Controlling Wine Malolactic Fermentation with Nisin and Nisin-Resistant Strains of *Leuconostoc oenos*

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The polypeptide nisin (100 U/ml) prevented malolactic fermentation in wines by indigenous or intentionally added lactic acid bacteria. Nisin (100 U/ml)-resistant mutants of *Leuconostoc oenos* were obtained and used with nisin in wine to carry out a pure-culture malolactic fermentation in the presence or absence of other lactic acid bacteria. Nisin degradation by mutants was not observed, and residual nisin was detectable in wines 4 months after it was added. Results indicated that nisin or nisin with resistant bacterial starter cultures can be used to control malolactic fermentation in wines.

Malolactic fermentation (MLF) by lactic acid bacteria (LAB) can play an important role (6) in governing the acidity and sensory characteristics of certain wines. Deacidification is achieved by the conversion of dicarboxylic malic acid to monocarboxylic lactic acid. In practice, the pH of wines undergoing MLF will rise 0.1 to 0.3 pH unit. The MLF may be advantageous for some wines but detrimental for others, depending on the style and composition of wine. Often, the MLF is difficult to manage when it is most needed, such as in wines of low pH, and most difficult to prevent when unwanted, such as in wines of high pH. The MLF can be promoted by inoculation of wine with selected strains of LAB (primarily *Leuconostoc oenos*), which can reduce inconsistencies associated with fermentations conducted by naturally occurring LAB. Growth of indigenous LAB can lead to spoilage such as ropiness (mucilaginous material) and the formation of excess acetic acid. Suppression of the MLF and the natural LAB can be achieved in some wines by maintaining a low pH (<3.2), a high alcohol content (>14%), and a high sulfur dioxide level (>50 mg/ml). However, these conditions are neither desirable or feasible for the majority of wines.

Recent studies (11, 12) have shown that wine LAB can be inhibited by nisin in both laboratory media and wine musts. The same studies also indicated that nisin did not affect the sensory characteristics of wine. Similar findings have also been found with nisin in beer (8-10), in which spoilage LAB were inhibited without affecting flavor. Nisin is a bactericidal polypeptide produced by *Lactococcus lactis* subsp. *lactis* (2), which is active against gram-positive bacteria and is used in the food industry to control the growth of *Clostridium botulinum*.

In this study we have investigated the use of nisin to specifically prevent or promote the MLF (i) by using nisin to inhibit naturally occurring LAB capable of MLF and (ii) by developing and using nisin-resistant mutants of *L. oenos* to conduct MLF in wines containing levels of nisin that are inhibitory to naturally occurring LAB.

L. oenos Erla and Ey2d, originally isolated from Oregon wines (3), and *Pediococcus pentosaceus* FBB-61-2 were from the authors' collection. Strains of *Pediococcus damnosus* were isolated from Oregon wines described as spoiled. A

commercial yeast preparation (Prise de Mousse, Lalvin EC-1118; Lallemend Inc., Montreal, Canada) was used as the inoculum for experimental wine.

Wine musts from the 1988 harvest (Pinot noir: 21.7° brix, 10.1 g/liter titratable acidity, pH 3.23; Chardonnay: 22.3° brix, 10.4 g/liter titratable acidity, pH 3.11) were produced from grapes obtained from the Oregon State University vineyard. SO₂ (25 ppm) was added to musts at the time of crush. After the primary yeast fermentation (10 days at 18°C), the new wines (Pinot noir, 11.8% alcohol by volume; Chardonnay, 12.5% alcohol by volume) were racked and frozen in air-tight vessels. In some experiments, wine was sterilized by filtration through membrane filters (0.22-μm pore size).

LAB were cultured and enumerated with a medium (herein called wine LAB [WLAB] medium) containing 27.5 g of MRS medium (Difco, Detroit, Mich.) per liter, 5 g of fructose per liter, 1 g of L-malic acid per liter, and 10% (vol/vol) unfiltered V-8 juice (Campbell Soup Co.). The medium pH (without adjustment) was 5.3 after sterilization (121°C, 15 min). Filter-sterilized cyclohexamide (50 μg/ml) was added to sterile WLAB to suppress yeast when present. Cultures were incubated at 30°C (except as noted) under a partial carbon dioxide atmosphere (Gas-Pak; BBL). In wine with both *L. oenos* and *P. damnosus* added, differential enumeration was by counting large colonies (>2 mm in diameter) as *P. damnosus* and small colonies (<1 mm in diameter) as *L. oenos* on WLAB agar spread plates incubated under carbon dioxide at 30°C for 96 h. Species differentiation was confirmed by microscopic observation. LAB cultures were added to wines at an inoculum level of 10⁶ to 10⁷ CFU/ml. Nisin (100 U/ml)-resistant mutants of *L. oenos* were obtained by stepwise exposure to increasing concentrations of nisin in 5- to 100-U/ml steps with WLAB medium. The initial minimum inhibitory concentration of nisin in WLAB for the parental strains was less than 1 U/ml. One mutant, designated *L. oenos* Ey2d-NR1, was used in the experiments with wine.

Nisin, obtained from Aplin and Barret Ltd. (Trowbridge, United Kingdom), contained 37 × 10⁶ international units/g (lot code 201H). Nisin activity was determined by a bioassay in which *P. pentosaceus* FBB-61-2 served as the sensitive indicator strain. Bioassay plates were prepared by adding a 0.1% inoculum of a log-phase culture of the indicator to 35 ml of tempered WLAB agar, which was then poured into

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TABLE 1. Inhibitory effect of nisin (100 U/ml) on the growth of *L. oenos* and malolactic fermentation in nonsterile new Pinot noir wine

Treatment of wine ^a	CFU of LAB per ml (mg of malic acid remaining per ml) on the following day after treatment:		
	0	28	56
<i>L. oenos</i> Erla	1.1×10^6 (3.7)	2.5×10^4 (2.5)	6.4×10^6 (<0.1)
<i>L. oenos</i> Erla, nisin	1.1×10^6 (3.7)	<10 (4.1)	<10 (3.6)
<i>L. oenos</i> Ey2d	1.1×10^6 (3.7)	3.4×10^4 (3.2)	1.0×10^7 (<0.1)
<i>L. oenos</i> Ey2d, nisin	1.1×10^6 (3.7)	<10 (3.9)	<10 (3.4)
Nisin	<10 (3.7)	<10 (4.0)	<10 (3.8)
No addition ^b	<10 (3.7)	<10 (ND ^c)	<10 (3.8)

^a The initial inoculum was approximately 1.1×10^6 CFU of *L. oenos* Erla or Ey2d per ml.^b LAB were detected ($>10^5$ CFU/ml) after 5 months.^c ND, Not determined.

petri plates. Wells (6.5 mm in diameter) were made within the agar with a sterile brass cork borer. Standard nisin solutions and nisin-containing wine samples were added to the wells in volumes up to 100 μ l.

Nisinase activity was determined as described by Collins-Thompson et al. (1); residual nisin levels were measured in cell-free supernatants from nisin-resistant and nisin-sensitive isolates of *L. oenos*. Controls consisted of uninoculated medium with and without nisin.

Wines were analyzed for degree brix (soluble solids by refractometer, grams of sucrose per 100 g), titratable acidity (grams of tartaric acid per 100 ml), and alcohol (ebuillimeter, volumes percent). Malic acid was assayed enzymatically as described by McCloskey (7) and is expressed as milligrams per milliliter. All expressed concentrations are means of duplicate determinations.

Nisin was able to prevent the growth of either intentionally added LAB that were capable of MLF or the naturally occurring LAB (Table 1). In the absence of nisin, both strains of added *L. oenos* were able to grow after an initial decline (at 28 days) to numbers greater than the initial populations. A decline in the inoculum population of *L. oenos* is typically observed (13) and is believed to be the result of acclimation to the wine environment. After 56 days, essentially all malic acid was degraded in treatments with added *L. oenos* but was intact in the same treatments with added nisin. In addition, at 56 days neither viable LAB or malic acid degradation was evident in noninoculated wines with or without nisin. The growth of the natural LAB in wine is often variable and is dependent on initial microbial populations, acid and alcohol concentrations, and availability of nutrients. In our study we did observe growth of the natural LAB at about 5 months (data not shown) in the absence of nisin but none when nisin was present. Our data are in agreement with those of Radler (12), who found that 100 U of nisin per ml of grape must was effective in suppressing the growth of intentionally added *L. oenos*.

In filter-sterilized wine, intentionally added spoilage LAB (*P. damnosus*) were able to partially degrade malic acid in the wine but were inhibited when nisin was present (Table 2, treatments 2 and 3). In wines with added nisin-resistant *L. oenos* Ey2d-NR1 and parent *L. oenos* (Table 2, treatments 8 and 10), both strains were able to degrade greater than 90% of the malic acid within 3 months. In the presence of nisin the *L. oenos* Ey2d-NR1 was able to grow and use greater than 95% of malic acid (treatment 9), whereas the sensitive parent was inhibited (treatment 11) and malic acid remained intact. When *L. oenos* Ey2d-NR1 was added with *P. damnosus* to wine containing nisin (treatment 5), only *L. oenos* was able to grow and degrade malic acid. This observation

demonstrated that nisin and nisin-resistant strains of desirable MLF bacteria can be used to promote a pure-culture MLF in the presence of other LAB, a situation representative of a commercial winery environment.

Several nisin-resistant mutants of *L. oenos* were tested for the ability to inactivate nisin. Nisin, a polypeptide, is subject to inactivation by certain proteolytic enzymes (4, 5). In this study, significant differences in nisin activity levels were not observed when nisin was added to WLAB supernatants in which *L. oenos*-resistant mutants or *L. oenos*-sensitive parent strains were grown. Nor were any significant differences observed in residual nisin levels in wines inoculated with nisin-resistant *L. oenos* and nisin-sensitive parent strains. This is an important observation, in that it would be counterproductive to have nisin degraded when nisin-resistant mutants are used to promote a pure-culture MLF.

A dramatic decrease in nisin activity in Pinot noir (red wine) was observed over a 4-month storage period (Fig. 1), but little, if any, decrease was observed in Chardonnay (white wine). The presence or absence of *L. oenos* cultures added to the initially sterile wines did not appear to have an effect on residual nisin activity. It is conceivable that nisin may be interacting with phenolic compounds such as anthocyanins that are present in red wines but absent in white wines.

The data presented indicate several potential applications

TABLE 2. Inhibitory effect of nisin on *P. damnosus*, nisin-resistant *L. oenos* Ey2d-NR1, and nisin-sensitive *L. oenos* Ey2d and MLF^a

Treatment	Nisin added	Bacteria (CFU/ml)		Malic acid (mg/ml)
		<i>P. damnosus</i>	<i>L. oenos</i>	
1. None	No	<10	<10	3.5
2. <i>P. damnosus</i>	No	1.6×10^4	<10	1.7
3. <i>P. damnosus</i>	Yes	<10	<10	3.7
4. <i>P. damnosus</i> , <i>L. oenos</i> NR1	No	9.0×10^5	5.2×10^7	<0.1
5. <i>P. damnosus</i> , <i>L. oenos</i> NR1	Yes	<10	9.3×10^7	<0.1
6. <i>P. damnosus</i> , <i>L. oenos</i>	No	1.3×10^6	8.4×10^7	<0.1
7. <i>P. damnosus</i> , <i>L. oenos</i>	Yes	<10	<10	3.2
8. <i>L. oenos</i> Ey2d-NR1	No	<10	1.4×10^7	<0.1
9. <i>L. oenos</i> Ey2d-NR1	Yes	<10	6.8×10^6	0.2
10. <i>L. oenos</i> Ey2d	No	<10	7.8×10^7	0.3
11. <i>L. oenos</i> Ey2d	Yes	<10	<10	3.5

^a Nisin (100 U/ml) was added to sterile new Pinot noir wine, which was held at 18°C for 3 months.

FIG. 1. Residual nisin activity (initial nisin concentration, 100 U/ml) in Pinot noir (A) and Chardonnay (B) after 4 months of storage. Treatments: 1, control (nisin added to wine just before assay); 2 and 3, *L. oenos* Ey2d cells added; 4 and 5, *L. oenos* Er1a cells added; 6 and 7, no cells added.

of nisin in commercial winemaking. If desired, MLF may be prevented by inclusion of nisin or may be promoted by its addition and deliberate inoculation of nisin-resistant mutants of *L. oenos*. Nisin may also serve to reduce the amount of sulfur dioxide that is used in winemaking to control bacterial spoilage.

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