove most of the bitter principle, followed by leaching in water to remove the lye, appears to have reduced the sugar content to a rather low level. This conclusion is based on the low acidity values of the controls (0.10 to 0.30%) compared to the acidities of 0.55 to 0.90% in inoculated lots to which sugar had been added.

In the above-discussed studies with Manzanillo olives where uninoculated corn sugar controls were included in the experiments, the final brine acidities for these lots were essentially the same as for the inoculated olives with added sugar; also, the rate of acid production was quite similar for both lots once fermentation was underway. This would indicate that the corn sugar additive was primarily responsible for the improved fermentation activity of the Manzanillo olives, not the pure-culture inoculation per se.

The Spanish workers have reported on the results of 12 years of experimentation with the use of pure cultures of lactobacilli in olive curing (5-7). Their first two reports indicated beneficial results from pure-culture inoculations for newly brined olives as well as for those in storage from the previous season. The 1964 report (5), which does not wholly substantiate their earlier work, summarizes a very extensive series of pure-culture inoculation tests involving almost 300 casks of olives representing six varieties (Rapasaya, Gordal, Serrana, Manzanillo, Carrasequena, Morona) brined under commercial conditions. Culture inoculations usually were made a few days after the olive-brining operation. In most cases the preserved lactobacilli used (but not identified as to species) were from commercial sources, although some cultures were prepared in

the laboratory. The data clearly demonstrate there was no significant difference between the final acidity and pH values of the inoculated and the control brines. For example, averaged values for maximal brine acidities for six casks each of inoculated and control Manzanillo olive fermentations 8 months after brining were 0.55 and 0.52%, respectively. In another experiment with this variety, involving 18 casks each of inoculated and control fermentations, the averaged brine acidities at 40 days were 0.41 and 0.46%, respectively. Also, there was no significant difference shown in rate or acceleration of acid production between the control and the inoculated fermentations. The lack of improvement in the fermentation behavior of the inoculated lots was attributed to the naturally occurring lactobacilli (i) providing sufficient inocula without the added culture and (ii) being more suitable for growth in the olive brines than the laboratory-grown cultures.

The pertinent literature indicates that pure-culture inoculation of brined olives, both in this country and abroad, can produce variable results as to fermentation control. Often little or no effect on lactic acid fermentation was achieved as to either rate or amount of acid produced. A number of factors listed by Vaughn et al. (29) may influence the desirable action of starter cultures in olive brines, such as (i) brine strength, (ii) brine acidity and pH, (iii) available brine sugar, (iv) brine temperature, (v) time of inoculation, and (vi) olive variety. Most of these can be regulated or controlled more or less. However, in the pure-culture studies discussed up to this point, the cultures of lactic acid bacteria were added directly to the heterogeneous natural microbial flora of the brined olives. With this practice, the dominance of the introduced culture cannot be assured, and competitive microbial groups already present in the brine may well gain ascendancy and effectively monopolize the fermentation. Under such conditions it would be difficult indeed to determine whether effective control of the fermentation proper actually was obtained as the direct result of development by the culture used for inoculation. Cruess (14) summarized the varied experience of the California workers with pure-culture starters for olive fermentations when he stated: "Homofermentative (nongas formers) are preferred; hence the addition of pure cultures would appear desirable, although experiments by R. Vaughn and the author have not been so successful as desired."

No previous attempts to study pure-culture fermentations of brined green olives in the absence of naturally occurring, undesirable, and

competitive microorganisms have come to our attention. However, a method for carrying out pure-culture fermentations of cucumbers and other vegetables has been described by Etchells and co-workers (21). We report here the results for our attempts to apply this technique to obtain pure-culture fermentations of Spanish-type green olives.

Included are data on fermentations induced by pure-culture inoculation of unheated olives as compared to lots subjected to a mild heat-shock treatment. The heat treatment was designed first to rid the fruit of naturally occurring, undesirable, asporogenous microflora prior to brining, followed by inoculation with the desired lactic acid bacteria. Both heated and unheated brined olives received four additive subtreatments: (A) no additive; (B) plus lactic acid; (C) plus lactic acid and dextrose; and (D) plus dextrose only.

MATERIALS AND METHODS

The green olives used consisted of 300 lb (136 kg) of freshly harvested, field-run grade Manzanillo variety shipped by air from the Department of Pomology, University of California, Davis. After being hand-sorted to remove leaves, trash, defective and ripe fruit, the remaining 266 lb (121 kg) of olives were processed by conventional procedures to remove most of their bitterness. This treatment, based on directions given by Cruess (12) and Preble (*personal communication*), consisted of soaking the fruit in a previously cooled (4.5 C) 2% lye solution for 14 hr in containers stored at room temperature (until the lye had penetrated two-thirds to three-fourths the way to the pit of the olives). This was followed by quickly pouring off the lye solution, rinsing the olives twice with cool water (11 C), and then leaching the fruit in two changes of cool water during a 14-hr period to remove the alkali. These olives were of excellent texture and color and were free from such defects as softening, blistering, and graying that might result from improper lye treatment and the subsequent washing (12, 29).

The debittered olives were divided into two equal lots. One was subjected to a heat-shock treatment by immersion for 3 min in hot water maintained at 74 C. This was accomplished by placing 7 to 8 lb of olives in a deep-fry basket fitted with a hardware cloth top and immersing them in about 50 gal (189 liters) of water kept at the desired temperature in a steam-jacketed kettle. To insure even heating of the olives, the basket was kept in constant motion during the exposure time and the water was circulated. This treatment was designed as a means of controlling competitive microorganisms occurring naturally on the olives. The remaining lot of olives was used in preparing the unheated controls.

Approximately 2.5-lb amounts of heat-shocked olives were packed hot, with aseptic precautions, into 0.5-gal glass jars previously sanitized with a 50-ppm chlorine solution, and then were covered with a pasteurized (74 C for 15 min) and cooled 40° salom-

eter brine (10.6% NaCl). The cover brine was cooled to about 4.5 C so as to reduce promptly the temperature of the heated olives as well as to provide suitable temperature conditions (ca. 33 C) for culture inoculation. The olives were held underneath the brine by use of a circular piece of sanitized, semirigid plastic netting that fitted snugly just under the shoulder of each glass jar. For acidified lots, 2.5 ml of 85% lactic acid were added per 0.5 gal of packed olives; this amount reduced the initial brine pH levels of heat-shocked and unheated olives to the levels shown in Table 1. For the 1.5% (w/v) dextrose lots, the sugar was added as a concentrated solution which previously had been slightly acidified (pH 5), pasteurized, and promptly cooled to room temperature. The sugar used was a product manufactured for industrial use, labeled "Clintose" Brand Dextrose (Clinton Corn Processing Co., Clinton, Iowa), and contained 8.5% moisture, 91.1% dextrose (syn. glucose), and 0.3% nondextrose carbohydrates. For proper identification, the material used is referred to herein as dextrose. The packed, brined, treated, and inoculated jars then were sealed with 82-mm diameter, six-lug "Twist-Off" metal closures (White Cap Co., Chicago, Ill.), which were sanitized in a 50-ppm chlorine solution before use. These jars then were divided into seven sets of eight each. Duplicate jars of each set received the four additive treatments listed earlier with respect to the addition of lactic acid or dextrose or both. The seven sets of jars, representing four treatments in duplicate, permitted the use of six different pure-culture inoculations (two jars each) plus one set reserved for uninoculated controls.

The procedure with the unheated olives was essentially the same, except that the jars and metal closures were not sanitized and aseptic precautions during packing were not employed. An insufficient amount of olives in this series necessitated omitting two inoculation treatments (the two- and three-species mixtures) in three of the four additive treatments.

The six pure-culture inoculation treatments used consisted of four single species of lactic acid bacteria, a two-species mixture, and a three-species mixture. The following cultures, all isolated from cucumber fermentations [Costilow et al. (11), all "FBB" designated cultures; Borg, Etchells, and Bell, *Bacteriol. Proc.*, p. 28, 1956, the single "L" designated culture]

were employed: *Lactobacillus plantarum* L-442, *Pediococcus cerevisiae* FBB-39, *Lactobacillus brevis* FBB-70, and *Leuconostoc mesenteroides* FBB-73. The two-species mixture contained *L. plantarum* and *P. cerevisiae*. The three-species mixture contained *L. plantarum*, *P. cerevisiae*, and *L. brevis*. All jars were allowed to stand overnight at room temperature. Those scheduled for inoculation then received 8 ml of a 30-hr culture grown at 32 C in Trypticase Sugar Broth (BBL) for single-species inoculations, and 4 ml of each broth culture when two- and three-species mixtures were used. At the time the cultures were added, the original cover brine strength of 10.6% NaCl had dropped to about 6.5% NaCl. This indicated that the brined material was approaching equilibration and was well within the salt-tolerance range for good growth by the cultures used for inoculation. All jars were incubated at 27 to 28 C for 72 hr and then stored at 21 to 24 C.

The metal jar closures were fitted previously with rubber serum stoppers to provide for repeated aseptic withdrawal of brine samples by means of sterile, disposable syringes equipped with 20- or 22-gauge needles. Of the total pack of 99 0.5-gal jars, 50, representing all treatments and all culture inoculations, were sampled 12 times during the fermentation and storage period. Sampling began 2 days after packing, six samples being taken in the first month, five more over the next 2 months, and a final one after 7.5 months. The remaining 49 jars were sampled three times, at 1, 3, and 7.5 months. The brine samples collected at 1 month and at 7.5 months from all 99 jars were examined microscopically under oil immersion as to cellular morphology (size, shape, and arrangement) of the predominating microbial types present.

Routine chemical and physical tests on the brine samples included those for acidity, pH, optical density (in percentage of light transmission), salt concentration, and visual turbidity. The procedures used were those described by Etchells et al. (21). Measurement of reducing sugars in the green olives before and after the lye treatment and in the olive brines was by the method of Sumner and Somers (27). Qualitative tests for carbohydrates were made by use of paper chromatograms of the alcoholic extracts of samples of raw and lye-treated fruit, and

TABLE 1. Effect of acidification on the initial pH and acidity values of Manzanillo olive brines

Treatment of brined olives ^a	Brines from heat-shocked olives				Brines from unheated olives			
	No.	pH		Avg acidity as lactic acid	No.	pH		Avg acidity as lactic acid
		Avg	Range			Avg	Range	
Nonacidified.....	14	8.25	8.1-8.5	% 0.00	10	7.50	7.1-7.6	% 0.010
Acidified.....	14	7.15	7.0-7.4	0.01	10	6.60	6.4-7.4	0.015

^a Acidified lots received 2.5 ml of 85% lactic acid per 0.5 gal of brined olives added at the time the jars were packed. The tests for brine acidity and pH were made after the jar contents had equalized (with respect to salt and added acid) but before fermentation started.

the compounds were detected by the benzidine-sugar reagent of Columbo et al. (9). When samples (15 to 20 ml) of unfermented brines were exposed to the air in 50-ml beakers, they rapidly increased in pH (Fig. 1). This pH change was accompanied by a marked color change of the brine from light amber to brown or dark brown and a decrease in the small amount of brine acidity usually present. The color change resulted in lower values for percentage of light transmission, but visual readings for brine turbidity were not affected.

After 7.5 months, olives from 24 of the heat-shocked, inoculated treatments, plus controls, were evaluated as to odor, flavor, color, freedom from fermentation defects, and degree of cure. Olives from each lot were tested for firmness with a special texture-measuring instrument. This device consisted of a 1,000-g capacity spring tester (model 516-1000, John Chatillon and Sons, New York, N.Y.) mounted vertically on an inexpensive, lever-operated, cast-aluminum, 0.25-inch drill press stand (Dayton Power Tools, Dayton, Ohio) made for home use. The unmounted tester was first tried by holding the plunger centered on the olive (perpendicular to its long dimension) and exerting a downward force until the olive tissue was pierced to the pit. The tester was hard to operate in this manner and produced considerable variability in results.

Mounting the tester on the drill press stand with lever operation exerted a constant downward pressure, and reduced the error substantially. The tester comes supplied with five small metal rods that serve as plungers. These can be attached by an adapter to the end of the original rod of the instrument. The diameters (in thousandths of an inch) for the plungers are: no. 1, 0.125 (original rod); no. 2, 0.063; no. 3, 0.058; no. 4, 0.0468; no. 5, 0.0320; and, no. 6, 0.0260. Plungers 3 and 4 were found most suitable for determining the texture of experimentally brined olives or

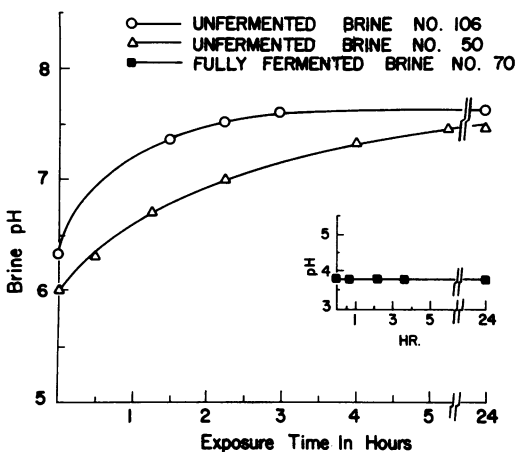


FIG. 1. Effect of exposure to air on the pH of unfermented and fully fermented Manzanillo olive brines. Brine samples were removed from original containers after storage for 7.5 months and exposed to the air in uncovered 50-ml beakers.

whole olives purchased from retail stores. In general, olives giving values below 200 g of resistance to the no. 3 plunger were considered soft to bordering on soft when chewed or eaten by a panel of judges. If tests are made with the no. 3 and no. 4 plungers on the same olives, the values obtained with the smaller one (no. 4) can be expected to be about 50 to 60 g lower than those with the larger plunger (no. 3). A single texture test requires a minimum of 15 to 20 uniformly sized olives and each olive is punctured twice. The results are recorded in grams of resistance of the olive tissue to the particular plunger being used.

RESULTS

Results for the determination of reducing sugars and certain other carbohydrates in the olives used in the current work are shown in Table 2. These tests, of necessity, were run on frozen samples of the same material employed in the brining experiments and are meant only to give a reasonable estimate of the sugar content of the olives before and after the lye treatment (and washing) to remove most of the bitterness. The results for total and reducing sugars of the raw olive flesh are comparable to the values reported by Nichols (26) for Manzanillo olives. Our data for reducing sugars in both the fresh and lye-treated and washed fruit fall within the ranges given by Cruess (12) for this variety. From the brine acidity values obtained in certain of the pure-culture fermentations, it is apparent that the lye-treated and washed olives contained an ample amount of readily fermentable carbohydrates and that the sugar additive treatment was not essential in obtaining a prompt and vigorous acid fermentation.

The striking effect of the heat-shock treatment on the fermentation behavior of Manzanillo olives by *L. plantarum* is presented in Fig. 2. The marked increase in both the amount and the rate of acid production in the brines of heated as compared with unheated olives is clearly evident. Microscopical examination of those brines showed large populations of bacteria whose

TABLE 2. Reducing sugar content of field-run grade green Manzanillo olives before and after lye treatment

Olive flesh sample ^a	Reducing sugars
	%
Raw, untreated ^b	3.2
Lye-treated and washed	1.5

^a Qualitative tests showed cellobiose, glucose, fructose, ribose, and galacturonic acid to be present in the olive samples.

^b Total sugar content was found to be 4.9%.

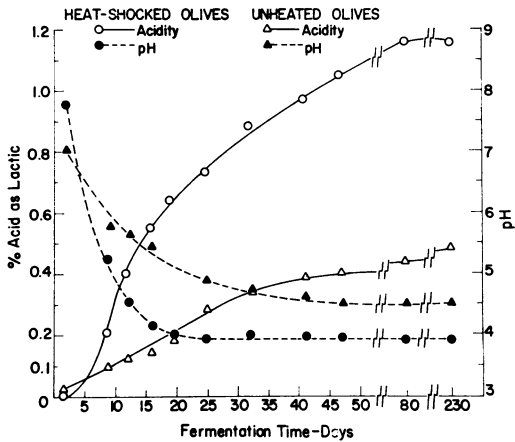


FIG. 2. Effect of heat-shocking Manzanillo olives on their subsequent acid fermentation by *Lactobacillus plantarum*. For heat-shocked lots, the lye-treated and washed olives were prepared for fermentation by immersion in hot water (74 C for 3 min) and, after packing into sanitized containers, were covered with a previously pasteurized and cooled 10.6% salt brine which equalized at 4.7 to 5.9%. Inoculation with the desired culture(s) followed a 12-hr brine equilibrating period; incubation was at 27 to 28 C for 72 hr and then at 21 to 24 C.

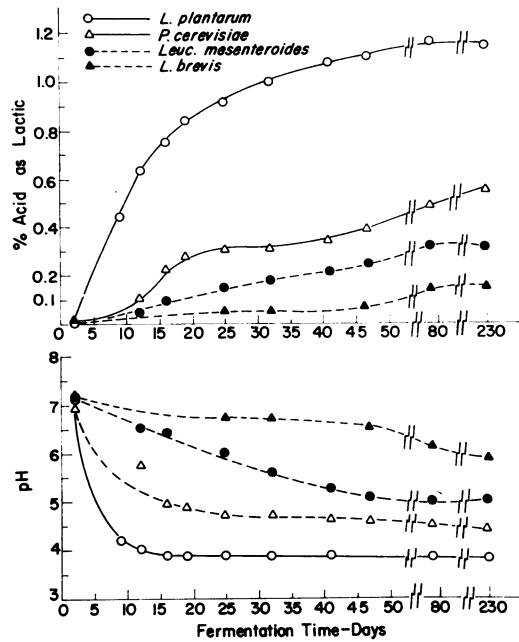


FIG. 3. Acid production by *Lactobacillus plantarum*, *Pediococcus cerevisiae*, *Leuconostoc mesenteroides*, and *L. brevis* during the fermentation of heat-shocked Manzanillo olives. See Fig. 2 for procedure.

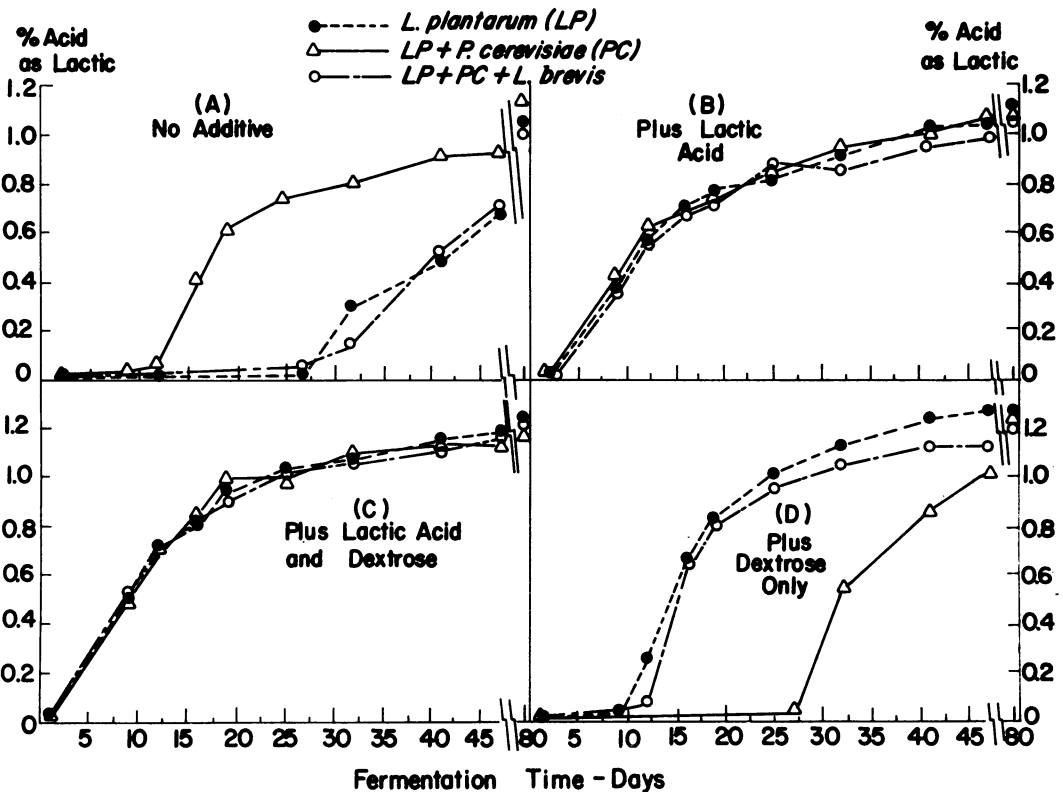


FIG. 4. Effect of the addition of lactic acid or dextrose, or both, on the fermentation of heat-shocked Manzanillo olives by *Lactobacillus plantarum* used singly, or in a two-species (with *Pediococcus cerevisiae*) or a three-species (with *P. cerevisiae* and *L. brevis*) mixture. See Fig. 2 for procedure.

cellular morphology was typical of the *L. plantarum* culture used. In comparable unheated lots, the rate of acid development was very slow. After 7.5 months of fermentation and storage, the brine acidity was less than half the amount produced with heat-shocked olives, but entirely comparable with reported maximal acidity and pH values developed during the natural fermentation of this variety both in this country (12, 29) and abroad (5). The microscopical examination of these brines showed conclusively that *L. plantarum* did not become established to any appreciable degree. Rather, pediococci, yeasts, ovoid cells usually in short chains (coccobacilli), and variously shaped rods, in the order named, were the principal morphological cell types present.

Of the four species of lactic acid bacteria used as inocula, *L. plantarum* was by far the most vigorous in fermentation activity as judged by the rate and amount of developed acidity and corresponding decrease in brine pH (Fig. 3). This

species consistently established itself in heat-shocked brined olives and produced final acidities between 1.0 and 1.2% with a brine pH of 3.8 to 3.9. *P. cerevisiae* was the next most active species, yet the maximal acidity developed was about one-half that resulting from fermentations by *L. plantarum*. Even so, this amount (0.57%) is about the same as that produced by *P. cerevisiae* in the pure-culture fermentation of brined cucumbers (21). *Leuconostoc mesenteroides* and *Lactobacillus brevis*, in that order, followed *P. cerevisiae* as to fermentation activity in the olive brines. *L. brevis* was almost wholly inactive, showing very little acid production or brine turbidity.

Modification of the curing brines by the addition of lactic acid, either alone or with dextrose, led to an early onset of the fermentation of heat-shocked brined olives (Fig. 4). However, in the two nonacidified treatments (A and D), individual fermentations were highly variable as to the onset of acid development. In contrast, the lactic acid-treated lots (B and C) initiated early acid development and showed brine acidity values during fermentation that appear to be almost identical; in fact, the three curves with each of the two treatments are almost superimposed. These results also serve to emphasize that *L. plantarum* becomes dominant when the inoculum consists of a mixture of this organism with *P. cerevisiae*, or with *P. cerevisiae* and *L. brevis* in equal numbers. This is clearly evident from the curves for acidified treatments (B and C). Microscopical examination of these brines during active fermentation revealed a large predominance of cells typical of the *L. plantarum* culture used as compared to the organisms added with it. In fact, in most instances the latter cell types could not be found.

The data for the individual curves shown in Fig. 4 were combined according to additive treatment and replotted for comparison with those of comparable fermentations of unheated olives. The results (Fig. 5) clearly emphasize the necessity of the basic heat-shock treatment in order to obtain fermentation benefits previously mentioned for acidified treatments (B and C). The unheated olives fermented rather poorly and slowly, regardless of treatment, and produced comparatively smaller amounts of total acid than did heat-shocked olives. The general shape and character of the curves for the two acidified treatments (B and C) of heat-shocked olives are rather similar, although a slight but consistent increase in both the amount and rate of acid formation is apparent for fermentations that received both lactic acid and dextrose (treatment C).

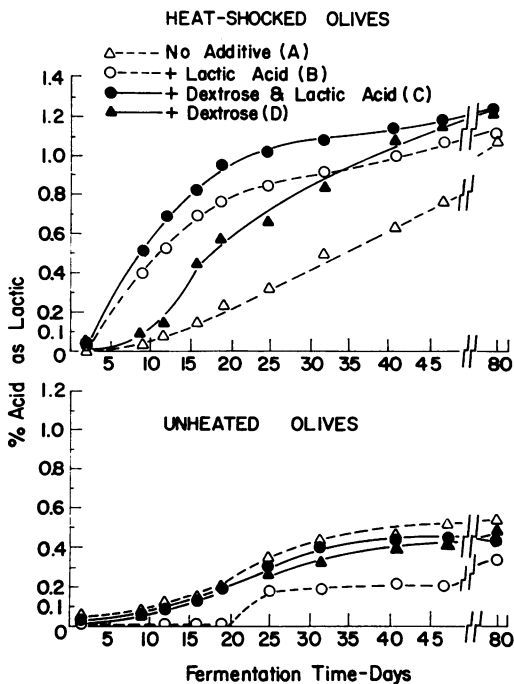


FIG. 5. Acid production by *Lactobacillus plantarum* (used either singly or as an equal part of the inocula; see Fig. 4 for species mixtures employed for heat-shocked olives) during the fermentation of Manzanillo olives to which lactic acid or dextrose, or both, were added at the outset. Upper part reflects acid production using heat-shocked olives; lower part gives acid production with unheated olives inoculated with *L. plantarum*. See Fig. 2 for procedure.

More specific information bearing on the marked stimulation of the onset of acid formation in heat-shocked olive brines is given in Table 3. In pure-culture fermentations involving *L. plantarum* (used either singly or in two- or three-species combinations), the addition of lactic acid at the outset reduced by approximately one-half the average time in days required for brine acidities to reach the 0.25 and 0.50% levels as compared to controls. Essentially the same was true for *P. cerevisiae* fermentations at the 0.25% acidity level, whereas at the next level, 0.50% (which is near the maximal amount produced by this species), the beneficial effect of the lactic acid treatment was not as great. Fermentations with brine acidities reaching the 1.00% level were restricted to those involving *L. plantarum* as inoculum. In these instances, the reduction in time required to reach this acid level ranged from one-fifth to one-third in acidified lots as compared to nonacidified controls. Only the 0.25% level of brine acidity was reached by fermentations with *Leuconostoc mesenteroides* and these were restricted to the lactic acid-treated lots. No data are shown for *Lactobacillus brevis*, as none of the eight fermentations with this species reached the 0.25% brine acidity level. Considering that Manzanillo olives are noted for their slow and long-drawn-out fermentation, the

marked reduction in fermentation time described in the foregoing is of special interest.

Although the acidification treatment greatly accelerated the onset of acid formation, it had essentially no effect on the total amount of acid produced in fermentations inoculated with *L. plantarum* (used either singly or in two- or three-species mixtures) or with *P. cerevisiae* (Table 4). Also, the close similarity of these acidity values was reflected in almost identical brine pH levels for both the acidified and the control treatments. The apparent acidity increase obtained with fermentations inoculated with *Leuconostoc mesenteroides* may not be a fair comparison, as this species did not become established in the non-acidified brines.

When dextrose was the brine additive, the brine acidity pattern was somewhat different (Table 4). A slight but consistent increase in maximal acidities (0.10 to 0.19%) over the controls was obtained in the fermentations with added dextrose with all culture inoculations except *L. mesenteroides*. Data for *Lactobacillus brevis* are not included in Table 4 because this species failed to become established in any of the brines.

The ability of the four species of lactic acid bacteria to establish themselves in heat-shocked olive brines is shown in Table 5. Summarized

TABLE 3. Effect of acidification treatment on the progress of the acid fermentation of heat-shocked, brined Manzanillo olives by different species of lactic acid bacteria

Species	Brines acidified ^a	Avg time required to reach brine acidities of		
		0.25%	0.50%	1.00%
		days	days	days
<i>Lactobacillus plantarum</i>	—	20 ^b	23	54
	+	11 (45) ^c	13 (43)	44 (19)
<i>L. plantarum</i> + <i>Pediococcus cerevisiae</i>	—	19	34	67
	+	11 (42)	13 (62)	43 (36)
<i>L. plantarum</i> + <i>P. cerevisiae</i> + <i>L. brevis</i>	—	33	34	65
	+	11 (67)	12 (65)	46 (29)
<i>P. cerevisiae</i>	—	50	133	NR ^d
	+	17 (66)	90 (32)	NR
<i>Leuconostoc mesenteroides</i>	—	NR	NR	NR
	+	38	NR	NR

^a Acidified lots received 2.5 ml of 85% lactic acid per 0.5 gal of brined olives; this was sufficient to reduce the average initial brine pH of 8.25, at equalization and before fermentation, to 7.15; minus sign in column means brines were not acidified.

^b All values shown in each acidity column represent averages of four fermentations.

^c Values in parentheses give percentage reduction in time for acidified lots to reach a given acidity level.

^d Acidity level not reached in 7.5 months.

TABLE 4. Effect of lactic acid and dextrose additives on the amount of acid produced during the fermentation of heat-shocked Manzanillo olives by several species of lactic acid bacteria^a

Species	Lactic acid added ^b	Maximal acidity as lactic acid	Final pH	Dextrose (1.5%) added	Maximal acidity as lactic acid	Final pH
		%			%	
<i>Lactobacillus plantarum</i>	— ^c	1.15	3.83	— ^c	1.08	3.88
	+	1.20	3.83	+	1.26	3.78
<i>L. plantarum</i> + <i>Pediococcus cerevisiae</i>	—	1.16	3.85	—	1.08	3.88
	+	1.17	3.83	+	1.24	3.80
<i>L. plantarum</i> + <i>P. cerevisiae</i> + <i>L. brevis</i>	—	1.14	3.85	—	1.06	3.90
	+	1.15	3.85	+	1.23	3.80
<i>P. cerevisiae</i>	—	0.58	4.43	—	0.52	4.45
	+	0.57	4.38	+	0.63	4.35
<i>Leuconostoc mesenteroides</i>	—	0.14	6.10	—	0.23	5.60
	+	0.33	5.50	+	0.24	5.50

^a Values shown for brine acidity and pH represent averages for four fermentations for each determination.

^b Acidified lots received 2.5 ml of 85% lactic acid per 0.5 gal of brined olives.

^c Minus indicates not added.

microscopical observations are presented for all inoculated lots in the heat-shocked olive series that received the four brine additive treatments. Results for the inoculated, unheated olive series were not tabulated but will be discussed; the same applies to the uninoculated controls for both series.

The findings (Table 5) indicate that the species *L. plantarum* became established and grew to a high degree of predominance with all brine additive treatments employed (A–D), whereas *P. cerevisiae* and *L. mesenteroides* became fully predominant only in the two acidified treatments (B and C). There was no evidence to show that *L. brevis* became established in any of the brines. The same was true for *L. mesenteroides* when inoculated into the nonacidified brine treatments (A and D). *P. cerevisiae* developed, but failed to predominate in one of the two brines receiving no additive (A).

In comparable, inoculated unheated lots, microscopy showed that the added pure cultures did not grow to an appreciable degree. Nearly all of the 35 inoculated brines in this series were placed in the "not established" category as far as growth of the introduced culture was concerned. In 4 of the 11 brines in which *L. plantarum* was used, there was evidence of some growth by this species which may have been responsible for the slightly higher brine acidities obtained in these fermentations (Table 6). In three other cases, all inoculated with *P. cerevisiae*, it was not possible

to determine whether the growth observed was the result of the inocula or whether it originated from the naturally occurring pediococci on the olive fruit. These organisms were usually the predominating cell types present during the fermentation of unheated brined olives in which appreciable microbial development was observed. In the 16 acidified brines of this series (B and C treatments), yeasts were found exclusively in 12 brines and present in another, but there was little evidence of their active development. Yeasts were also found in 14 of the 19 remaining brines in the other two additive treatments (A and D).

In the uninoculated, unheated control brines, yeasts again were noted almost exclusively in the two acidified treatments (B and C) and again showed no appreciable development during the 7.5-month observation period. However, in the two nonacidified treatments (A and D), active growth took place chiefly by pediococci, yeasts, coccobacilli, and, to a lesser extent, moderate sized rods. The occurrence of these cell types as to estimated populations in the brine was in the order named. This would indicate that these microbial groups were naturally occurring on the green olives used in the brining experiments, as they were consistently observed in the brines of the unheated olive series in which the inoculated lactic species failed to become established.

Before discussing the microscopy of the uninoculated, heat-shocked controls, it is necessary

TABLE 5. Ability of certain species of lactic acid bacteria to become established in heat-shocked, brined Manzanillo olives

Species	No. of fermentations	No. of fermentations where culture growth in brines was							
		Fully or effectively predominant with brine additive treatment ^a				Not predominant or not established with brine additive treatment ^a			
		A	B	C	D	A	B	C	D
<i>Lactobacillus plantarum</i>	24 ^b	6	6	6	6	0	0	0	0
<i>Pediococcus cerevisiae</i>	8	1 ^c	2	2	2 ^c	1	0	0	0
<i>Leuconostoc mesenteroides</i>	8	0	2	2	0	2	0	0	2
<i>Lactobacillus brevis</i>	8	0	0	0	0	2 ^d	2	2	2

^a A = no additive; B = plus 2.5 ml of 85% lactic acid per 0.5 gal of brined olives; C = plus lactic acid and 1.5% dextrose; D = plus dextrose (1.5%) only.

^b Includes eight fermentations of *L. plantarum* alone and eight each with two- and three-species mixtures.

^c These fermentations were in the "effectively predominant" category, meaning that there was slight to moderate development of other morphological cell types in the brine at one time or another during the fermentation period. All other fermentations listed in this portion of the table were in the "fully predominant" category, meaning that the inoculated culture was present in extremely large numbers to the exclusion or near exclusion of any other morphological cell types.

^d One fermentation developed a 5+ brine turbidity but the growth could not be ascribed to the inoculated culture. The remaining seven brines inoculated with this species (*L. brevis*) showed 0 to ± brine turbidities throughout the 7.5-month observation period; also, the amount of brine microflora did not appear to exceed that contributed by the inoculum.

to review certain pertinent points. First, the heat-shock treatment was, as mentioned earlier, developed in our pure-culture fermentation studies of brined cucumbers (21) to rid the fruit of naturally occurring competitive microorganisms so that the introduced species of lactic acid bacteria would be provided ample opportunity to become established and develop properly. Second, the acidification treatment was included to preclude or inhibit the development of bacterial spores surviving the heat-shock treatment in the possible event the added lactic culture was slow to establish itself.

The studies on brined cucumbers revealed that uninoculated, heat-shocked controls that were not acidified usually developed a vigorous, malodorous fermentation typical of that resulting from butyric acid-producing anaerobes; when the brines were acidified with lactic acid or inoculated with the appropriate lactic culture, no such butyric acid fermentations took place. Acidification with sufficient lactic acid to reduce the initial brine pH to 4.50 to 4.75 at equalization evolved as the standard treatment for cucumbers; this amounted to about 2.5 to 3.0 ml of 85% lactic acid per 0.5 gal of brined material. When this treatment was applied to olives in the present study, the equalized brine pH was considerably higher than found for cucumbers because of the much higher initial pH of the olives after the debittering treatment and of their greater buffering capacity. Actually, about twice the amount of

lactic acid was required for brined olives as for cucumbers to achieve the desired pH range. This amounted to 5 to 6 ml of 85% lactic acid per 0.5 gal of brined olives. This amount, and two other levels, were used in subsequent fermentation studies on brined olives (1965 season). Also, a sufficient amount of 50% lactic acid was added to two of the four uninoculated, heat-shocked controls in the present work to obtain equalized brine pH of 4.8 (Table 6, treatments B-1 and C-1). The latter brines remained optically clear throughout the 7.5-month observation period, and no microbial cells were seen upon microscopical examination. Two of the six remaining lots in this control series developed active growth sometimes between 1 and 7.5 months of storage. This was understandable, as one of the brines was not acidified and the other was not acidified sufficiently to preclude latent development of spores surviving heat-shock treatment. The morphological cell types seen in both brines were the same and consisted of rods of moderate size in abundance. Cell types typical of those predominating in unheated controls (pediococci, yeasts, and coccobacilli) were not observed, and both fermentations were free of butyric acid odor.

Maximal growth and acid production obtained for all inoculated lots in both the heat-shocked and unheated olive series that received the four brine additive treatments (A and D) are summarized in Table 6. In the heat-shocked series, *L. plantarum* consistently produced the most

TABLE 6. Summarized results for all inoculated fermentations of heat-shocked and unheated Manzanillo olives^a conducted with several species of lactic acid bacteria

Species	Additive treatment ^b	Brines from heat-shocked olives					Brines from unheated olives				
		Equalized NaCl content	Visual opacity ^c	Light transmission	Acidity ^d	Final pH	Equalized NaCl content	Visual opacity	Light transmission	Acidity	Final pH
<i>Lactobacillus plantarum</i> .	A	5.9	5+	5	1.04	3.85	5.6	4+	19	0.36	4.95
	B	5.7	5+	6	1.13	3.90	5.6	2+	52	0.25	5.35
	C	4.9	5+	8	1.27	3.75	5.0	3+	40	0.42	4.50
	D	4.9	5+	6	1.25	3.80	4.9	5+	15	0.49	4.40
<i>Pediococcus cerevisiae</i> ...	A	5.8	5+	18	0.50	4.50	5.7	3+	35	0.20	5.55
	B	5.8	5+	21	0.54	4.40	5.6	0	63	0.19	5.80
	C	4.8	5+	19	0.61	4.35	5.0	0	64	0.17	5.80
	D	4.9	5+	10	0.66	4.35	5.9	3+	25	0.21	5.40
<i>Leuconostoc mesenteroides</i>	A	5.4	<2+	46	0.11	6.20	5.7	<2+	41	0.19	5.85
	B	5.8	4+	31	0.35	5.00	5.7	0	63	0.17	5.85
	C	5.0	4+	22	0.32	5.00	4.8	0	63	0.17	5.80
	D	4.9	<1+	45	0.16	6.00	4.9	0	52	0.20	5.70
<i>Lactobacillus brevis</i>	A	5.6	2+	37	0.16	5.85	5.9	3+	35	0.21	5.55
	B	5.9	0	66	0.15	5.85	5.7	0	61	0.18	5.80
	C	5.5	0	59	0.17	5.75	4.9	0	65	0.18	5.75
	D	5.2	0	66	0.11	6.30	4.8	3+	34	0.25	5.55
<i>L. plantarum</i> + <i>P. cerevisiae</i>	A	5.8	5+	4	1.09	3.90	5.6	2+	35	0.23	5.55
	B	5.8	5+	5	1.08	3.85					
	C	4.9	5+	6	1.26	3.80					
	D	4.7	5+	4	1.23	3.80					
<i>L. plantarum</i> + <i>P. cerevisiae</i> + <i>L. brevis</i>	A ^e	5.5	5+	5	1.03	3.90	5.7	3+	33	0.23	5.40
	B	5.8	5+	7	1.08	3.90					
	C	5.1	5+	7	1.22	3.80					
	D	5.1	5+	4	1.24	3.80					
Uninoculated controls	A	5.8	<1+	45	0.11	6.15	5.7	2+	31	0.19	5.55
	B ^f	5.7	0	63	0.13	6.00	5.7	0	60	0.16	5.85
	C ^f	5.7	4+	20	0.35	4.90	4.9	<1+	58	0.24	5.70
	D	4.9	2+	23	0.12	6.00	4.9	3+	36	0.26	5.55
	B-1	5.9	0	71	0.34	4.80					
C-1	4.8	0	77	0.31	4.80						

^a Heat-shocked (74 C for 3 min) and unheated olives were packed in 0.5-gal glass jars, covered with a 10.6% NaCl brine plus the desired additives, and allowed to stand overnight prior to inoculation. All values shown represent averages of duplicate fermentations except for the three treatments indicated. Maximal values shown for growth and acid production.

^b A = no additive; B = plus 2.5 ml of 85% lactic acid per 0.5 gal of brined olives; C = plus lactic acid and 1.5% dextrose; D = plus 1.5% dextrose only.

^c Values with plus signs indicate the amount of growth as reflected by the degree of brine turbidity (5+ = very heavy; 0 = none).

^d As lactic acid.

^e Values for this treatment, with unheated olives, represent a single fermentation.

^f Values shown for these two treatments, with heat-shocked olives, represent single brined lots with the regular amount of added lactic acid; values shown for treatments B-1 and C-1 are for single lots that each received an additional 12 ml of 50% lactic acid.

abundant growth, the highest brine acidities, and the lowest pH values of the cultures tested; this was true for all brine additive treatments and in all instances in which this species was used alone or as an equal part of mixed species inocula. Also, the data shown for the three sets of fer-

mentations each employing *L. plantarum* singly, or in a two-species, or a three-species mixture are almost the same when like brine additive treatments are compared. Of the three remaining species used as inocula for heat-shocked olives, *P. cerevisiae* was the only one which achieved a

5+ growth rating and produced sufficient acid to drop the brine pH to the 4.35 to 4.50 range.

Evaluation of the brine-cured olives from most of the heat-shocked, inoculated treatments, after 7.5 months of fermentation and storage, revealed that the overall quality of the pickled fruit from fermentations induced by *L. plantarum* was superior to that obtained with the other three species, *P. cerevisiae*, *L. mesenteroides*, and *L. brevis* (listed in descending order as to quality of the brine-cured product produced). The quality characteristics of brine-cured olives from fermentations with *P. cerevisiae* were rather similar in most respects to those obtained with *L. plantarum*, differing chiefly in that a higher and more desirable degree of acidification occurred with the latter species. The failure of *L. brevis* to become established and to develop in the brined olives was clearly reflected in an unacceptable final product that was almost wholly unfermented and had a raw olive color. Essentially the same was true for the quality of brined olive samples from fermentations that had been inoculated with *L. mesenteroides*; this species became established only in brines that were acidified at the outset, and even then it produced a rather weak acid fermentation.

Measurements for the firmness of brined olives from 16 inoculated and 8 control treatments are shown in Table 7. Based on the values obtained, all lots could be placed in the "firm" category as to texture. There were no differences noted in olive firmness obtained within a given set of additive treatments for both inoculated and control lots, but there was a definite trend for lower readings for olives from inoculation treatments which resulted in vigorous acid fermentations (i.e., *L. plantarum* used singly or with *P. cerevisiae*). The values for olives from the two sets of uninoculated controls were almost the same; this indicated that the prebrining heat-shock treatment of 74 C for 3 min did not decrease the firmness of brine-cured olives.

For comparison with the firmness values of our brine-cured olives, measurements were made on 10 jars of imported Spanish green olives, representing eight different packers, and purchased in retail stores located in the Chicago and Minneapolis areas. The values obtained, with a plunger of the same size (no. 4), ranged from 110 to 290 g, with two lots (110 and 155 g) being rated as soft to bordering on soft when eaten or chewed. We continue to gather data pertinent to evaluation of the olive pressure tester used herein and believe it has promise in both experimental and commercial areas for measuring olive texture.

In the experiments described, we used olives which were lye-treated and washed in the conventional manner to remove most of their bitter-

TABLE 7. Firmness of heat-shocked Manzanillo olives from fermentations inoculated with different species of lactic acid bacteria^a

Species	Pressure of olives (in grams) from additive treatment ^b				
	A	B	C	D	Mean ^c
<i>Lactobacillus plantarum</i>	355	340	330	330	340
<i>L. plantarum</i> + <i>Pediococcus cerevisiae</i>	360	350	360	350	355
<i>P. cerevisiae</i>	350	380	385	380	375
<i>Leuconostoc mesenteroides</i>	400	395	365	370	385
Heat-shocked controls (uninoculated).....	400	390	395	400	390
Unheated controls (uninoculated).....	400	385	385	365	380

^a Olives, 98 to 113 per pound, after a 7.5-months fermentation and storage period, were examined as to firmness with a Chatillon spring tester equipped with the no. 4 plunger. The individual firmness values represent the average of 30 to 40 readings (15 to 20 olives, two punctures each).

^b A = no additive; B = plus 2.5 ml of 85% lactic acid per 0.5 gal of brined olives; C = plus lactic acid and 1.5% dextrose; D = plus dextrose (1.5%) only.

^c Least significant difference at *P* 0.05, 19; at *P* 0.01, 26.

ness. In contrast, and as a matter of curiosity, a single one-quart jar was filled with green raw olives just as received from California and covered with a 40° salometer salt brine (10.6% NaCl). At equalization, the brine strength was 6.5% (NaCl) with a light transmission of 85%; the brine acidity, pH, and reducing sugar content were 0.13% (calculated as lactic acid), 5.0%, and 2.3%, respectively. These values remained fairly constant throughout the observation period (7.5 months); the complete absence of microbiological activity during the same period prompted a companion study, to be reported separately, on the inhibitory properties of several varieties of green olives for different species of lactic acid bacteria.

DISCUSSION

The method previously developed in our laboratory for the pure-culture fermentation of brined cucumbers and other vegetables has been successfully applied to Manzanillo olives. The relative abilities of *L. plantarum*, *P. cerevisiae*, *Leuconostoc mesenteroides*, and *Lactobacillus brevis* to become established and to produce acid in both heat-shocked and unheated brined olives

were evaluated. When extended to olives, the heat-shock treatment not only proved effective in ridding the fruit of competitive interfering microbial groups prior to brining and inoculation, but also made the olives highly fermentable with respect to growth and acid development by the introduced culture, particularly *L. plantarum*. Inoculation of heat-shocked olives with *L. plantarum* led to early and vigorous acid fermentations; when unheated olives were used, this species failed to grow, or at best grew only slightly. Lack of growth was even more complete for the other lactic species tested.

Manzanillo olives are one of the more difficult varieties to ferment properly. Hence, the great improvement in their fermentability resulting from the heat-shock treatment is an important finding that needs further inquiry for proper explanation. We are suggesting that the heat treatment probably destroys or attenuates a naturally occurring inhibitory substance in the olive flesh. Preliminary results reinforce the idea that Manzanillo olives and other varieties do indeed contain a bacteriostatic material (Fleming and Etchells, unpublished data) and that there exists a selective inhibitory action according to species, with *L. plantarum* being the most resistant, followed by *P. cerevisiae*, *L. mesenteroides*, and *L. brevis*, in the approximate order of increased sensitivity.

Manzanillo olives are comparatively rich in the bitterness principle (15) and low in fermentable sugars (26), which contribute to their fermentation difficulties (28, 29). Frequently, to reduce the bitterness to an acceptable level, the lye treatment (and subsequent leaching in water to remove the alkali), as employed by processors, may be sufficiently severe to reduce the sugar content below that required to support an active acid fermentation. Such treatment could result in fermentations having low levels of brine acidity. Although the above explanation is acceptable for lack of acid development under the condition described, it hardly applies to the problem of "stuck" fermentations which, according to Vaughn et al. (29), commonly occur with Manzanillo olives. These workers stated that the brine sugar content of "stuck" fermentations is sufficient to support active yeast growth, which predominates, and, for some unknown reason, the lactic acid bacteria fail to become established. Addition of sugar only further accentuated yeast development; replacing part or all of the original brine with an actively fermenting normal brine was considered necessary to correct the abnormal condition. We would suggest that the lactic acid bacteria failed to grow properly in the "stuck" brines for much the same reason that our inoculated cultures of lactic acid bacteria failed to grow

in the brines of unheated olives in the present study: the inhibitory action of a soluble substance present in the olives.

Yeasts were the most common single microbial group observed in the brines of our unheated olives, despite the fact they had been inoculated with pure cultures of lactobacilli. Assuming that the yeasts were more tolerant than the lactic acid bacteria to the inhibitor level in the "stuck" Manzanillo brines, these undesirable organisms could grow and predominate to the near exclusion of the desired lactics.

The corrective procedure recommended by the California researchers for "stuck" fermentations consisted of removal of part or all of the original brine. This would serve to reduce or dilute the residual inhibitor content of the brined olives to a level tolerated by the lactic acid bacteria introduced with the normally fermenting replacement brine. In this more favorable environment, active growth and subsequent acid production by the newly added lactobacilli would be expected, provided sufficient fermentable sugar was available.

The total amount of fermentable carbohydrates available in olive brines to support microbial activity may depend on residual amounts of the bitter compound remaining in the olives that are brined. The glucoside responsible for the bitterness [discovered and named "oleuropein" by Bourquelot and Vintilesco (8) and studied in the purified state by Cruess and Alsberg (15)] is hydrolyzed by weak acids to yield D-glucose. Lactobacilli could cause this reaction during the acid fermentation and slowly continue to add a secondary supply of fermentable sugar to the brine during the full course of the fermentation, even after the primary source of sugars from the olives is depleted. The slow release of glucose from the hydrolyzed glucoside could, in part, be responsible for the prolonged microbiological activity associated with brined olives, particularly the Manzanillo variety.

As mentioned earlier, our curiosity first became aroused concerning a possible microbial inhibitor in olives when the brine on raw Manzanillo olives remained optically clear for months and microscopic examination repeatedly revealed no evidence of microbial cells. The notion of a microbial inhibitor was not original with us. Probably most researchers and technologists in the olive industry have given the matter thought at one time or another; Vaughn (28) mentioned the possibility of antibiotic substances in olives, as did Webster (*personal communication*).

Even so, no experimental studies establishing the presence of inhibitors for lactic acid bacteria in olives have come to our attention. It is our opinion that the fermentability of different varieties of olives depends more on their inhibitor

content than on any other single property they might possess.

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