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Behavior of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in Fresh and Thermally Processed Orange Juice

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ABSTRACT

Lactobacillus plantarum and *Saccharomyces cerevisiae* are acid-tolerant microorganisms that are able to spoil citrus juices before and after pasteurization. The growth of these microorganisms in orange juice with and without pasteurization was investigated. Two samples of orange juice were inoculated with ca. 10^5 CFU/ml of each microorganism. Others were inoculated with ca. 10^7 CFU/ml of each microorganism and then thermally treated. *L. plantarum* populations were reduced by 2.5 and <1 $\log_{10}$ CFU/ml at 60°C for 40 s and at 55°C for 40 s, respectively. For the same treatments, *S. cerevisiae* populations were reduced by >6 and 2 $\log_{10}$ CFU/ml, respectively. Samples of heated and nonheated juice were incubated at 15°C for 20 days. Injured populations of *L. plantarum* decreased by ca. 2 $\log_{10}$ CFU/ml during the first 70 h of storage, but those of *S. cerevisiae* did not decrease. The length of the lag phase after pasteurization increased 6.2-fold for *L. plantarum* and 1.9-fold for *S. cerevisiae,* and generation times increased by 41 and 86%, respectively. The results of this study demonstrate the differences in the capabilities of intact and injured cells of spoilage microorganisms to spoil citrus juice and the different thermal resistance levels of cells. While *L. plantarum* was more resistant to heat treatment than *S. cerevisiae* was, growth recovery after pasteurization was faster for the latter microorganism.

The microbial contamination level and the storage temperature stability before and after pasteurization are major factors determining the quality and shelf life of citrus juices. Microbial populations were estimated to be $10\sim10^6$ CFU/ml in single-strength orange juice *(18, 27).* Lactic acid bacteria, especially *Lactobacillus* spp. and *Leuconostoc* spp., and yeasts such as *Saccharomyces* spp. and *Candida* spp. have often been reported as spoilage microorganisms of citrus juices *(1, 6–8, 13, 19–21, 25).* These microorganisms produce an undesirable buttermilk flavor due to diacetyl production, a fermented flavor due to the presence of ethanol and organic acids (lactic acid and acetic acid), and swelling of packages due to the production of carbon dioxide and trace fermentation products. Two types of pasteurization are traditionally applied to citrus juices: full pasteurization at 76 to 99°C for a few seconds to 1 min and light pasteurization at 66 to 75°C for 10 to 16 s *(17, 22, 23).* These regimens have historically been used to eliminate or reduce microbial charge and to inactivate enzymes involved in juice alterations (pectinmethylesterase and polyphenoloxydase). The storage temperature before and after pasteurization plays an essential role in determining the shelf life of a citrus juice. Single-strength orange juice was found to be chemically, visually, and microbially stable for 20 to 23 days at −1.7°C, for 16 to 22 days at 1.1°C, for 10 to 16 days at 4.4°C, and for 5 to 8 days at 7.8°C *(5).* Heat treatment increases shelf life by up to 4 weeks at 4°C for lightly pasteurized juice and by 3 to 6 months at room temperature for fully pasteurized juice *(22, 23).* Previous studies have investigated the shelf life and quality of orange juice at several storage temperatures *(4, 5, 10, 14, 22)*; other studies have dealt with the thermal resistance of citrus juice spoilage microorganisms *(9, 15, 18).* There is a lack of detailed information about the behavior of spoilage microorganisms before and after thermal treatment when storage temperature is abusive. This study was conducted to investigate the growth of intact and heat-injured cells of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in orange juice at an abusive temperature.

MATERIALS AND METHODS

Microbial strains and culture conditions. A strain of *L. plantarum* isolated from fermenting, unpasteurized orange juice was obtained from the Citrus Research and Education Center (Lake Alfred, Fla.) *(19),* and a strain of *S. cerevisiae* (CBS 8066) was obtained from the CentralBureau voor Schimmelcultures (The Netherlands). Colonies from agar plates were transferred into 0.1% sterile peptone water. *L. plantarum* was plated on deMan Rogosa Sharpe (MRS) agar *(2)* and incubated at 37°C for 48 h. Similarly, *S. cerevisiae* was plated on yeast malt (YM) broth agar (Difco Laboratories, Elancourt, France) and incubated at 30°C for 48 h. To prepare precultures, a colony was transferred from each plate into 10 ml of MRS broth or YM broth and then incubated for 24 h at 37°C (for *L. plantarum*) or 30°C (for *S. cerevisiae*). The precultures were then subcultured in 100 ml of the respective growth media and statically incubated for 20 to 24 h to the stationary phase. The cells were spun in a Beckman J2-HC centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) with a JA-10 rotor for 5 min at 4,420 × *g* for *L. plantarum* and for 10 min at 8,670 × *g* for *S. cerevisiae*. The pellets were washed with sterile buff-

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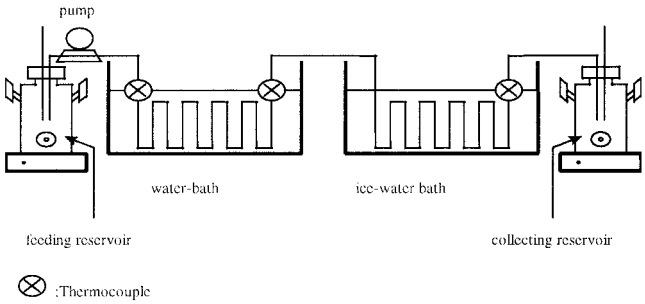

FIGURE 1. *Continuous-flow system for thermal treatment.*

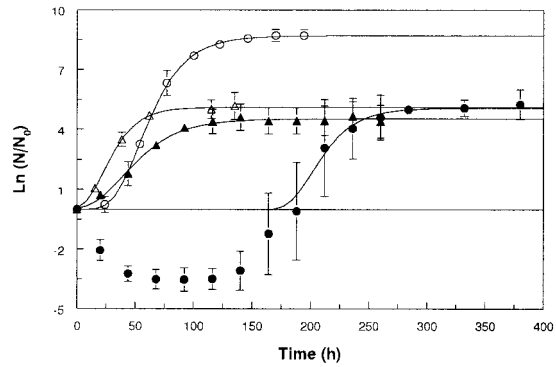

FIGURE 2. *Growth of intact cells (open symbols) and injured cells (closed symbols) of* L. plantarum (○, ●) *and* S. cerevisiae (△, ▲) *in orange juice at a storage temperature of 15°C and at an inoculum concentration of ca. 10^5 CFU/ml. Curves were fitted with the modified Gompertz equation.*

ered peptone water (pH 7), resuspended separately in 1 ml of orange juice (pH 3.6 ± 0.05), and inoculated into 100-ml aliquots of juice (cell densities were ca. 10^5 CFU/ml). Subsamples were taken from each orange juice sample and analyzed on MRS and YM agar plates to determine initial populations. The remaining juice was incubated at 15°C for 20 days. At suitable time intervals, 0.1 ml was plated on MRS agar (for *L. plantarum*) or YM agar (for *S. cerevisiae*) and incubated at 37°C for 72 h or at 30°C for 72 h, respectively.

Orange juice preparation. A fully pasteurized commercial orange juice with a pH of 3.6 ± 0.05 (Joker Co., Macon, France) was used, and subsamples were plated onto orange serum agar (Biokar Diagnostics, Beauvais, France) at 30°C for 5 days to determine the initial microbial contamination of the juice. The total microbial counts were less than 1 CFU/ml.

Juice samples for thermal treatment experiments were prepared to obtain an initial microbial population of ca. 10^7 CFU/ml. The pH was measured with a combined electrode (Inlab 427, Metler Toledo, S.A.R.L., Paris, France).

Continuous-flow thermal treatment. The experimental system, illustrated in Figure 1, consisted of two glass reservoirs, a 316 stainless steel coil (length, 3.66 m; internal diameter, 4.8 mm; wall thickness, 0.8 mm), a variable-speed peristaltic tubing pump (Model 7518-10, Cole-Parmer Inc., Chicago, Ill.), and thermocouples (Fisher Bioblock Scientific, Illkrich, France). The first glass reservoir was used to feed the inoculated orange juice to the system. The orange juice in the stainless steel coil that was immersed in a water bath was heated to the desired temperature and was then passed through a second stainless steel tube (1 m long, 8 mm in diameter) that was immersed in an ice-water bath to cool the heated orange juice to 25°C. Flow rates were measured at the outlet. The average residence time was obtained by dividing the total volume of the test sample inside the heating section by the steady-state volumetric flow rate of orange juice through the system. Inlet and outlet temperatures were continuously measured with thermocouples. Two regimens of pasteurization, 60°C for 40 s and 55°C for 40 s, were applied for each microorganism separately. The treated and cooled orange juice was aseptically collected in the second reservoir, hermetically closed, and stored at 15°C for 20 days. Samples (0.1 ml) of treated orange juice were analyzed on nonselective media (MRS agar for *L. plantarum* and YM agar for *S. cerevisiae*) at t (time) = 0 and at periodic intervals during storage.

Statistical analysis. Experiments were repeated at least three times. The number of survivors recovered immediately after pasteurization for any microorganism at any given temperature and residence time was statistically analyzed by a two-way analysis of variance (species × temperature) to determine differences in the thermotolerance of each microorganism. These analyses were carried out with StatBox software (Grimmer Logiciels, Paris, France). Significant differences were calculated to determine differences between individual means. Similarly, the kinetic parameters of growth, lag phase time in hours (λ), maximum specific growth rate per hour (μ_{max}), and increase in population (A) were estimated by fitting experimental data with the modified Gompertz equation *(28)*:

$$\ln(N_t) = \ln(N_0) + \exp\left\{-\exp\left[\frac{\mu_{max}e}{A}(\lambda - t) + 1\right]\right\}$$

where t is time (h), N_t is the number of microorganisms at time t (CFU/ml), N_0 is the asymptotic number of microorganisms at time 0 (CFU/ml), $A = \ln(N_{max}) - \ln(N_0)$ (where N_{max} is the maximum number of microorganisms [CFU/ml]), and $\mu_{max} = \ln 2/t_g$ (where t_g is the generation time [h]). Slide Write software (Advanced Graphics Software, Inc., Encinitas, Calif.) was used to determine these parameters.

RESULTS AND DISCUSSION

The growth of *L. plantarum* and *S. cerevisiae* in orange juice is shown in Figure 2. *S. cerevisiae* adapted in orange juice better than *L. plantarum* did. For orange juice stored at 15°C, the intact populations of *S. cerevisiae* increased sooner than populations of *L. plantarum* did, with lag phase times of 7.1 and 29 h, respectively. During the logarithmic phase, *L. plantarum* grew faster than *S. cerevisiae* did, with generation times of 5.3 and 5.8 h, respectively. After ca. 125 h of storage, *L. plantarum* became dominant (Table 1 and Fig. 2). Similar results were observed by Murdock and Hatcher *(14),* who reported that the lag phase for yeasts at 10°C was ≤1 day, whereas it was >4 days for *Lactobacillus.* Parish *(16)* reported that the minimum temperature and pH for *S. cerevisiae* growth are lower than those for *Lactobacillus* spp. growth.

Data presented in Table 2 show the lethality of two pasteurization regimens (at 55°C for 40 s and at 60°C for 40 s) to *L. plantarum* and *S. cerevisiae*. These different pasteurization regimens were used to obtain similar numbers of survival populations for each microorganism at time 0 of storage (ca. 10^5 CFU/ml). These data demonstrate that *S. cerevisiae* is more sensitive to heat than *L. plantarum* is. Similar results were obtained for apple juice by Tajchakavit

TABLE 1. *Kinetic growth parameters for heated and nonheated cells of* L. plantarum *and* S. cerevisiae *in orange juice stored at 15°C*

Parameter[a]	Value (95% confidence limit) for: *L. plantarum* No pasteurization (R^2 = 0.99)	*L. plantarum* Pasteurization (R^2 = 0.85)	*S. cerevisiae* No pasteurization (R^2 = 0.99)	*S. cerevisiae* Pasteurization (R^2 = 0.99)
Lag phase (h)	29.0 (25.1–33.0)	181 (163–199)	7.1 (1.8–12.4)	13.7 (1.4–26.0)
Generation time (h)	5.3 (4.8–6.0)	7.5 (4.6–23.1)	5.8 (4.7–7.7)	10.8 (8.1–16.1)
$\ln(N_t/N_0)_{max}$	8.7 (8.6–8.8)	5.0 (4.4–5.7)	5.1 (4.9–5.3)	4.6 (4.4–4.7)

[a] The lag phase includes the decline phase and finishes when $N_t = N_0$ after pasteurization; $\ln(N_t/N_0)_{max}$ starts when $N_t = N_0$ after pasteurization (N_t and N_0 are the numbers of microorganisms at storage times t and 0, respectively).

et al. *(24),* who reported that *S. cerevisiae* was more sensitive to heat treatment than was *L. plantarum* and that the D_{60}-value of *L. plantarum* was 21.9 s and the D_{55}-value of *S. cerevisiae* was 25.1 s. Also, Parish *(16)* reported that *S. cerevisiae* was more sensitive to heat treatment in grapefruit serum than *L. plantarum* was. Analysis of variance showed that the lethalities of the heat treatments to *L. plantarum* (55°C for 40 s) and *S. cerevisiae* (60°C for 40 s) were homogenous within a 95% confidence interval.

During the first 70 h of storage of treated orange juice at 15°C, populations of *L. plantarum* were reduced to 10^{-3} CFU/ml. Surviving cells resumed active growth after 160 h of storage. The times required to reach initial population levels (N_0) and the end of the growth phase were 163 to 199 h and 300 h, respectively (Table 1 and Fig. 2). In contrast, sublethally heat-damaged cells of *S. cerevisiae* reached the end of the growth phase within 120 h without any population decline (Fig. 2) and with a lag phase time of 13.7 h. Lag phases (λ) increased 6.2-fold for *L. plantarum* and 1.9-fold for *S. cerevisiae* after pasteurization.

The yeast cells heated to 60°C for 40 s (<1 log cell survivors) showed no dramatic decline in survival during storage at 15°C, but those of *L. plantarum* heated to 55°C for 40 s (>6 logs cell survivors) showed a dramatic decline in survival (ca. 0.8 to 1.3 logs) during the first 50 h of storage, and surviving cells resumed active growth after 95 h of storage (data not shown). These data suggest that damaged *L. plantarum* cells need more time to repair heat injury than do damaged *S. cerevisiae* cells. Generation times increased by 41% for *L. plantarum* and by 86% for *S. cerevisiae* after pasteurization. This increase may be a consequence of physicochemical changes in the medium due to the Maillard reaction between reducing sugars and proteins *(12)* and to intrinsic modifications of macromolecules, i.e., ribosomes, nucleic acids, enzymes, and proteins in the cell and in the membrane *(3).* In addition, the maximum number of populations [$\ln(N_t/N_0)_{max}$] was lower for injured cells than for intact ones by 43% for *L. plantarum* and by 10% for *S. cerevisiae*. Sadler et al. *(22)* reported that yeast and mold were better adapted to the orange juice environment at 4°C than were bacteria. Juice pH decreased after 20 days of storage for all samples by 0.2 to 0.3 units for intact and injured populations of *L. plantarum,* and by 0.05 to 0.1 units for intact and injured cells of *S. cerevisiae.*

TABLE 2. *Lethality of two pasteurizations to* L. plantarum *and* S. cerevisiae *in orange juice*

	Flow rate/ residence time (ml min^{-1}/s)	Outlet juice temperature (°C)	Microbial reduction (log_{10} CFU/ml)[a]
L. plantarum	100/40	60	2.5 ± 0.2 A
		55	<1.0 B
S. cerevisiae	100/40	60	>6.0 C
		55	2.0 ± 0.5 A

[a] Different letters indicate significant differences among the samples (determined by 95% confidence intervals).

These results demonstrate that lightly pasteurized citrus juice could be spoiled in storage at abusive temperatures by injured cells of spoilage microorganisms. One of the major problems caused by these cells is the production of an off-flavor. Undesirable flavors could therefore be produced in orange juice containing relatively low microbial populations *(14).* Sadler et al. *(22)* reported that lightly and fully pasteurized orange juices were microbially stable at 4°C for 4 weeks but not for 6 weeks. A loss of *L. plantarum* cell viability after two different pasteurization regimens is not understood. Torreggiani and Toledo *(26)* suggested that the environment surrounding a cell can affect the ability of the cell to repair heat injury, and they reported that the type of heating and recovery media used affected the degree of inactivation and repair capability of a Chablis strain of *S. cerevisiae*. Maillard reaction products are known to be inhibition factors for some microorganisms *(11).* As assumed by Patrick and Hill *(21),* the interactive effects of pH and cold storage of orange juice might be less favorable for the survival of heat-injured cells of *L. plantarum* than for those of *S. cerevisiae* and could explain the decline in *L. plantarum* populations directly after pasteurization. The results of this study show that increased heat treatment of lightly pasteurized juices may select for an aciduric bacterial spoilage flora rather than yeasts. Conversely, unpasteurized or less heat treated juices may support the growth of yeasts that appear to be better adapted to acid and can quickly spoil juice. However, despite the superior yeast adaptation in acidic fruit juices, lactic acid bacteria are those that increase to high numbers and eventually predominate at spoilage. Storage temperature control (at 2 to 4°C) could delay the growth and recovery of these bacteria, which have a

minimum growth temperature that is higher than that of yeast. Increasing marketing of lightly pasteurized citrus juices gives rise to the need to better understand the behavior of specific spoilage microorganisms. Since sublethally heat-injured cells may survive in lightly pasteurized citrus juices, it is imperative to control the product environment, especially the storage temperature during the concentration, transportation, reconstitution, and distribution steps of citrus juice manufacture.

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