Evaluation of Processing Methods to Control the Growth of *Lactobacillus kunkeei*, a Micro-organism Implicated in Sluggish Alcoholic Fermentations of Grape Musts*

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Processing strategies that could be used to control the growth of the spoilage bacterium *Lactobacillus kunkeei* during vinification were evaluated. These strategies included the use of SO_2, low temperatures, and adjustment of must pH. On the one hand, *Lactobacillus kunkeei* was sensitive to SO_2 in that growth was not observed at concentrations greater than 0,1 mg/L molecular SO_2. On the other hand, the organism remained viable at 5° and 10°C for up to 10 days in a synthetic medium. Regarding low pH, the organism did not grow well in a synthetic medium at less than pH 3,5. Thus *Lactobacillus kunkeei* was inoculated into fermenting Chardonnay musts adjusted to pH 3,16; 3,50 and 3,80. Although a decrease in must pH resulted in less growth of *Lactobacillus kunkeei*, the bacterium achieved populations in excess of 10^8 CFU/mL and all alcoholic fermentations were slowed. Thus, SO_2 remains the best method for winemakers to control growth of this spoilage bacterium.

Sluggish/stuck alcoholic fermentations are defined as the delayed or imcomplete utilization of sugars during grape juice fermentation. This condition is undesirable and may result in financial losses for winemakers. This problem can be the result of nutritional deficiencies or the presence of inhibitory substances for the yeast or winemaking practices (Lafon-Lafourcade *et al.*, 1984; Tromp, 1985; Ingledew & Kunkee, 1985; Houtman & Du Plessis, 1986; Rasmussen *et al.*, 1995). Recently, Huang *et al.* (1996) demonstrated that uncontrolled growth of certain lactic acid bacteria including a novel species of *Lactobacillus, Lactobacillus kunkeei* (Edwards *et al.*, 1998a), and wild strains of *Oenococcus oeni* (Edwards *et al.*, 1998b) can also retard fermentations.

One method used by winemakers to control the growth of undesirable lactic acid bacteria is through the addition of SO_2 (Fugelsang, 1997). While some wineries have successfully experimented in producing wines without SO_2, some have experienced problems due to uncontrolled bacterial growth. Thus, research investigating alternative processing methods that improve microbiological control during vinification would be advantageous to wineries. For instance, the pH of wines from such regions as Washington State and South Africa can exceed 3,5 (Van Wyk, 1976; Nagel & Spayd, 1989), a factor favourable to the growth of lactic acid bacteria (Wibowo *et al.*, 1985; Davis *et al.*, 1986, 1988; Edwards *et al.*, 1993; Liu *et al.*, 1995). Higher temperatures of musts/wines can also favour bacterial growth (Van der Westhuizen & Loos, 1981; Lafon-Lafourcade *et al.*, 1983).

The objectives of this study were to evaluate the growth of the bacterium, *Lactobacillus kunkeei*, in the presence of SO_2 and under conditions of low temperatures and different pH levels as means to control its growth during winemaking.

MATERIALS AND METHODS

Cultivation media/micro-organisms: *Lactobacillus kunkeei* ATCC 700308 (strain YH-15), *Oenococcus oeni* (strains YH-24 and YH-37), and *Lactobacillus plantarum* (strain WS-23) were previously isolated from commercial wines (Huang *et al.*, 1996; Edwards *et al.*, 1993; 1998b). All bacterial cultures were maintained on modified Rogosa (MR) agar plates (Beelman, 1982) incubated anaerobically at 25°C using the BBL Gas Pak system (Becton Dickinson, Cockeysville, MD, USA) and as lyophilized cultures.

Saccharomyces cerevisiae (strain Epernay) was obtained in the active dry form (Uvaferm CEG, Danstar Ferment AG, Switzerland) and was cultivated on wort agar as described by King & Beelman (1986) using diastatic diamalt (Premier Malt Products, Inc., Grosse Point, MI, USA).

Characterization: Growth of bacteria at different SO_2 concentrations, temperatures, and pH levels was initially evaluated using MR medium. Strains were inoculated into 10 mL MR broth and incubated at 25°C until the end of exponential growth (3 days for YH-15, 5 days for YH-24 and YH-37). The cells were then harvested by centrifugation (2 000 x g, 15 min). After washing the cells twice using 5 mL sterile phosphate buffer (pH 7; 0,023 M NaH_2PO_4/0,030 M $Na_2HPO_4 \cdot 7H_2O$), the cells were resuspended in 5 mL sterile MR broth at either pH 3,5 (SO_2 and pH characterizations) or pH 3,7 (temperature characterizations) prior to inoculation. Bacterial growth was monitored by determining turbidity using a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., New York, NY, USA) or viability using the spread plate method. For the SO_2 characterization, growth

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was evaluated after 16 days of incubation and defined as no growth (maximum turbidity achieved ≤20 Klett units), weak growth (21 to 50 Klett units), growth (51 to 99 Klett units) or strong growth (≥100 Klett units).

Sulfur dioxide studies/assay: MR broth was prepared and adjusted to pH 3,5 with 50% H_3PO_4. After filtration through glass prefilters to remove any precipitate, the media were divided into five aliquots. A 10 g/L $K_2S_2O_5$ (Fisher Scientific, Fair Lawn, NJ, USA) solution was prepared and different volumes were added to the media to yield different concentrations of SO_2. The media were sterilized by filtration through 0,45 µm Nalgene disposable filters and aseptically transferred into sterile screw-top bottles. After 24 h, the pH of each aliquot was rechecked. The free and total SO_2 concentrations present in each medium were determined by the aeration-oxidation method as described by Buechenstein & Ough (1978). All media were aseptically dispensed into sterile 15 x 125 mm capped test tubes (6 mL/tube) and inoculated with 0,1 mL resuspended culture for each SO_2 treatment in triplicate. Treatments were incubated at 25°C.

Temperature studies/assay: Growth of *Lactobacillus kunkeei, Oenoccoocccus oeni, and Lactobacillus plantarum* were evaluated at 5°, 15°, 25°, 32° and 37°C in MR broth that was adjusted to pH 3,7 using 50% H_3PO_4. The media were dispensed into 15 x 125 mm test tubes (6 mL/tube) and sterilized by autoclaving. The tubes were inoculated with 0,1 mL resuspended culture in triplicate for each temperature treatment and turbidity was monitored daily for at least 15 days. In a second experiment, *Lactobacillus kunkeei* was grown in 10 mL MR broth and transferred into 100 mL MR for an additional 24 h prior to harvest as previously described. The strain was inoculated into flasks containing 100 mL MR broth (pH 3,7) at an initial population of 5 x 10^7 CFU/mL. The flasks were incubated at 5°, 10°, 15° and 25°C and viability was determined over 10 days.

pH studies/assay: MR broth was prepared and adjusted to pH 2,7 using 50% H_3PO_4. The precipitate that formed at this pH was removed by filtration through glass prefilters (Millipore, Bedford, MA, USA). The filtered media were separated into six aliquots and adjusted to pH 2,7; 2,9; 3,1; 3,3; 3,5 and 3,7 using 5 M NaOH. These media were filter sterilized through 0,45 µm Nalgene membrane filters (Nalgene Co., Rochester, NY, USA) and aseptically transferred into sterile screw-top bottles. After 24 h, 10 mL was aseptically dispensed into sterile 15 x 125 mm capped test tubes (6 mL/tube) and inoculated with 0,1 mL resuspended culture for each pH treatment in triplicate. Treatments were incubated at 25°C.

Fermentation trial: Seven cans of Chardonnay grape juice concentrate (California Concentrate Company Acampo, CA, USA) were reconstituted with approximately 53 L tap water and brought to 21°Brix with equal weights D(+) glucose and D(-) fructose (Sigma, St. Louis, MO, USA). Diammonium phosphate (J.T. Baker, Phillipsburg, NJ, USA) was added to a concentration of 0,35 g/L. The amount of total SO_2 in the juice was reduced to less than 3 mg/L by adding H_2O_2 and confirmed by the aeration-oxidation method (Buechenstein and Ough, 1978). The juice was divided into three lots and these were adjusted to pH 3,81; 3,48; or 3,29 using L(+) tartaric acid (Sigma). The juices were filtered through a Durapore® 0,45 µm filter (Millipore) into sterile 3,8 L amber carboys (2 kg juice/carboy). Powdered cellulose (0,1% w/v Sigmacell® Type 20, Sigma) was added to the carboys to simulate particulate matter normally present in grape juice for fermentation (Huang *et al.*, 1996).

Lyophilized cultures of YH-15 were rehydrated with 10 mL sterile phosphate buffer and inoculated (0,1 mL) into 10mL MR broth. Cultures were incubated at 25°C until the end of exponential growth (3 days), aseptically transferred into 500 mL MR broth and incubated for the same period of time. Cells were harvested by centrifugation (5 000 x g, 25 min) and washed twice with 25 mL sterile rehydration buffer. The cells were then resuspended in sufficient rehydration buffer to achieve an approximate cell population of 10^5 CFU/mL in the juice through inoculation of 10 mL of culture. Juices without bacterial inoculation received 10 mL sterile rehydration buffer.

Two days after bacterial inoculation, yeasts were inoculated into the juice. *Saccharomyces cerevisiae* was rehydrated with prewarmed (40°C) sterile rehydration buffer at a rate of 4,1 g yeast/200 mL buffer and allowed to stand for 20 min. Ten milliliters of this suspension was inoculated into the juice to achieve an initial population of 10^5 CFU/mL. All treatments were performed in triplicate. Juices were fermented at 22°C and sampled using a CO_2 siphon system.

Analytical methods: Viable populations of bacteria and yeast were determined by the spread plate method on either MR agar containing 0,01% cycloheximide (Sigma) or wort agar, respectively. Samples were serially diluted in sterile 0,1% peptone prior to plating and incubated for 3 days at 25°C. The progress of the fermentations was monitored by a gravimetric method utilizing a vacuum oven at 20" Hg (80°C) for 28 h (Ingledew & Kunkee, 1985).

The fermentations were completed when the control wines contained <0,2% reducing sugar as determined by Clinitest® (Edwards, 1990). Chemical analyses of the Chardonnay wines were performed according to the methods of Edwards (1990). Statistical analysis was accomplished by analysis of variance (ANOVA) and Fischer's least significant differences at $p<0,05$ level.

RESULTS AND DISCUSSION

The growth of bacteria in grape juices and wines is influenced by various factors including SO_2, temperature and pH (Bousbouras & Kunkee, 1981; Lafon-Lafourcade *et al.*, 1983; Liu & Gallander, 1983; Wibowo *et al.*, 1985; Davis *et al.*, 1986; 1988; Querol *et al.*, 1990; Britz & Tracey, 1990; Guerzoni *et al.*, 1995; Liu *et al.*, 1995). As strains of *Lactobacillus kunkeei* and *Oenococcus oeni* used in this study were previously shown to retard alcoholic fermentations (Huang *et al.*, 1996), the effects of the above factors on the growth of these strains were evaluated.

Sulfur dioxide has been used as a means to control growth of undesirable bacteria, although these micro-organisms vary in their sensitivities (Beelman *et al.*, 1977; Davis *et al.*, 1988; Britz & Tracey, 1990; Edwards *et al.*, 1991; 1993). Tolerances of strains to SO_2 are illustrated in Table 1 using a synthetic medium adjusted to pH 3,5, a similar pH as some grape juices and wines from Washington State and South Africa (Van Wyk, 1976; Nagel

& Spayd, 1989). Results indicated that *Lactobacillus kunkeei* was most sensitive to SO_2 in that growth was not observed at concentrations of ≥0,1 ml/L molecular SO_2. The strains of *Oenococcus oeni* studied were more tolerant than *Lactobacillus kunkeei* but less tolerant than *Lactobacillus plantarum.*

Given the sensitivity of *Lactobacillus kunkeei* to SO_2, this strain probably will not grow in sulfited wines. Therefore, the use of sulfites remains a viable method to prevent infection of this micro-organism. However, there is a current practice by some winemakers to reduce or eliminate the use of sulfites during winemaking, conditions that are inductive to growth of *Lactobacillus kunkeei.* Thus, other methods of controlling this undesirable bacterium such as use of low temperatures or low pH were investigated.

Lactobacillus kunkeei grew at temperatures between 21° and 32°C (Fig. 1). Some growth was also observed at 15°C or at 37°C while growth was inhibited at 5°C. However, turbidity does not give an indication whether growth was retarded or the organism died. A decrease in viability at 5° or 10°C could potentially be used by winemakers to decrease the viability of *Lactobacillus kunkeei* prior to yeast inoculation and fermentation. Thus, the viability of *Lactobacillus kunkeei* incubated at various temperatures was also determined (Fig. 2). As suggested by Fig. 2, the viability of *Lactobacillus kunkeei* remained at 10^7 CFU/mL for a number of days and did not decrease. In fact, the micro-organism eventually achieved populations approaching 10^9 CFU/mL at 15° and 25°C. These data indicate that it is unlikely that reduction in must temperature prior to fermentation would result in death of *Lactobacillus kunkeei* to such a degree as to reduce the risk of slow/sluggish fermentations. The other strain of *Lactobacillus* studied, *Lactobacillus plantarum*, grew well at 15° and 37°C unlike *Lactobacillus kunkeei* (Fig. 1) *Oenococcus oeni* YH-37 grew well between 21° to 32°C, higher optimal ranges than the previously reported 20° to 25°C for other strains of *Oenococcus oeni* (Garvie, 1984).

TABLE 1
Growth of *Lactobacillus kunkeei*, *Oenococcus oeni* and *Lactobacillus plantarum* in MR broth (pH 3,5) with different amounts of sulfur dioxide.

Total SO_2 (mg/L)	Molecular SO_2(mg/L)	Microorganism			
		Lb. kunkeei (ATCC 700308)	*O. oeni* (YH-24)	*O. oeni* (YH-37)	*Lb. plantarum* (WS-23)
0	0	++	++	++	++
13	0	+	++	++	++
30	0,096	–	++	++	++
49	0,26	–	–	–	++
66	0,42	–	–	–	±
88	0,56	–	–	–	–

No growth = (–); Weak growth = (±); Growth = (+); Strong growth = (++)

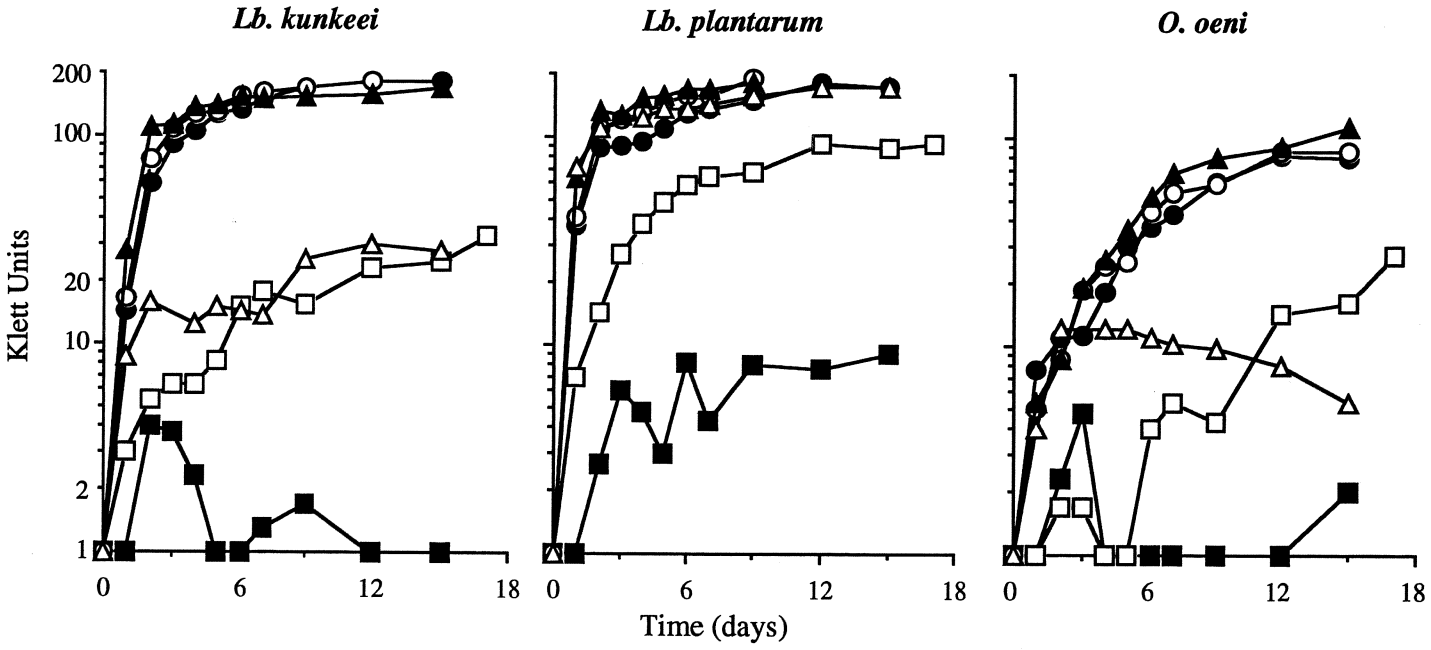

FIGURE 1
Growth of *Lb. kunkeei, Lb. plantarum*, and *O. oeni* (YH-37) in MR broth at 5° (■), 15°(□), 21°(●), 25°(○), 32° (▲) or 37°(△)

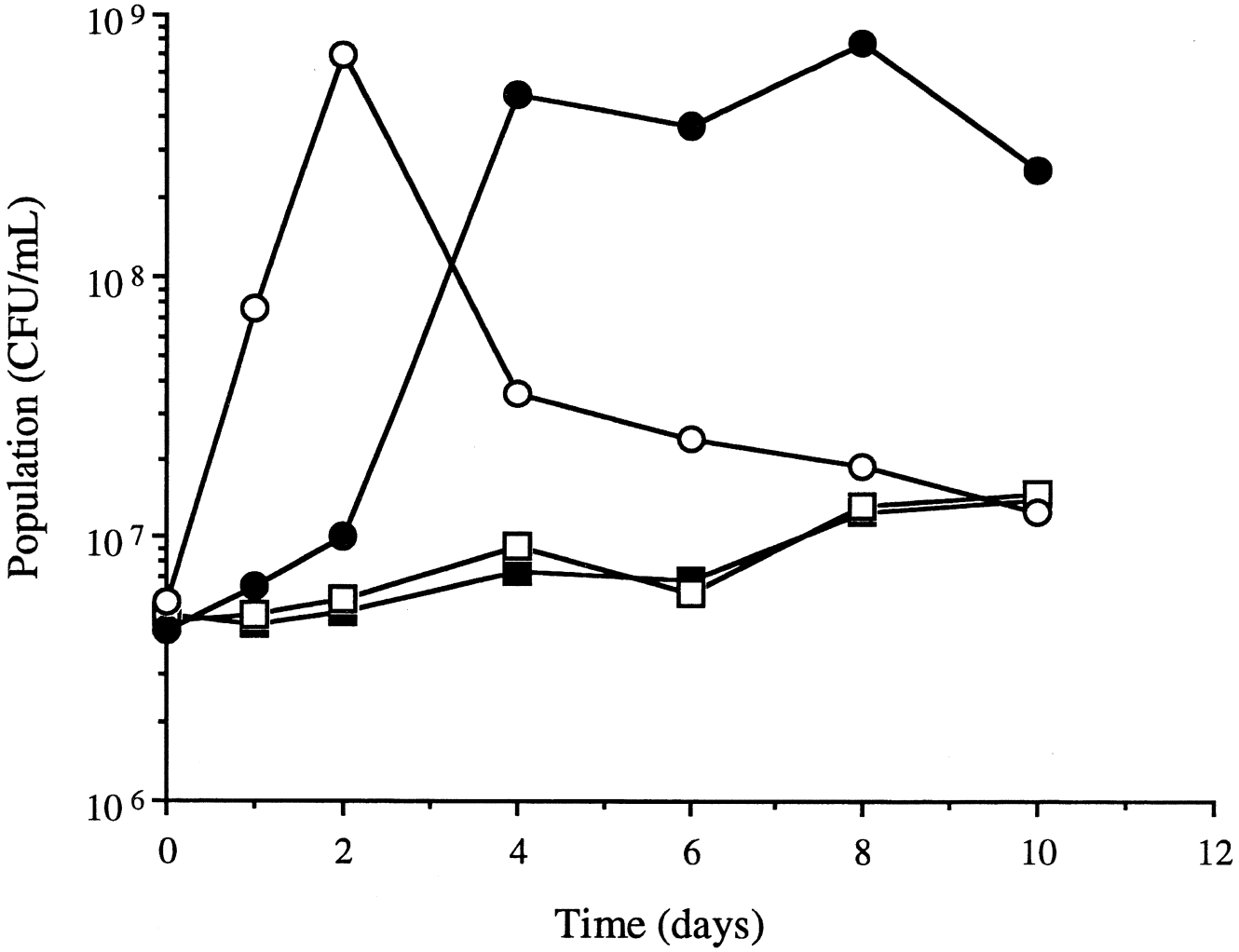

FIGURE 2
Viability of *Lb. kunkeei* grown in MR broth incubated at 5°(■), 10°(□), 15°(●) or 25°(○).

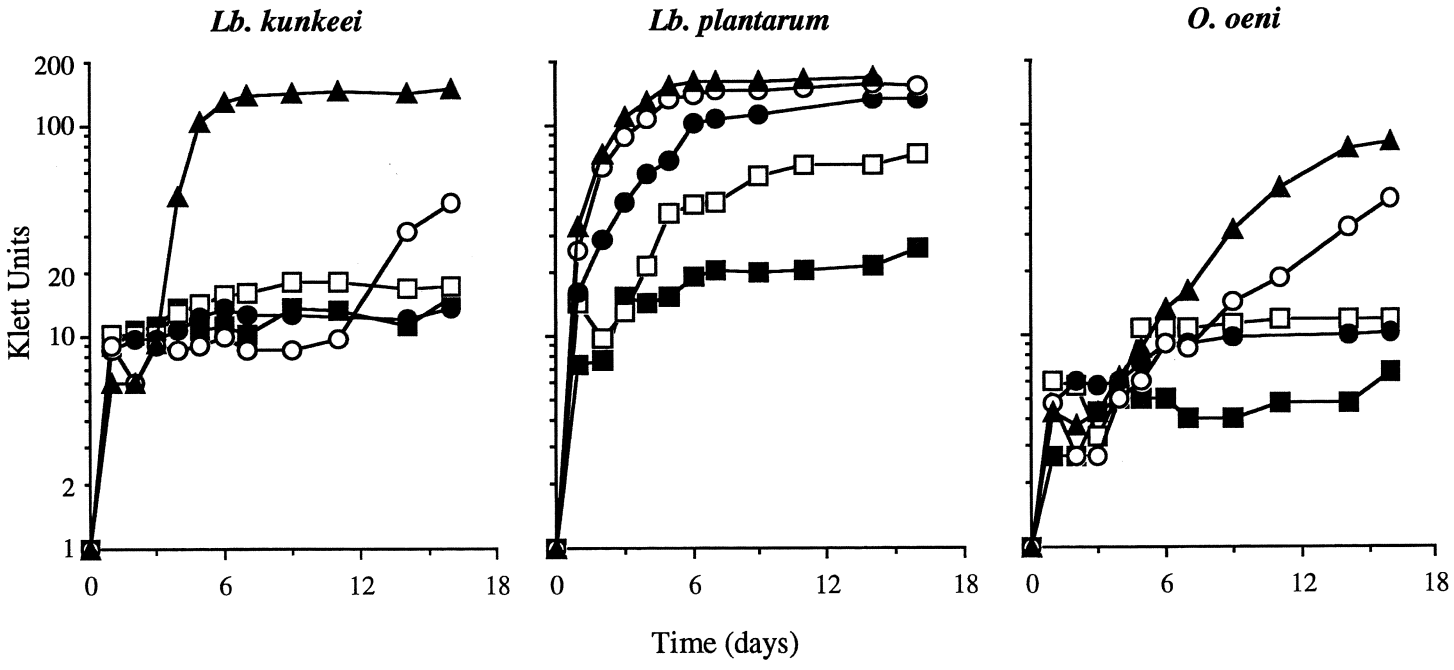

FIGURE 3
Growth of *Lb. kunkeei, Lb. plantarum*, and *O. oeni* (YH-37) in MR broth at pH 2,9 (■), 3,1 (□), 3,3 (●), 3,5 (○) or 3,7 (▲).

In addition to low temperatures, another possible strategy available to winemakers would be alteration of must pH prior to fermentation. Several studies have concluded that wines below pH 3,5 do not support growth of lactobacilli (Wibowo *et al.*, 1985; Davis *et al.*, 1986; Liu *et al.*, 1995), although some strains are capable of growing at lower pH levels (Dakin & Radwell, 1971; Davis *et al.*, 1988; Edwards *et al.*, 1993). In agreement, *Lactobacillus kunkeei* did not tolerate low pH as *Lactobacillus plantarum* (Fig. 3). *Lactobacillus kunkeei* grew at pH ≥3,5, but was inhibited in media below this pH, while *Lactobacillus plantarum* exhibited growth at all pH levels studied including pH 2,9, in agreement to the initial characterization of this strain (Edwards *et al.*, 1993). Similar to *Lactobacillus kunkeei*, *Oenococcus oeni* grew at pH 3,5 or above but not at 3,3 or below. The optimum growth range for *Oenococcus oeni* has been reported to be between 4,3 and 4,8 (Garvie, 1967), although some strains can grow under low pH conditions (Beelman *et al.*, 1977; Izuagbe *et al.*, 1985; Davis *et al.*, 1986; 1988).

As Fig. 3 indicates that *Lactobacillus kunkeei* may not grow well at pH ≤3,3, it was hypothesized that this bacterium would be inhibited in musts adjusted to low pH. To test this hypothesis, Chardonnay grape juices were adjusted to pH 3,3; 3,5 and 3,8; respectively, and inoculated with *Lactobacillus kunkeei* and *Saccharomyces cerevisiae*. In order to prevent yeast dominance of the fermentations which can occur in inoculated musts (Fornachon, 1968; Beelman *et al.*, 1982; Fleet *et al.*, 1984; King & Beelman, 1986), and to provide optimal bacterial growth conditions, the yeasts were inoculated two days after bacteria inoculation.

Musts inoculated with yeasts alone achieved populations in excess of 10^7 CFU/mL irrespective of initial must pH (Fig. 4). These fermentations fermented to dryness and contained less than 0,2% residual sugar (data not shown). In contrast to the growth of *Lactobacillus kunkeei* in broth (Fig. 3), the strain grew better in grape juice and achieved populations of 10^9 CFU/mL in the medium pH (3,5) and high pH (3,8) juices by day two (Fig. 5). These peak populations were similar to those of *Lactobacillus kunkeei* observed by Huang *et al.*, (1996). In addition, *Lactobacillus kunkeei* grew in the low pH juice, achieving a population of nearly 10^8 CFU/mL on the second day. By day 8, the viability of *Lactobacillus kunkeei* had decreased to <300 CFU/mL in the low and medium juices and by day 12 in the high pH juices. All fermentations inoculated with yeast only proceeded to dryness while fermentations inoculated with *Lactobacillus kunkeei* did not (Fig. 6). As the pH of the juices was increased, *Lactobacillus kunkeei* exhibited more sustained growth and this growth resulted in slower fermentations (Fig. 6).

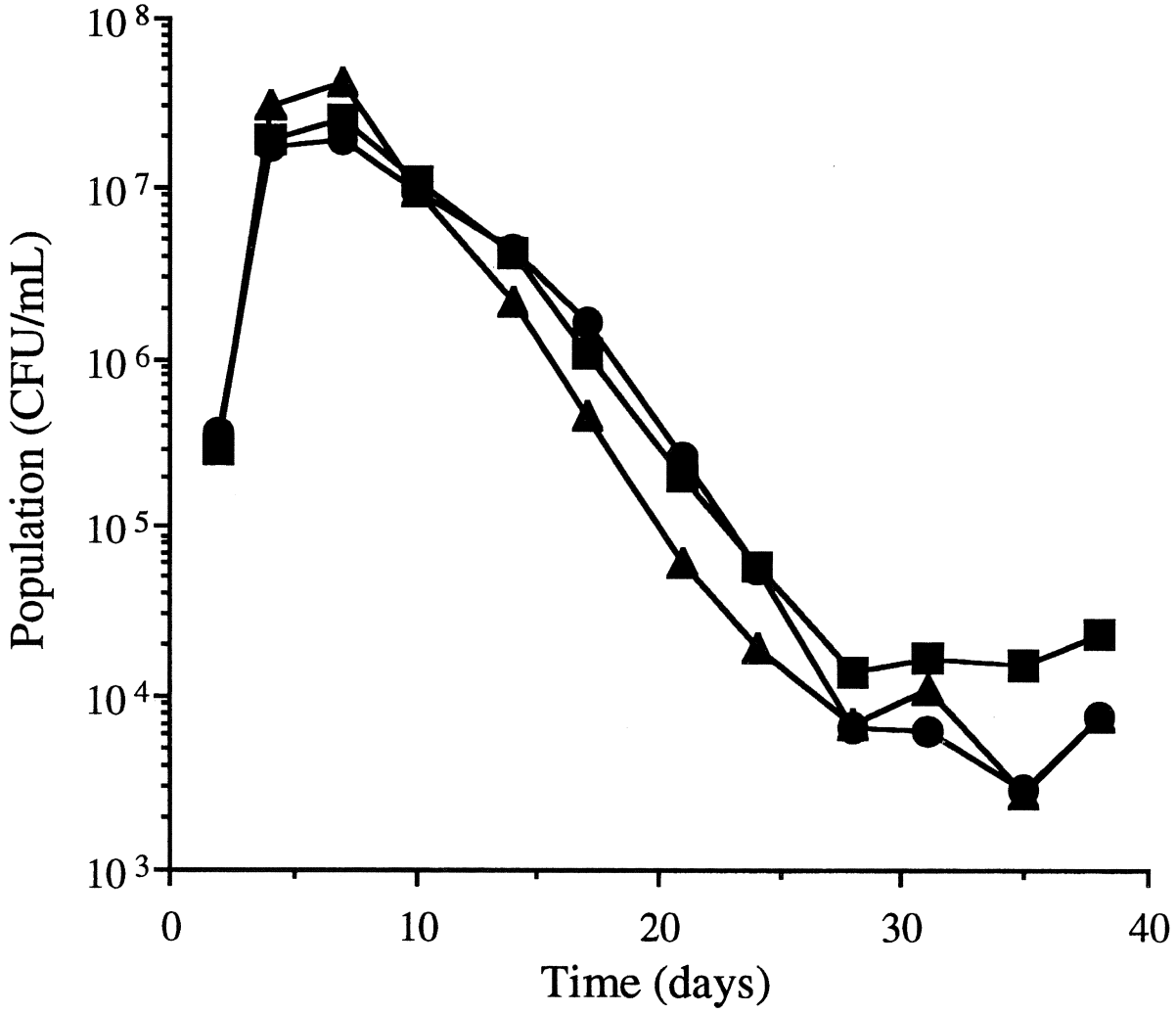

FIGURE 4
Growth of *S. cerevisiae* in Chardonnay musts initially adjusted to pH 3,3 (■), 3,5 (●) or 3,8 (▲).

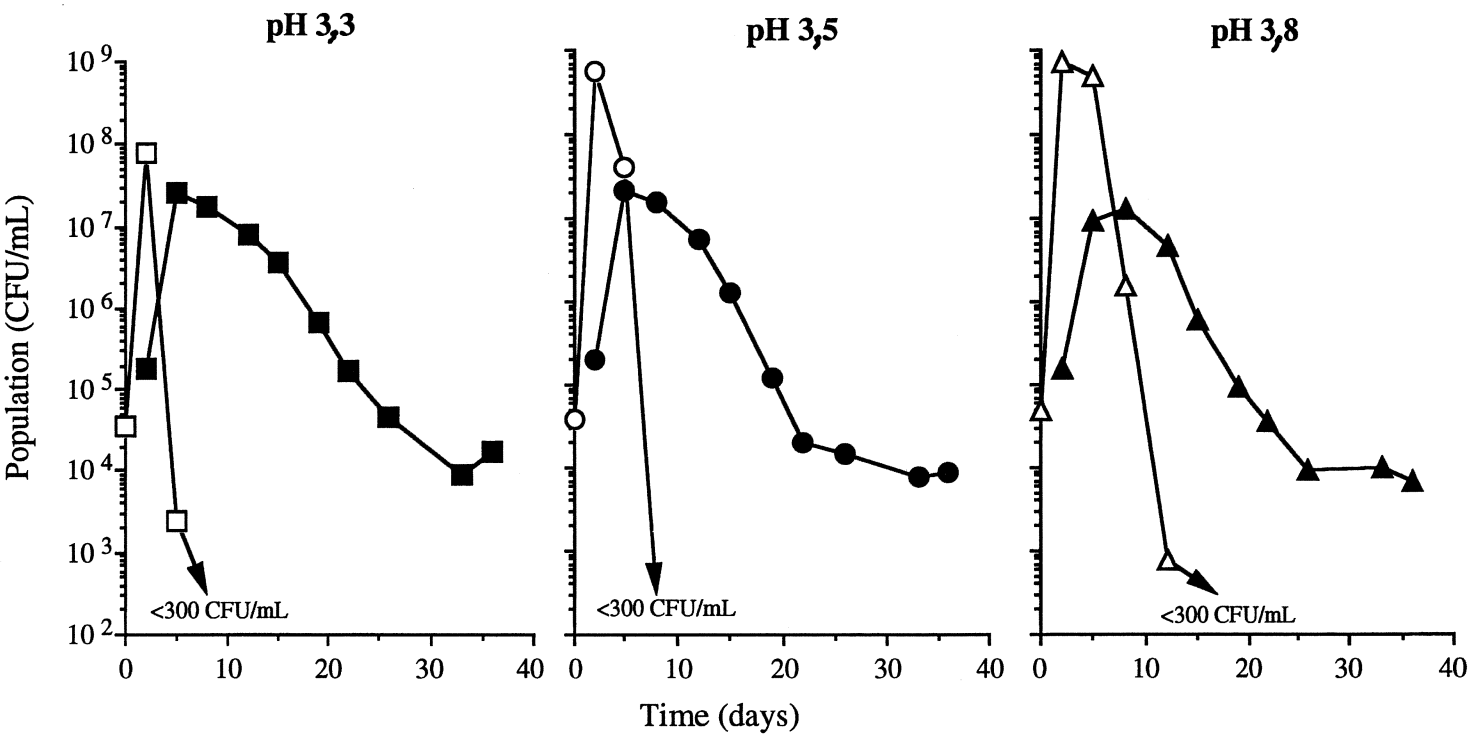

FIGURE 5
Viability of *S. cerevisiae* (■,●,▲) and *Lb. kunkeei* (□,○,△) during fermentation of Chardonnay musts initially adjusted to pH 3,3; 3,5 or 3,8.

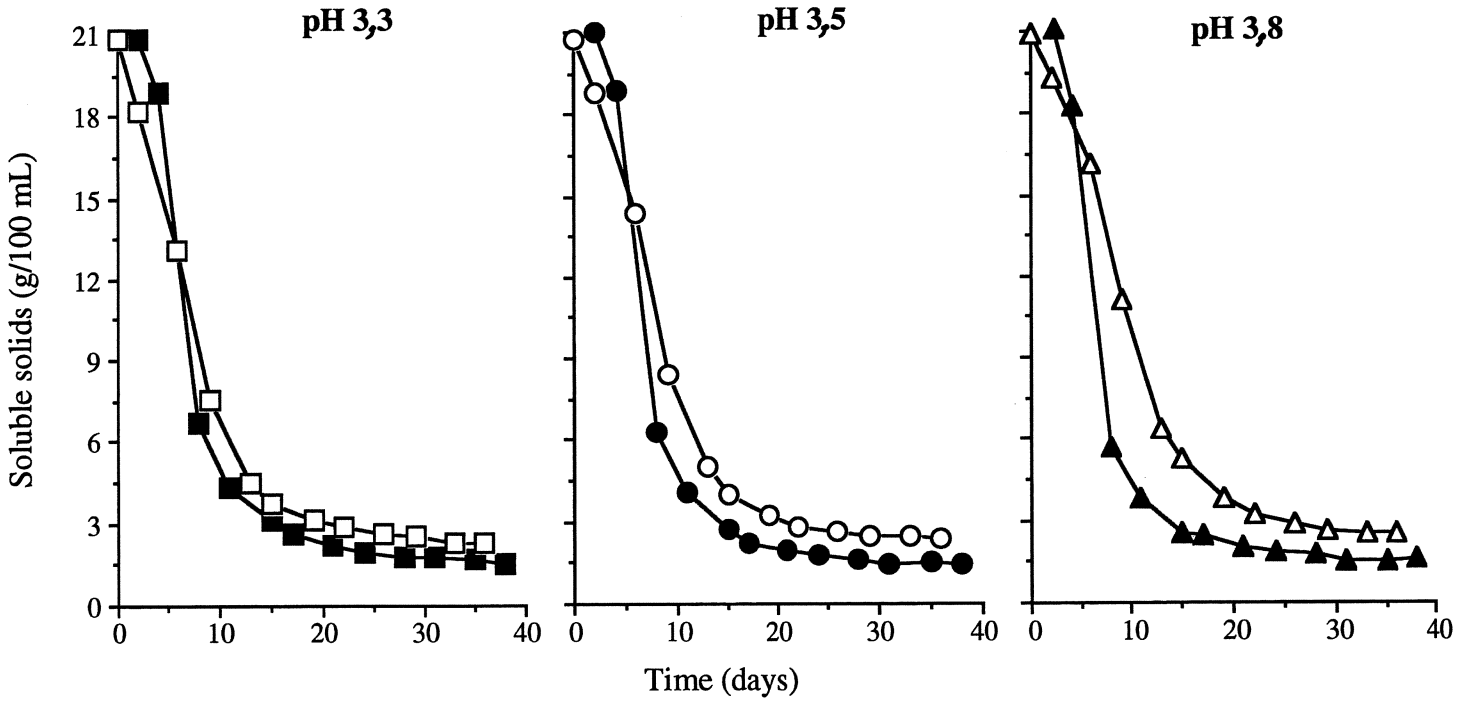

FIGURE 6
Decline of soluble solids during fermentation of Chardonnay musts initially adjusted to pH 3,3; 3,5 or 3,8 inoculated with *S. cerevisiae* alone (■,●,▲) or *S. cerevisiae* and *Lb. kunkeei* (□,○,△).

TABLE 2
Chemical analyses of Chardonnay wines inoculated with *S. cerevisiae* alone or with *S. cerevisiae* and *Lactobacillus kunkeei**.

	pH 3,3 Must		pH 3,5 Must		pH 3,8 Must	
	S. cerevisiae alone	*S. cerevisiae* & *Lb. kunkeei*	*S. cerevisiae* alone	*S. cerevisiae* & *Lb. kunkeei*	*S. cerevisiae* alone	*S. cerevisiae* & *Lb. kunkeei*
pH	3,13e	3,11f	3,26b	3,16d	3,41a	3,24c
Titratable acidity (g/100mL)	0,53d	0,64c	0,49e	0,69b	0,42f	0,73a
Volatile acidity (g/100 mL)	0,052d	0,10c	0,046d	0,15b	0,047d	0,22a

* Means within a row with different superscripts are significantly different at p<0,05.

The titratable acidity, pH, and volatile acidity of each of the Chardonnay wines were determined after the fermentations in the control wines were complete (Table 2). The wines inoculated with *Lactobacillus kunkeei* and *Saccharomyces cerevisiae* had significantly higher titratable acidities and lower pH values than the wines inoculated with *S. cerevisiae* alone. This was probably due to production of lactic and acetic acids by *Lactobacillus kunkeei*, a heterofermentative bacterium (Edwards *et al.*, 1998a).

The volatile acidity concentrations of the control wines at the low, medium, and high pH levels were not significantly different. These volatile acidity concentrations are within the range commonly found in wines (Lafon-Lafourcade, 1983). However, each of the wines inoculated with *Lactobacillus kunkeei* had significantly higher volatile acidity concentrations than the control wines. In fact, the volatile acidity concentrations increased with an increase in the pH of the must (Table 2). For instance, the low pH must contained 0,10 g acetic acid/100 mL while the high pH must contained twice the amount, an observation which was probably a result of extended viability of *Lactobacillus kunkeei* in musts of higher pH (Fig. 5).

CONCLUSION

Although lowering the pH of a grape must prior to fermentation may slow the growth of spoilage bacteria like *Lactobacillus kunkeei*, it is apparent that the use of SO_2 remains an effective means to control the growth of these undesirable micro-organisms.

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