Growth Rates of *Lactobacillus* and *Leuconostoc* Species in Orange Juice as Affected by pH and Juice Concentration

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Bacteria of the *Lactobacillus* and *Leuconostoc* species are frequently encountered in concentrated orange juice. Several strains have been identified with a type of spoilage known as "buttermilk off-flavor" characterized by the presence of diacetyl in the product. The processing conditions under which this substance is formed by the bacteria are not known. It is assumed, however, that the bacterial populations are probably large and that the environment in some stage of the evaporation process must be particularly favorable for the synthesis and accumulation of diacetyl. In support of this assumption, Hays and Riester (1952) observed lactobacilli and leuconostoc in commercial citrus juice evaporators, particularly in the early stages where the concentration of the juice is relatively low.

From orange concentrate showing this type of spoilage, Hays (1951) isolated *Lactobacillus brevis* and *Lactobacillus plantarum* var. *mobilis* strains which were capable of growing in 35 Brix concentrate. He states that these organisms came from the fruit, gained access to the evaporators with the feed juice, and multiplied in the citrus solids which were not being continually washed free from the surfaces of the evaporators.

Murdock *et al.* (1952b) found two of the most prevalent spoilage organisms causing this type of off-flavor to be species of the genera *Lactobacillus* and *Leuconostoc*. They found that the development of off-flavor depended on the strain of organisms, the size of the inoculum, and the period of incubation. They believed it could be possible for off-flavors to develop in some types of evaporators where recirculation allows the juice to stay in the first effect for unusually long periods of time. However, they considered that the condenser was the most likely point for a buildup of contaminants to occur.

Hays and Riester (1952) described the cultural characteristics of *Lactobacillus* and *Leuconostoc* species recovered from spoiled orange concentrate. They reported *Lactobacillus* to be the predominant spoilage type in high-acid juices, and the less-acid-tolerant *Leuconostoc* in the lower acid juices. These species produced diacetyl as one of the products of fermentation when cultured in orange juice in the laboratory. Concentrations of orange juice suitable for the growth of these organisms were found in the early stages of evaporation in the vacuum concentrators.

Murdock *et al.* (1953) observed that the majority of true gum-forming organisms, coccoid in shape, catalase negative, and belonging to the genus *Leuconostoc*, grew in orange juice and produced off-flavors. Most catalase positive coccoid organisms failed to grow in orange juice. The former type of organism was isolated from unsound fruit, and the latter from sound fruit.

Faville *et al.* (1951) studied the ability of *Leuconostoc mesenteroides* to grow at 30 C in orange juice and concentrate. They took hourly platings for seven hours and found that this organism remained static at pH 3.68 in 10.5 Brix juice and died rapidly at pH 3.5 in 42 Brix concentrate.

In their studies on the detection of diacetyl, Hill *et al.* (1954) reported on the growth of *L. plantarum* and of a gum-forming spherical organism in recirculated 20 Brix concentrate at pH 3.9. Off-flavors were produced in conjunction with a large increase in numbers for both organisms.

Materials and Methods

Sufficient Valencia orange concentrate to complete the proposed study was prepared in a pilot plant evaporator and adjusted to 42 Brix with distilled water. After thorough mixing, it was sealed in 46-ounce cans and stored at −20 C. When needed, the concentrate was thawed quickly in running tap water. The desired concentration was obtained by dilution with distilled water and the pH adjusted with sodium hydroxide or citric acid. Erlenmeyer flasks were half-filled, that is, 250 ml of 12 Brix orange juice in 500-ml flasks, 150 ml of 18 or 24 Brix concentrate in 300-ml flasks, and 50 ml of 32, 37 or 42 Brix concentrate in 125-ml flasks. For economy of materials smaller flasks were used as the concentration was increased, but in each case, the ratio of surface to volume remained of the same order. The flasks of juice were placed for 30 minutes in flowing steam, then cooled and stored at −20 C. Control plates demonstrated that this procedure produced sterility. They were allowed to warm to 21 C before inoculation.

In order to insure a vigorously growing culture each

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test organism was inoculated into orange serum broth, incubated for 24 hours then transferred to 12 Brix orange juice, and incubated overnight. Subcultures thus prepared were used to seed the orange juice or concentrate for growth rate determinations. The size of the inoculum was chosen to obtain initial counts in the range 100,000 to 300,000 organisms per ml. The inoculated flasks used in the growth rate studies were shaken continuously on a Kahn-type shaker at 40 oscillations per minute while incubated at 21 C. This temperature was chosen as representative of that existing in commercial evaporators during operation, and shaking was used to simulate the agitation of the juice during concentration.

Aliquots of the seeded orange juices or concentrates were taken at the time of inoculation and at hourly intervals for seven hours. These samples were diluted and plated in duplicate on orange serum agar (Murdock *et al.*, 1952a). The plates were incubated at 30 C for *Lactobacillus* and 21 C for *Leuconostoc*. Plate counts were made at 24 to 36 hours for *Leuconostoc* and 48 hours for *Lactobacillus*.

Hays and Riester (1952) isolated *L. brevis*, strains B27 and B28, *L. plantarum* var. *mobilis*, B29 and B32, *Leuconostoc dextranicum*, B34 and B35, and *L. mesenteroides*, B42 and B47 from orange concentrate showing "buttermilk" spoilage. Subcultures of these strains were obtained from these investigators and used for these studies. When grown in orange juice, all of these organisms produced off-flavors characterized as being of the "buttermilk" type.

Growth rates for these organisms were determined in the following order: (1) in portions of 12 Brix juice at pH 3.4, 3.6, 3.8, and 4.0; (2) in portions of 18, 24, 32, 37, and 42 Brix concentrate at pH 3.8; and (3) in portions of 18 Brix concentrate at pH 3.4, 3.6, 3.8, and 4.0. All experiments were in triplicate except where cross checks were made (18 Brix at pH 3.8). In this case there was a total of six replicates. Both strains of each organism were treated in this manner; therefore, the values reported are averages of 3 to 6 determinations.

The $\log_{10}$ of the number of organisms per ml was plotted against time. Usually the points fell along a straight line, indicating regular logarithmic growth. In those few cases where appreciable deviation from logarithmic growth was observed, the results of that fermentation were discarded and the experiment repeated.

The most probable line of logarithmic growth was calculated for each series of platings, and the replicates of each strain were averaged. The following formulas were used (Youden, 1951):

$$a = y - bx$$

where a is a constant representing the $\log_{10}$ of the initial microbial population, y is the $\log_{10}$ of the number of organisms per ml at time x in hours, and b is the slope of the line; and

$$b = \frac{n\Sigma xy - \Sigma x\Sigma y}{n\Sigma x^2 - (\Sigma x)^2}$$

where n is the number of platings in a series. The increase in the $\log_{10}$ of the number of organisms per ml per hour will be referred to hereafter as the growth rate. In order to allow for a lag period during which the organisms became adjusted to the change in osmotic pressure of the medium, the initial counts were not used in the calculations. Instead, calculations were based upon the plate counts beginning with the end of the first hour of incubation.

Generation times are based on the equation:

$$g = \frac{t \log 2}{\log b - \log B} \qquad \text{(Porter, 1946)}$$

where g is the generation time, t is the time in hours, b is the number of bacteria per ml at time t, and B is the number of bacteria per ml at the beginning of the experimental period. If t is considered to be one hour, then $\log b - \log B$ is the growth rate, and

$$g = \frac{\log 2}{\text{growth rate}}$$

Results and Discussion

In order to illustrate the course of the plate counts, a typical series of determinations is presented for *L. plantarum* var. *mobilis* in figure 1. In this case, the fermentations at pH 3.4, 3.8, and 4.0 were started from the same inoculum. The pH 3.6 fermentation was made on another day and the initial counts were different.

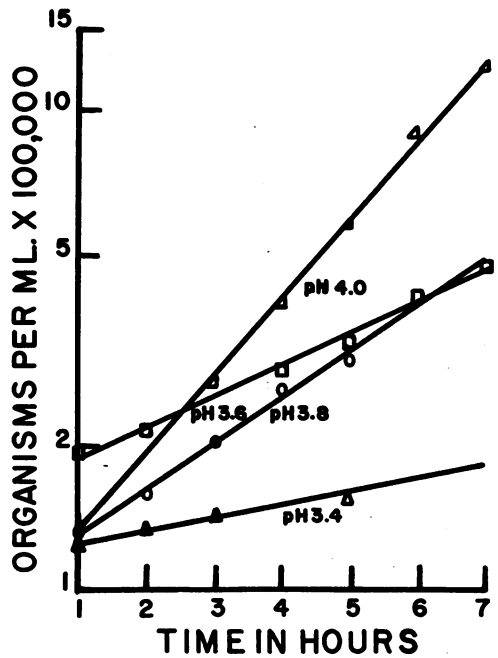

FIG. 1. Typical growth curves of *Lactobacillus plantarum* var. *mobilis* in 12 Brix orange juice.

The counts have been plotted on a logarithmic scale, and it will be noted that straight lines are formed indicating logarithmic growth.

The growth rates of two strains each of two species of *Lactobacillus* and of two species of *Leuconostoc* in orange juice at several concentrations and pH values are summarized in table 1.

In general, both strains of each organism were very much alike in growth rates. With the exception of *L. brevis* at 37 Brix and pH 3.8, the differences are very small. It is of interest to note that much more erratic results were obtained when sterile media were stored for several days at about 4 C before use. This kind of result decreased when the sterile media were stored at −20 C. Evidently changes take place in orange juices at refrigerator temperatures which affect its quality as a culture medium.

The highest growth rate found was that for *L. mesenteroides* in 12 Brix juice at pH 4.0. This organism was the only one studied which showed a negative growth rate in 12 Brix juice (at pH 3.4), indicating that this species is most sensitive to changes in pH of the four studied. In general, the two *Lactobacillus* species were very similar to each other, with *L. plantarum* var. *mobilis* growing a little faster under all conditions. The genus *Lactobacillus* is known to be more acid tolerant

TABLE 1. *Effect of pH and concentration of orange juice on the growth rates of four species of bacteria*

Orange Juice		*Lactobacillus brevis*		*Lactobacillus plantarum* var. *mobilis*		*Leuconostoc dextranicum*		*Leuconostoc mesenteroides*	
		Strain no.							
Degrees Brix	pH	B27	B28	B29	B32	B34	B35	B42	B47
12	3.4	.032	.037	.044	.043	.062	.061	−.026	−.025
	3.6	.052	.050	.064	.067	.11	.11	.051	.047
	3.8	.077	.079	.094	.093	.14	.14	.13	.13
	4.0	.10	.12	.13	.13	.20	.20	.21	.23
18	3.4	.027	.028	.035	.036	.033	.033	−.037	−.035
	3.6	.038	.037	.047	.044	.051	.056	−.025	−.024
	3.8	.067	.063	.076	.073	.089	.09	.081	.081
	4.0	.086	.086	.098	.095	.11	.11	.10	.10
24	3.8	.054	.054	.065	.062	.071	.073	.065	.067
32	3.8	.031	.037	.048	.048	.042	.044	.037	.037
37	3.8	.006	.015	.025	.023	.023	.029	.024	.022
42	3.8	−.021	−.026	.014	.012	.014	.014	−.006	−.003

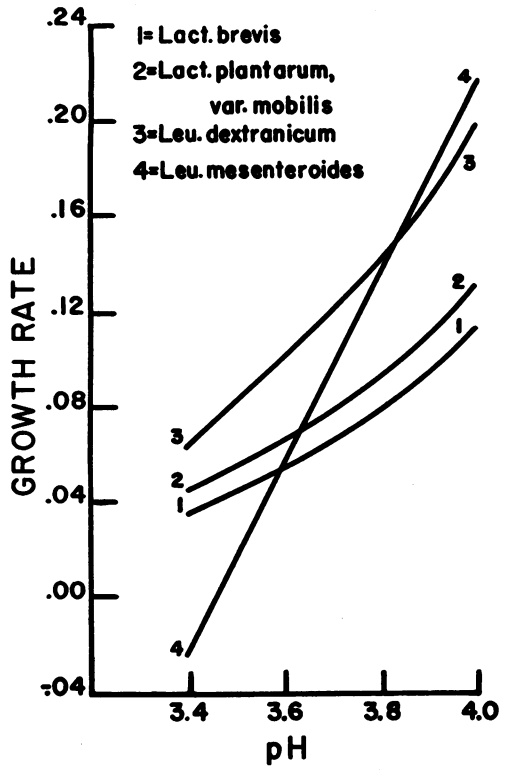

FIG. 2. Growth rates of *Leuconostoc* and *Lactobacillus* species in orange juice of 12 Brix at different pH values.

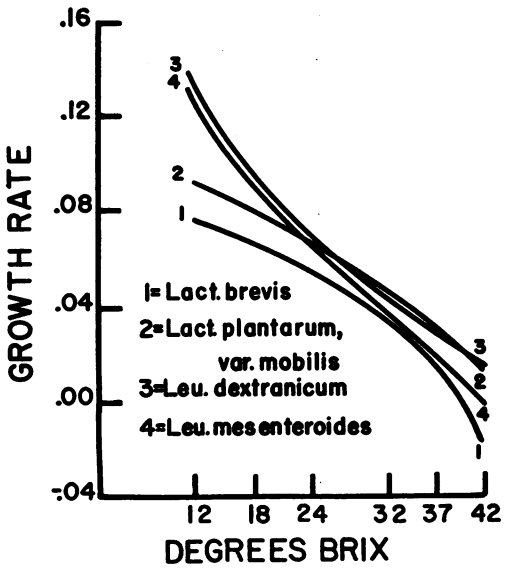

FIG. 3. Growth rates of *Leuconostoc* and *Lactobacillus* species in pH 3.8 orange juice or concentrates at different concentrations.

TABLE 2. *Generation times in hours of Lactobacillus and Leuconostoc species in orange juice or concentrates at various pH values*

Generation time in hours

Orange Juice		*Lactobacillus brevis*	*Lactobacillus plantarum* var. *mobilis*	*Leuconostoc dextranicum*	*Leuconostoc mesenteroides*
Degrees Brix	pH				
12	3.4	8.8	6.8	4.9	*
	3.6	5.9	4.6	2.8	6.1
	3.8	3.9	3.2	2.2	2.3
	4.0	2.7	2.3	1.5	1.4
18	3.4	10.7	8.4	9.1	*
	3.6	7.9	6.7	5.6	*
	3.8	4.6	4.0	3.3	3.7
	4.0	3.5	3.1	2.7	3.0
24	3.8	5.6	4.7	4.2	4.6
32	3.8	8.8	6.3	7.0	8.1
37	3.8	27.	12.5	11.6	13.1
42	3.8	*	23.	22.	*

* Negative growth rates were encountered under these conditions.

than is *Leuconostoc*, and this is confirmed for the species examined in this study, since increasing acidity reduced the growth rates of the *Leuconostoc* species more than those of the *Lactobacillus* species.

Negative growth rates are indicative of a decrease in viable (plate) count due to unfavorable environmental conditions. These conditions for *L. mesenteroides* were present in 12 Brix juice at pH 3.4, in 18 Brix concentrate at pH 3.4 and 3.6, and in 42 Brix concentrate at pH of 3.8. The least favorable conditions for this species occurred in 18 Brix concentrate at pH 3.4. The decrease in viable cells was about the same in 12 Brix at pH 3.4 as in 18 Brix at pH 3.6.

For all strains examined, the growth rate decreased as the pH decreased. Figure 2 illustrates the results obtained with 12 Brix juice as the pH was varied. Only *L. mesenteroides* shows a response to pH markedly different from the others. The two *Lactobacillus* species show essentially parallel behavior. *L. dextranicum* was somewhat more sensitive to pH change than the *Lactobacillus* species, but only *L. mesenteroides* was extremely sensitive to the pH of orange juice.

All organisms grew more slowly as the concentration of orange juice was increased. Negative growth rates were observed at 42 Brix for *L. brevis* and *L. mesenteroides*, but not for the other two species.

While curves shown in figure 3 for each genus are approximately parallel, there is a somewhat greater rate of change of the response of the *Leuconostoc* species than of the *Lactobacillus* species between 12 and 18 Brix. *L. brevis* is somewhat more sensitive to increase in concentration above 37 Brix than are any of the other species.

In as much as generation times may be of somewhat greater utility in interpreting plant experience than are growth rates, these were computed for each of the four species studied. The average growth rates for the two strains of each species were used because they did not differ significantly. The values obtained are presented in table 2.

If the highest growth rate is taken as an example, it would be necessary for *L. mesenteroides* to remain in the evaporator at 12 Brix and pH 4.0 for 1.4 hours in order for the population to double. Because juice in the operating evaporator is concentrated rapidly and the over-all retention time is a matter of a few minutes, not hours, it becomes difficult to explain the development of significant numbers of these organisms on the basis of the growth rates observed, unless other factors are considered. Such factors might be the propagation of the microorganisms in static films on the walls of condensers or evaporators, or in pockets of relatively undisturbed juice. The possibility that the organisms had already developed in the fruit has not been ruled out entirely.

Barreto's Master of Science thesis (1953), on *L. brevis*, *L. plantarum*, and *L. mesenteroides* in orange juice media indicates essentially the same growth rates under microaerophilic and aerobic conditions. The differences were insignificant when considering the time necessary for off-flavors to develop.

SUMMARY

Growth rates were determined in orange juice and in orange concentrates for 8 strains of bacteria which had been isolated from commercial frozen orange concentrates exhibiting buttermilk off-flavor spoilage. The concentrations of orange juice varied from 12 to 42 Brix and the pH from 3.4 to 4.0. *Leuconostoc* species were more affected by changing pH than were *Lactobacillus* species. *Leuconostoc mesenteroides* was especially sensitive to pH change. Negative growth rates were observed for *Leuconostoc mesenteroides* at pH 3.4 in both 12 and 18 Brix, at pH 3.6 in 18 Brix, and at pH 3.8 in 42 Brix. *Lactobacillus brevis* also had a negative growth rate of pH 3.8 in 42 Brix. The most rapid growth observed was that of *Leuconostoc mesenteroides* at pH 4.0 in 12 Brix. This growth rate was equivalent to a generation time of 1.4 hours. It is believed that there is little chance of off-flavor development in commercial evaporators as a result of the growth of these organisms except in pockets or films where juice turnover is abnormally slow.

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