BACTERIA IMPORTANT DURING WINEMAKING

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Numerous bacterial species are present during the vinification process. The extent to which these species grow determines the types and concentrations of many substances that contribute to the aroma and flavor of a wine. Growth of bacterial species such as *Acetobacter*/*Gluconobacter, Lactobacillus*, and *Pediococcus* may cause spoilage of a wine through the production of

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off-flavors and aromas. Certain bacterial strains may also produce substances in wine such as biogenic amines and ethyl carbamate precursors that are of public health concern. Conversely, growth of *Oenococcus oeni* may be desirable because this bacterium conducts malolactic fermentation (an enzymatic conversion of malic acid to lactic acid that decreases wine acidity) and contributes desirable flavors and aromas to a wine. Additionally, many interactions occur between different bacterial species and with the wine yeast *Saccharomyces.* These interactions may be beneficial or detrimental to wine quality depending on the species involved. This chapter discusses current information regarding the bacteria important during the winemaking process including the impact these microorganisms have on wine quality, public health concerns, and the interactions that occur between these microorganisms and *Saccharomyces cerevisiae.*

I. INTRODUCTION

The fermentation of grape juice into wine represents a complex biochemical process involving many microorganisms. Because of this diversity, a complicated microbial ecology evolves that greatly affects overall wine quality. The microorganisms present in grape musts before fermentation have varying growth requirements and tolerances to inhibitory compounds and, thus, dominate at different times during the course of natural fermentation (Amerine *et al.*, 1980; Lonvaud-Funel, 1999). Microbial diversity also results in complex ecological interactions with both beneficial and antagonistic relationships being exhibited. For example, *Saccharomyces* has been reported to inhibit and stimulate growth of certain lactic acid bacteria (LAB) (Beelman *et al.*, 1982; Cannon and Pilone, 1993; Guilloux-Benatier *et al.*, 1985; King and Beelman, 1986). Conversely, LAB have been reported to inhibit *Saccharomyces* (Boulton *et al.*, 1996; Edwards *et al.*, 1999). As such, the interactions between microorganisms in wine are important because they may influence the success or failure of the alcoholic or malolactic fermentation, which in turn will influence the final quality of a wine.

In many countries, alcoholic fermentation is induced by inoculation with a yeast starter culture of *Saccharomyces* selected for its desirable winemaking qualities (Kunkee, 1984; Kunkee and Bisson, 1993; Rainieri and Pretorius, 2000; Reed and Chen, 1978; Reed and Nagodawithana, 1988). Starter cultures of *S. cerevisiae* strains are generally used because of to their increased ethanol and sulfur dioxide resistance and production of desirable aromas and flavors (Boulton *et al.*, 1996; Ebeler, 2001; Nykänen, 1986; Reed and Chen, 1978; Reed and Nagodawithana, 1988).

Besides *Saccharomyces*, different bacterial species increase or decrease wine quality, depending on the microorganisms involved. Because grape juice and wines are harsh environments for microbial growth due to low pH, minimal levels of oxygen, the presence of ethanol, and high osmotic pressure, only a few bacterial species are able to grow. As examples, LAB belonging to the genera *Lactobacillus, Oenococcus*, and *Pediococcus* are commonly found in wine because of tolerance to these factors (Amerine *et al*., 1980; Dicks *et al*., 1995; Fleet, 2003; Henick-Kling, 1993; Lonvaud-Funel, 1999). Other bacteria such as *Acetobacter* and *Gluconobacter* are present in grape musts, with the former being a concern in wines (Drysdale and Fleet, 1988; Du Toit and Lambrechts, 2002; Joyeux *et al*., 1984a).

Many LAB found in wines can improve wine quality by metabolizing malic acid to lactic acid in a process called malolactic fermentation (MLF). This fermentation is an enzyme-mediated decarboxylation of the dicarboxylic acid, L (−) malic acid, to the monocarboxylic L (+) lactic acid (Amerine *et al*., 1980; Kunkee, 1967; Lonvaud-Funel, 1999). MLF decreases wine acidity and is particularly important in wines produced from grapes grown in cool climates, which often have high acidity (Beelman and Gallander, 1979; Kunkee, 1967, 1974).

However, some LAB can be associated with spoilage problems including stuck alcoholic fermentations (Edwards *et al*., 1999; Huang *et al*., 1996), production of off-flavors or off-odors (Costello and Henschke, 2002; Drysdale and Fleet, 1989a; Sponholz, 1993), excessive volatile acidity (VA) (Drysdale and Fleet, 1989a; Huang *et al*., 1996), synthesis of polysaccharides responsible for ropiness (Manca de Nadra and Strasser de Saad, 1995), or other defects.

This chapter summarizes current information regarding the bacteria important during the winemaking process including *Acetobacter*/*Gluconobacter, Lactobacillus, Oenococcus*, and *Pediococcus*, as well as the impact these microorganisms have on wine quality and public health concerns. Furthermore, the interactions that occur between these microorganisms and *S. cerevisiae* and their influences on wine quality are discussed.

II. *ACETOBACTER* AND *GLUCONOBACTER*

A brief overview of the importance of the acetic acid bacteria (AAB) *Acetobacter* and *Gluconobacter* in winemaking is given here, but readers are directed to a comprehensive review by Du Toit and Pretorius (2002) for a more detailed discussion.

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

AAB are Gram negative, aerobic, catalase-positive rods that belong to the family Acetobacteraceae, which is divided into the genera *Acetobacter, Acidomonas, Gluconobacter*, and *Gluconacetobacter* (Holt *et al*., 1994; Ruiz *et al*., 2000). *Acetobacter* and *Gluconobacter* have been isolated from flowers, fruits, wine, beer, and brewers yeast and are the primary microorganism involved in vinegar production (De Lay *et al*., 1984; Drysdale and Fleet, 1988; Holt *et al*., 1994). *Acetobacter* can also be found in garden soil and canal water (De Lay *et al*., 1984).

Gluconobacter oxydans, Acetobacter aceti, Acetobacter pasteurianus, Gluconacetobacter liquefaciens, and *Gluconacetobacter hansenii* are associated with grapes and wine (Drysdale and Fleet, 1988; Du Toit and Lambrechts, 2002; Joyeux *et al*., 1984a). *Gluconobacter* species are commonly isolated from grapes and musts but disappear as alcoholic fermentation begins, perhaps because of low ethanol tolerance or a lack of oxygen (Joyeux *et al*., 1984a). *Acetobacter* are more ethanol tolerant than *Gluconobacter* and so may survive through alcoholic fermentation (Drysdale and Fleet, 1984, 1988; Joyeux *et al*., 1984a). Commonly, *A. aceti* and *A. pasteurianus* are isolated from grapes and musts and may reach populations of 10^6 cells/g on damaged grapes (Drysdale and Fleet, 1984, 1989a; Joyeux *et al*., 1984b). Yeast present on damaged grapes can metabolize grape sugars, producing ethanol, which is oxidized to acetic acid by these bacteria (Joyeux *et al*., 1984b). Acetic acid concentrations as high as 3.9 g/L may be found in juices made from infected grapes (Drysdale and Fleet, 1989a).

Because of their aerobic nature, AAB are normally inhibited in the anaerobic environment associated with alcoholic fermentation (Joyeux *et al*., 1984b). However, *A. pasteurianus* and *A. aceti* are frequently isolated from wines stored in barrels or other vessels in the winery under semi-aerobic conditions (Joyeux *et al*., 1984a). The survival of AAB in wines may be due to the exposure of wine to air during pumping and transfer operations. For instance, Drysdale and Fleet (1989b) reported AAB populations of up to 10^8 cfu/ml in wine exposed to air during pumping-over operations. Besides the presence of oxygen, the growth of AAB is greatly influenced by the pH of the must. In a study by Du Toit and Lambrechts (2002), AAB populations decreased from 10^5 cfu/ml to 10^2 cfu/ml in musts with a pH less than 3.5, whereas higher cell viability was found in musts with a pH of 3.7.

AAB produce acetic acid through the oxidation of ethanol by two membrane-bound enzymes: an alcohol dehydrogenase and an aldehyde dehydrogenase (Saeki *et al*., 1997). The alcohol dehydrogenase oxidizes ethanol to

acetaldehyde, which is further oxidized to acetic acid by the aldehyde dehydrogenase (Saeki *et al*., 1997). Some strains of AAB can produce more than 50 g/L from ethanol, making them important in the production of vinegar (Lu *et al*., 1999).

During growth in wine, AAB use glucose as a carbon source, although it is a better carbon source for *Gluconobacter* than *Acetobacter* because not all strains of *Acetobacter* can use glucose effectively (De Lay *et al*., 1984). *Acetobacter* species metabolize sugars via the hexose-monophosphate pathway, as well as the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways, producing acetate and lactate as byproducts. These compounds can be further oxidized by some strains to CO_2 and water via the tricarboxylic acid (TCA) pathway. Unlike *Acetobacter, Gluconobacter* species use only the pentose-phosphate pathway to generate energy. These organisms do not contain a functional TCA cycle and, therefore, cannot oxidize acetate or lactate to CO_2 and water (De Lay *et al*., 1984). Besides glucose, AAB are able to use other carbohydrates, which may be found in wine such as arabinose, fructose, galactose, mannitol, mannose, ribose, sorbitol, and xylose (De Lay *et al*., 1984).

B. WINE SPOILAGE

A major cause of wine spoilage by AAB is the production of excessive acetic acid. The legal limit for acetic acid in wine is 1.2–1.4 g/L, concentrations that also greatly reduce wine quality (Drysdale and Fleet, 1989a; Sponholz, 1993). Drysdale and Fleet (1989a) reported that as much as 50–60% of the ethanol content of a wine could be oxidized by these bacteria with the production of 1.5–3.75 g/L of acetic acid.

Another major byproduct that affects wine quality is ethyl acetate. This compound is highly undesirable and possesses a low flavor threshold of 10 mg/L (Berg *et al*., 1955). AAB have been shown to produce ethyl acetate concentrations of up to 140 mg/L in wine and 30 mg/L in must (Drysdale and Fleet, 1989a).

Other than acetic acid and ethyl acetate, AAB produce other compounds detrimental to wine quality including acetaldehyde, acetoin, and dihydroxyacetone. Drysdale and Fleet (1989a) reported increased concentrations of acetaldehyde in wine in which AAB had been grown. Acetaldehyde concentrations above the sensory threshold in wine (100–125 mg/L) are undesirable due to "green," "grassy," and "vegetative" off-aromas (Kotseridis and Baumes, 2000; Liu and Pilone, 2000). Dihydroxyacetone may be produced by *A. aceti* and *G. oxydans* through the metabolism of glycerol under aerobic conditions (Drysdale and Fleet, 1989a; Fugelsang, 1997). This compound

can affect the sensory quality of the wine with a "sweet/esterish" property and may also react with proline to produce a "crustlike" aroma (Boulton *et al.*, 1996; Drysdale and Fleet, 1988). Furthermore, acetaldehyde and dihydroxyacetone can bind with SO_2 in wine to produce compounds that are ineffective as antimicrobial agents (Fornachon, 1963; Hood, 1983; Romano and Suzzi, 1993). In addition, some *Acetobacter* species can metabolize lactate producing acetoin, a precursor of the flavor compound diacetyl (Romano and Suzzi, 1996).

III. *LACTOBACILLUS*

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

Lactobacillus represents a highly diverse group of species that are gram-positive microaerophilic bacteria that vary from long to short rods or even coccobacilli (Kandler and Weiss, 1986). Lactobacilli are catalase and cytochrome negative, although a few strains decompose peroxide by a non–heme-containing pseudocatalase (Beyer and Fridovich, 1985; Johnston and Delwiche, 1962; Kandler and Weiss, 1986; Kono and Fridovich, 1983). *Lactobacillus* can have complex nutritional requirements for amino acids, nucleic acid derivatives, vitamins, fatty acids, and fermentable carbohydrates (Kandler and Weiss, 1986). *Lactobacillus* species are either homofermentative or heterofermentative with regards to hexose metabolism. Homofermenters convert glucose to DL-, D-(–), or L-(+) lactic acid via the Embden-Meyerhof-Parnas pathway without the production of CO_2, whereas heterofermenters metabolize hexoses via the phosphoketolase pathway producing DL-, D-(−), or L-(+) lactic acid, CO_2, and ethanol or acetate (Kandler and Weiss, 1986).

Species of *Lactobacillus* isolated from grapes and wines worldwide have included *L. brevis, L. buchneri, L. casei, L. cellobiosus, L. curvatus, L. fermentum, L. fructivorans, L. hilgardii, L. homohiochii, L. jensenii, L. leichmannii, L. plantarum, L. sake*, and *L. trichodes* (Chalfan *et al.*, 1977; Costello *et al.*, 1983; Davis *et al.*, 1986a,b; Dicks and Van Vuuren, 1988; Douglas and Cruess, 1936; Du Plessis and Van Zyl, 1963; Fornachon, 1957; Kandler and Weiss, 1986; Lafon-Lafourcade *et al.*, 1983; Maret and Sozzi, 1977, 1979; Pilone *et al.*, 1966; Sieiro *et al.*, 1990; Vaughn, 1955). *L. cellobiosus* is regarded as a biotype of *L. fermentum*, whereas *L. leichmannii* is now referred to as *L. delbrueckii* subspecies *lactis* (Kandler and Weiss, 1986). *L. trichodes*, is considered a synonym of *L. fructivorans* (Weiss *et al.*, 1983). Edwards *et al.*

(1998, 2000) isolated two novel *Lactobacillus* species from commercial grape wines undergoing sluggish/stuck alcoholic fermentations. Based on phenotypic and phylogenetic evidence, *L. kunkeei* and *L. nagelii* were proposed as new species (Edwards *et al.*, 1998, 2000).

The occurrence and survival of *Lactobacillus* species in wine is highly pH and ethanol dependent (Davis *et al.*, 1986a). In high pH wines (>3.5), *Lactobacillus* species often are predominant, whereas at lower pH values, other LAB such as *Oenococcus oeni* dominate (Davis *et al.*, 1986b; Henick-Kling, 1993). Ethanol tolerance varies among *Lactobacillus* species. For example, growth of *L. plantarum* ceases at ethanol concentrations of 5–6% v/v, whereas the more ethanol tolerant *L. casei* and *L. brevis* have been successfully used to induce MLF (Kosseva *et al.*, 1998; Wibowo *et al.*, 1985). Finally, *L. fructivorans* is extremely ethanol tolerant and has been isolated from high alcohol (>20% v/v) dessert wines (Amerine and Kunkee, 1968; Fornachon *et al.*, 1949).

B. WINE SPOILAGE

Lactobacillus is generally considered undesirable in wine because uncontrolled growth can lead to increases in VA or formation of other adverse odors or flavors. Some species produce excessive amounts of acetic acid (Davis *et al.*, 1986b; Edwards *et al.*, 1999; Huang *et al.*, 1996). As evidence, *L. kunkeei* can produce between 3 and 5 g/L of acetic acid in wines (Edwards *et al.*, 1999; Huang *et al.*, 1996).

In addition to its sensory effect on wine, *Lactobacillus* species have been implicated in causing stuck or sluggish fermentations. Some winemakers have observed rapid wine spoilage by microorganisms dubbed the "ferocious" lactobacilli (Boulton *et al.*, 1996). Boulton *et al.* (1996) characterized this spoilage as being very swift with abundant bacterial growth during the early stages of vinification. Huang *et al.* (1996) demonstrated that uncontrolled growth of certain LAB including *L. kunkeei* (Edwards *et al.*, 1998) could cause stuck or sluggish alcoholic fermentations. Some strains of *L. hilgardii* have also been implicated in this spoilage (Mills, 2001). In many cases in which "ferocious" lactobacilli had caused spoilage, the winemakers had not used any addition of SO_2 and the initial pH of the wines was above 3.5 (Huang *et al.*, 1996), conditions favorable to the growth of *Lactobacillus.* Further discussion of the inhibition of *Saccharomyces* by *Lactobacillus* can be found in Section VIII.C.

Heterofermentative lactobacilli have been associated with the "mousy" defect in wines (Costello *et al.*, 2001; Heresztyn, 1986). This type of spoilage is characterized by the development of an offensive odor that renders a wine

unpalatable (Costello and Henschke, 2002). Lactobacilli associated with this taint *L. brevis, L. hilgardii*, and *L. cellobiosus* (Costello *et al*., 2001), can synthesize *N*-heterocyclic bases such as, 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine, and 2-acetyl-1-pyrroline (Costello and Henschke, 2002; Heresztyn, 1986). Because synthesis of these compounds requires the presence of ethanol, this defect is associated with wines rather than musts (Heresztyn, 1986).

Certain *Lactobacillus* are also implicated in spoilage of fortified wines. Known as "Fresno mold," this spoilage is characterized by mycelial/fiber like growth in wines (Amerine and Kunkee, 1968; Fornachon *et al*., 1949) and is caused by *L. trichodes* (*L. fructivorans*) (Gini and Vaughn, 1962; Vaughn, 1955). The species is relatively sensitive to SO_2, so use of this antiseptic can prevent the growth of this microorganism (Fornachon *et al*., 1949).

IV. *OENOCOCCUS*

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

Wine strains belonging to the genus *Leuconostoc* were originally classified as *Leuconostoc oenos* by Garvie (1967a). Later, phylogenetic studies revealed that *L. oenos* represented a distinct subline separate from other *Leuconostoc* species (Martinez-Murcia *et al*., 1993) and resulted in reassignment of this microorganism to a new genus *Oenococcus* (Dicks *et al*., 1995). Strains of *O. oeni* are described as Gram-positive, nonmotile, facultatively anaerobic, catalase-negative, ellipsoidal to spherical cells that usually occur in pairs or chains (Dicks *et al*., 1995; Garvie, 1967a). Similar to *Lactobacillus, O. oeni* are chemoorganotrophs requiring a rich medium containing complex growth factors and amino acids (Dicks *et al*., 1995; Garvie, 1967b). These microorganisms are heterofermentative and convert glucose to equal molar amounts of D-(−) lactic acid, CO_2, and ethanol or acetate via the phosphoketolase pathway (Cocaign-Bousquet *et al*., 1996; Cogan and Jordan, 1994; Krieger *et al*., 1993).

B. MALOLACTIC FERMENTATION

Numerous articles have been published documenting the enological importance of malolactic fermentation (Davis *et al*., 1985; Kunkee, 1967; Lonvaud-Funel, 1999; Van Vuuren and Dicks, 1993; Wibowo *et al*., 1985). During this

process, malic acid is metabolized to L-(+) lactic acid and CO_2, reducing wine acidity (Korkes *et al.*, 1950; Pilone and Kunkee, 1970, 1972; Wibowo *et al.*, 1985). Early work by Korkes *et al.* (1950) demonstrated that MLF was a decarboxylation catalyzed by a NAD^+ specific "malic" enzyme requiring Mn^{2+}. This enzyme was first purified from *L. plantarum* (Korkes *et al.*, 1950) and then from other LAB (Cox and Henick-Kling, 1990; Lonvaud-Funel, 1995; Lonvaud-Funel and Strasser de Saad, 1982; Schutz and Radler, 1973).

The biochemical benefit of MLF to the microorganism was puzzling at first as no apparent adenosine triphosphate (ATP) or other direct energy was detected (Pilone and Kunkee, 1972). During the conversion of malic to lactic acid, pyruvate was not formed as an intermediate, and this prompted researchers to report that MLF must serve a non–energy-yielding function (Kunkee, 1967; Pilone and Kunkee, 1972). Pilone and Kunkee (1972) observed that MLF accelerated the rate of growth of *O. oeni* and theorized that the decarboxylation reaction stimulated the utilization of carbon sources by LAB. To the contrary, Cox and Henick-Kling (1989) demonstrated that MLF yielded ATP and proposed that the ability of a cell to expel lactate and protons through a symport could theoretically generate a proton motive gradient (Δp), which in turn would yield ATP through a membrane-bound adenosine triphosphatase (ATPase).

However, there is a lack of evidence for a lactate/proton symport in *O. oeni* (Cox and Henick-Kling, 1995; Olsen *et al.*, 1991; Ten Brink *et al.*, 1985). Cox and Henick-Kling (1995) reported that lactate efflux did not produce ATP during MLF at low pH levels. Instead, the authors suggested that ATP produced during malate catabolism was linked to Δp formed during malate transport and lactic acid diffusion. In support, Poolman *et al.* (1991) reported that *Lactococcus lactis* produced a Δp composed of a membrane potential and pH gradient through the electrogenic uptake of malate, together with proton consumption as a result of decarboxylation of L-malate. Salema *et al.* (1994) proposed a model showing uptake of L-malate in the monoanionic form (the dominant species at low pH) via a uniport (Figure 1). This would cause a net negative charge to be moved inwards, thereby creating an electrical potential. L-Malate is then decarboxylated inside the cell to L-lactic acid and CO_2 in a reaction that requires one proton. The consumption of a proton in the cytoplasm would generate a pH gradient that, together with the change in electrical potential, would form a Δp across the cytoplasmic membrane. ATP generation would then occur via a membrane-bound ATPase. Salema *et al.* (1994) suggested that L-lactic acid and CO_2 leave the cell as neutral species rather then being actively transported. Later work by Salema *et al.* (1996) supported this model, as a Δp was generated *in vitro* by the action of an electrogenic uniport in conjunction with proton consumption by L-malate decarboxylation.

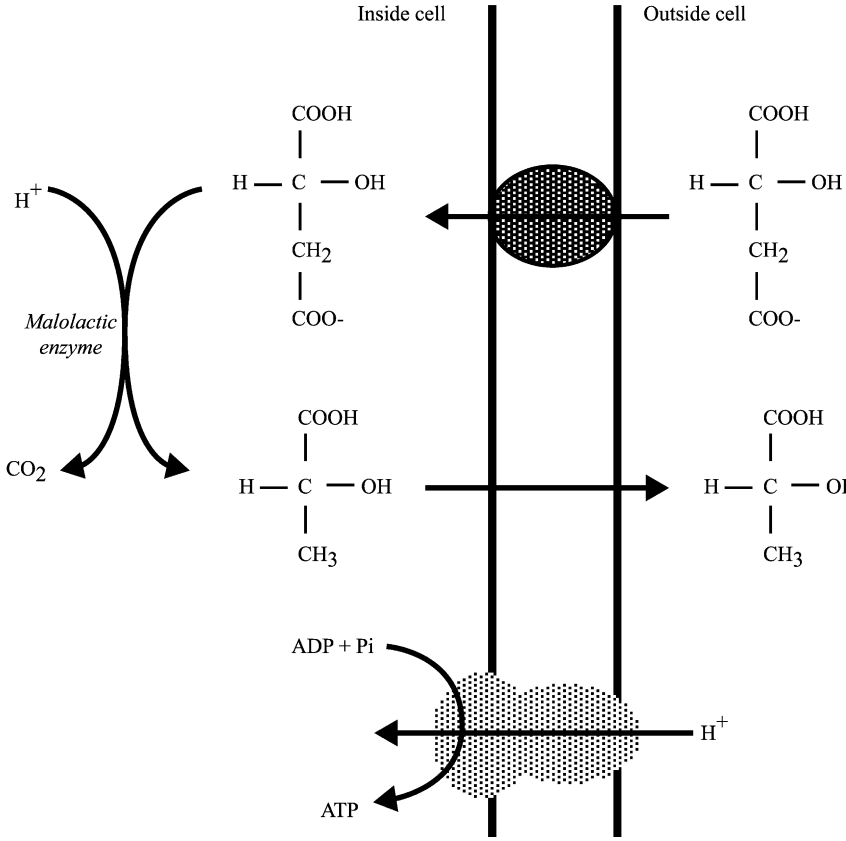

FIG. 1 Proposed model for energy generation (ATP) by *Oenococcus oeni* through conversion of malic acid to lactic acid and carbon dioxide. (Adapted from Poolman *et al*., 1991 and with permission of the Journal of Bacteriology.)

MLF most commonly occurs after alcoholic fermentation but may occur simultaneously with the primary fermentation. Because relying on natural microflora can be unpredictable and difficult to control, starter cultures of pure strains of bacteria have been developed (Henick-Kling, 1993; Krieger *et al*., 1993; Kunkee *et al*., 1964; Nielsen *et al*., 1996; Pilone, 1995). Although selected strains of *Lactobacillus* can be inoculated, *O. oeni* is the primary species to conduct MLF because of acid tolerance and the flavor profile produced (Guzzo *et al*., 1994; Krieger *et al*., 1993; Kunkee *et al*., 1964; Liu, 2002; Nielsen *et al*., 1996; Wibowo *et al*., 1985).

Exactly when to inoculate *O. oeni* during vinification is a point of contention among researchers and enologists. Some have argued that starter

cultures should be inoculated only after completion of the alcoholic fermentation, to prevent possible yeast antagonism and production of undesirable metabolites (Lafon-Lafourcade and Ribéreau-Gayon, 1984; Lafon-Lafourcade *et al*., 1983; Ribéreau-Gayon, 1985). Others contend that the inducement of simultaneous alcoholic and malolactic fermentations is feasible (Beelman, 1982; Beelman and Kunkee, 1985). Semon *et al.* (2001) concluded that the optimal time for inoculation of malolactic bacteria was dependent on the specific yeast and bacterial strains used. These authors postulated that problems associated with inoculation of bacteria before completion of alcoholic fermentation, such as excessive volatile acid and/or sluggish alcoholic fermentations, are most probably due to incompatibilities between specific strains of yeast or malolactic bacteria.

C. FLAVOR PRODUCTION

Aside from deacidification, MLF may also influence the sensory qualities of a wine by the production of many flavor and aroma compounds (Davis *et al*., 1985, 1988; Henick-Kling, 1993; Kunkee, 1967; Rankine, 1977). However, there is still debate regarding the contribution of MLF to the sensory properties of a wine. Early work by Kunkee *et al.* (1964) and Rankine (1972) indicated that MLF may not have a measurable effect on the sensory properties of a wine. On the other hand, many other studies have shown that MLF causes significant changes in wine aroma and flavor (Boido *et al*., 2002; Delaquis-Pascal *et al*., 2000; De Revel *et al*., 1999; Gambaro *et al*., 2001; Henick-Kling, 1995; Laurent *et al*., 1994; Maicas *et al*., 1999; McDaniel *et al*., 1987; Nielsen and Richelieu, 1999).

Although the exact contribution of MLF to wine flavor is still debatable, *O. oeni* is known to produce flavor and aroma compounds in wine. One of the most important is 2,3-butanedione or diacetyl (Collins, 1972; Fornachon and Lloyd, 1965; Martineau and Henick-Kling, 1995a,b; Nielsen and Richelieu, 1999; Rodriguez *et al*., 1990). Diacetyl has a distinct buttery aroma and is synthesized by wine LAB from citrate or other carbohydrates via pyruvate (Martineau and Henick-Kling, 1995a). Sensory threshold values range from 0.2 mg/L in Chardonnay to 0.9 mg/L in Pinot Noir to 2.8 mg/L in Cabernet Sauvignon wine (Martineau *et al*., 1995), with the final concentration in wine affected by many factors including bacterial strain, wine type, and redox potential (Martineau and Henick-Kling, 1995a,b; Nielsen and Richelieu, 1999). Although the presence of diacetyl at low concentrations (1–3 mg/L) is described sensorially as being "buttery" or "nutty," the compound will dominate wine aroma at higher concentrations (5–7 mg/L), resulting in spoilage (Rankine *et al*., 1969).

In addition to diacetyl, *O. oeni* produces esters, flavor compounds also important for wine flavor and aroma. Esters are primarily produced by *Saccharomyces* during alcoholic fermentation (Mason and Dufour, 2000; Nykänen, 1986; Nykänen and Nykänen, 1977; Soles *et al.*, 1982), although evidence shows that esters such as ethyl acetate, ethyl lactate, ethyl hexanoate, and ethyl octanoate can be synthesized by *O. oeni* (De Revel *et al.*, 1999; Delaquis-Pascal *et al.*, 2000; Edwards and Peterson, 1994; Maicas *et al.*, 1999; Tracey and Britz, 1989). For example, Edwards and Peterson (1994) reported that strains of *O. oeni* synthesized relatively large amounts of ethyl lactate (183–1280 μg/L) during growth in microbiological medium. In agreement, Maicas *et al.* (1999) reported that 50 mg/L of ethyl lactate was produced in wines fermented with *O. oeni*, as well as isoamyl acetate and ethyl caproate, compounds important for a pleasant fruity note in wine (Gil *et al.*, 1996; Mason and Dufour, 2000; Nykänen, 1986).

O. oeni may also influence wine flavor through the liberation of monoterpenes. These flavor compounds are often present in grapes and wine as nonvolatile flavorless glycosylated compounds (Ebeler, 2001). The liberation of monoterpenes is important for the development of certain wine aromas; however, the hydrolysis of monoglucosides requires the action of a β-glucosidase. It is known that wine yeast, in particular non-*Saccharomyces* yeast, have glycosidic activities (Charoenchai *et al.*, 1997; Delcroix *et al.*, 1994; Maicas *et al.*, 1999; McMahon *et al.*, 1999). Evidence indicates that some strains of *O. oeni* also possess β-glucosidase activity and, thus, may hydrolyze glycoconjugates and alter the sensory characteristics of wine (Boido *et al.*, 2002; Grimaldi *et al.*, 2000; Mansfield *et al.*, 2002; Ugliano *et al.*, 2003).

O. oeni may also influence the concentrations of aldehydes such as acetaldehyde. Acetaldehyde is the most abundant aldehyde found in wine and affects wine aroma, aging, and color stability (Liu and Pilone, 2000). Osborne *et al.* (2000) found that *O. oeni* can metabolize acetaldehyde, producing ethanol and acetic acid. Degradation of acetaldehyde may be desirable in some cases, because excess acetaldehyde causes an off-aroma in wine (Kotseridis and Baumes, 2000; Liu and Pilone, 2000), but undesirable in other cases because this compound plays a role in the color development of red wines (Somers and Wescombe, 1987; Timberlake and Bridle, 1976).

Aside from influencing flavor and aroma, MLF may increase the body and mouthfeel of a wine, possibly because of the production of polyols such as glycerol and erythritol (Henick-Kling *et al.*, 1994). Veiga da Cunha *et al.* (1993) demonstrated the production of glycerol and erythritol from glucose using a ^{13}C-nuclear magnetic resonance (NMR) technique, whereas

Liu *et al.* (1995) and Firme *et al.* (1994) observed the production of glycerol and erythritol by *O. oeni.*

V. *PEDIOCOCCUS*

A. TAXONOMY, OCCURRENCE, GROWTH, AND METABOLISM IN WINE

Pediococci are characterized as spherical, Gram-positive, nonmotile, catalase-negative, aerobic to microaerophilic microorganisms (Carr *et al.*, 2002; Garvie, 1986). Pediococci are the only LAB that divide in two planes, which form tetrads or large clumps of cells (Axelsson, 1998; Garvie, 1986). Currently approved species are *P. acidilacti, P. damnosus, P. dextrinicus, P. halophilus, P. inopinatus, P. parvulus, P. pentosaceus*, and *P. urinae-equi* (Garvie, 1986). The International Committee on Systematic Bacteriology has ruled that the species *P. cerevisiae* was not validly used in publications because it represented at least two species, *P. damnosus* and *P. pentosaceus* (Garvie, 1974, 1986; Raccach, 1987).

Pediococci are homofermentative, with glucose being converted to DL- or L-(+) lactate via the Embden-Meyerhof-Parnas pathway without the production of CO_2 (Garvie, 1986). *Pediococcus* are chemoorganotrophs having complex growth factor and amino acid requirements. All species require nicotinic acid, pantothenic acid and biotin, but none require thiamine, *p*-aminobenzoic acid, or cobalamin (Garvie, 1986). Growing cultures also have the ability to form L-(+) lactate from malic acid (Edwards and Jensen, 1992; Raccach, 1987).

Pediococci are commonly found associated with various plants and their products such as cabbage and sauerkraut, cucumbers and pickles, grapes and wine, and wort and grain mashes (Carr, 1970, 2002; Costello *et al.*, 1983; Edwards and Jensen, 1992; Flemming *et al.*, 1975; Mundt *et al.*, 1969; Solberg and Clausen, 1973). They may enter wine through being present on soil, grape berries, or winery equipment, but their survival is favored when pH is greater than 3.5 (Davis *et al.*, 1986a; Wibowo *et al.*, 1985). Species of *Pediococcus* isolated from wine include *P. damnosus, P. inopinatus, P. pentosaceus*, and *P. parvulus* (Davis *et al.*, 1986b; Edwards and Jensen, 1992; Garvie, 1986; Lafon-Lafourcade *et al.*, 1983).

B. WINE SPOILAGE

Growth of *Pediococcus* species in wine has been considered undesirable because of the production of off-aromas and off-flavors. Pediococci are capable of producing excessive acetoin and diacetyl, which can give undesirable aromas

and flavors at high concentrations (Sponholz, 1993). In addition, some species of *Pediococcus* are capable of degrading glycerol to acrolein, a compound that reacts with the phenolic group of anthocyanins producing a bitter taint in wine (Davis *et al.*, 1988; Du Toit and Pretorius, 2000; Sponholz, 1993).

Besides producing off-flavors, *Pediococcus* species have been implicated in the production of extracellular polysaccharides characterized as β-D-glucans (Llaubéres *et al.*, 1990). These homoglucans are produced from glucose and consist of a trisaccharide repeating unit having a (1 → 3)-linked backbone and a (1 → 2)-linked branch of one of the D-glucopyranosyl groups (Llaubéres *et al.*, 1990). Besides being visually unappealing, these polymers cause an increase in viscosity of the wine (Fugelsang, 1997; Manca de Nadra and Strasser de Saad, 1995). Pediococci associated with "ropiness" have higher tolerances to ethanol than other strains (Du Toit and Pretorius, 2000). Thus, this defect occurs in wines either during alcoholic fermentation or after bottling (Du Toit and Pretorius, 2000).

P. damnosus is the bacterium primarily implicated in the production of polysaccharides in wine (Lonvaud-Funel, 1999; Manca de Nadra and Strasser de Saad, 1995), although Manca de Nadra and Strasser de Saad (1995) isolated two strains of *P. pentosaceus* from "ropy" Argentinean wines. Strains of *P. damnosus* that produce exopolysaccharides contain an unique 4-Kb plasmid (Lonvaud-Funel, 1999; Manca de Nadra and Strasser de Saad, 1995). Taking advantage of this, Lonvaud-Funel *et al.* (1993) developed a DNA probe to detect the presence of extracellular polysaccharide-producing pediococci strains by labeling a 1.2-Kb part of this plasmid. Furthermore, Gindreau *et al.* (2001) devised a direct polymerase chain reaction (PCR) detection method to detect these strains of *P. damnosus*. The PCR detection method negated the need for the time-consuming culture and colony-isolation steps required for the DNA probe method.

Although growth of certain *Pediococcus* species in wines is undesirable, Edwards and Jensen (1992) reported that several wines from which pediococci had been isolated were not spoiled. In agreement, Edwards *et al.* (1994) reported that *P. parvulus* altered the bouquet of a Cabernet Sauvignon wine that had not undergone MLF but was not considered spoiled. Therefore, the growth of pediococci in wine may add desirable flavors and aromas under certain circumstances. Further research elucidating the impact of these microorganisms on the chemical composition, bouquet, and flavor of wines is warranted as *Pediococcus* species have been isolated from wines worldwide (Costello *et al.*, 1983; Edwards and Jensen, 1992; Fleet *et al.*, 1984; Manca de Nadra and Strasser de Saad, 1995).

VI. IDENTIFICATION OF BACTERIA IN WINE

Most bacterial species present during the vinification process have been identified by traditional microbiological techniques based on cell morphological and physiological differences. All LAB with their biochemical characteristics are described in *Bergey's Manual* (Kandler and Weiss, 1986). However, the use of physiological and biochemical criteria to identify LAB strains can yield ambiguous results because many of the bacteria have very similar nutritional requirements and grow under similar environmental conditions (Sohier and Lonvaud-Funel, 1998; Vandamme *et al.*, 1996). Furthermore, cultivation-dependent methods often exhibit biases resulting in an incomplete representation of the true bacterial diversity present (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998).

As a result, much research has been undertaken to develop more specific and reliable techniques to identify bacteria present during winemaking. Method development has focused on the use of molecular biology techniques allowing direct molecular characterization of bacterial strains through DNA-DNA hybridization (Lonvaud-Funel *et al.*, 1991). Some techniques include total cell DNA hybridization (Lonvaud-Funel *et al.*, 1991; Sohier and Lonvaud-Funel, 1998), PCR methods with species-specific primers targeted at the gene encoding the malolactic enzyme (Zapparoli *et al.*, 1998) or targeted at 16S rDNA (Bartowsky and Henschke, 1999; Maria Rodas *et al.*, 2003), with separation of PCR-amplified DNA by pulsed-field gel electrophoresis (Kelly *et al.*, 1993; Zapparoli *et al.*, 2000) or denaturing gradient gel electrophoresis (Lopez *et al.*, 2003), and randomly amplified polymorphic DNA techniques (Holt and Cote, 1998; Johansson *et al.*, 1995; Reguant and Bordons, 2003; Zapparoli *et al.*, 2000). These techniques are valid tools for the study of bacterial population dynamics during MLF and for the detection of spoilage bacterial species because they allow specific identification, detection, and enumeration of bacterial species in wine.

VII. PUBLIC HEALTH CONCERNS

A. BIOGENIC AMINES

Biogenic amines are low-molecular-weight organic bases that have undesirable physiological effects on humans when absorbed at too high a concentration (Arena and Manca de Nadra, 2001; Lonvaud-Funel, 2001; Silla Santos, 1996). These compounds are formed by decarboxylation of the

corresponding amino acids by microorganisms such as LAB (Arena and Manca de Nadra, 2001; Halasz *et al*., 1994). In wine, several amino acids can be decarboxylated producing histamine, tyramine, putrescine, cadaverine, and phenylethylamine (Arena and Manca de Nadra, 2001; Lonvaud-Funel, 2001; Moreno-Arribas *et al*., 2003). The presence of high concentrations of histamine, tyramine, and phenylethylamine in wine is thought to be responsible for headaches and flushing sometimes experienced after consumption (Granerus *et al*., 1969; Ough *et al*., 1987; Rivas-Gonzalo *et al*., 1983; Sandler *et al*., 1974; Silla Santos, 1996). In addition, putrescine and cadaverine may enhance the toxicity of histamine to humans by depressing histamine oxidation (Arena and Manca de Nadra, 2001; Jarisch and Wantkle, 1996; Taylor, 1986; Taylor and Lieber, 1979; Torrea and Ancin, 2002).

Histamine is one of the most frequently found biogenic amines in wine (Lonvaud-Funel, 2001; Ough *et al*., 1987; Soufleros and Bertrand, 1988; Zee *et al*., 1983). Histamine levels ranging from undetectable to 30 mg/L have been found in wines from both European and American origins (Baucom *et al*., 1986; Ough, 1971). In general, the level of histamine in wine is usually below the toxic dose of 8 mg/L, a concentration thought to induce headaches when large amounts of wine are ingested (Ough, 1971). However, the exact toxic threshold of histamine is difficult to determine because of the presence or absence of potentiating compounds such as ethanol, aldehydes, and polyamines (such as putrescine and cadaverine), as well as the relative histamine sensitivity of different individuals (Jarisch and Wantkle, 1996; Lowenberg *et al*., 1981; Marquardt and Werringloer, 1965).

It was thought that spoilage bacteria, mainly *Pediococcus* species, were solely responsible for the production of histamine in wine (Delfini, 1989). However, some *O. oeni* strains are also able to produce histamine from histidine in wine (Lonvaud-Funel and Joyeaux, 1994). Additionally, Arena and Manca de Nadra (2001) and Moreno-Arribas *et al.* (2003) isolated strains of *L. plantarum, L. hilgardii*, and *L. brevis* capable of producing certain biogenic amines under vinification conditions. Elevated levels of biogenic amines may be found in wines that have undergone MLF due to the presence of these biogenic amine-producing LAB (Bauza *et al*., 1995; Cilliers and Van Wyk, 1985). Higher concentrations of biogenic amines have also been observed if wines remain in contact with yeast lees, probably due to the increased amount of peptides and free amino acids available for decarboxylation by LAB (Lonvaud-Funel, 2001).

The presence of biogenic amines in wines is currently not regulated worldwide (Olga *et al*., 1996). However, because of their potential health implications, wines with high concentrations may be rejected from some markets. This concern has led to the production of malolactic starter cultures that do not contain amino acid decarboxylase (Lonvaud-Funel, 2001).

B. ETHYL CARBAMATE FORMATION

Ethyl carbamate is a weak carcinogen found in many fermented foods and beverages including wine (Canas *et al*., 1989; Liu *et al*., 1994; Ough, 1976; Zimmerli and Schlatter, 1991). Ethyl carbamate is formed through the spontaneous reaction between ethanol and an ethyl carbamate precursor such as citrulline, urea, or carbamyl phosphate (Ough *et al*., 1988). Wine LAB can produce citrulline via arginine degradation, which may then react with ethanol present in the wine to form ethyl carbamate. Mira de Orduna *et al.* (2000) observed that commercially available wine LAB strains used for induction of MLF were capable of excreting citrulline from arginine degradation. In addition, Liu *et al.* (1994) demonstrated a good correlation between the excretion of citrulline and the formation of ethyl carbamate during the degradation of arginine by *O. oeni* and *L. buchneri.*

These studies demonstrate that wine LAB may contribute to ethyl carbamate formation. In the United States, there is a voluntary concentration limit of 15 ng/g for ethyl carbamate in table wines (Canas *et al*., 1994). One suggested way to achieve this goal is the development of non–arginine-degrading *O. oeni* strains for the induction of MLF (Mira de Orduna *et al*., 2001).

VIII. INTERACTIONS BETWEEN BACTERIA AND OTHER WINE MICROORGANISMS

A. ECOLOGY OF WINE MICROORGANISMS DURING WINEMAKING

Several genera of yeasts and bacteria are naturally present on grapes at the time of harvest or present on winery equipment (Fleet *et al*., 1984; Kunkee *et al*., 1965; Lafon-Lafourcade *et al*., 1983; Wibowo *et al*., 1985). During the course of alcoholic and malolactic fermentations, there is a successional growth of microorganisms (Figure 2) due to differing tolerances of inhibitory substances and varying growth requirements (Costello *et al*., 1983; Davis *et al*., 1986b; Fleet *et al*., 1984; Wibowo *et al*., 1985). Initially, non-*Saccharomyces* yeast grow during the early stages of alcoholic fermentation, but their viability rapidly decreases because of lack of oxygen and elevated ethanol concentrations, leaving *S. cerevisiae* as the dominant species to complete the fermentation (Fleet *et al*., 1984; Heard and Fleet, 1985; Holm Hansen *et al*., 2001; Nissen and Arneborg, 2003). Toward completion of alcoholic fermentation when *Saccharomyces* enter stationary/death phase, populations of *Oenococcus* can increase to conduct malolactic fermentation. Other bacteria such as *Acetobacter, Lactobacillus*, and *Pediococcus* can grow after MLF during the conservation or aging of wine.

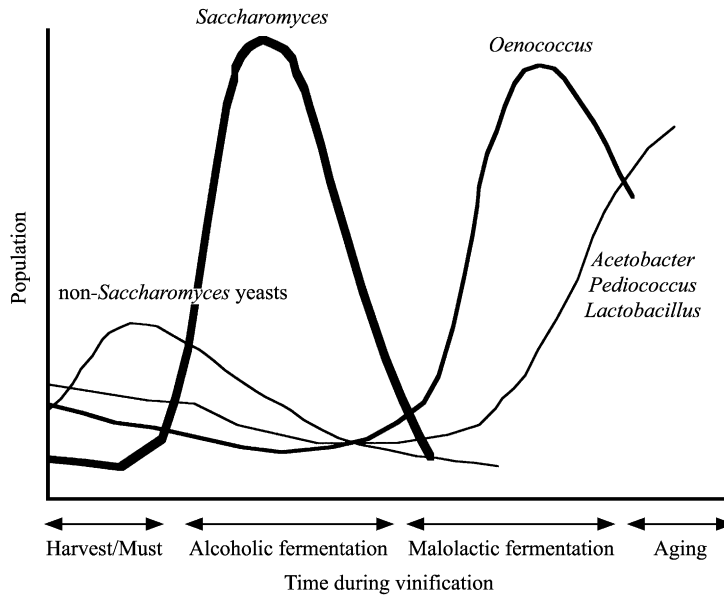

FIG. 2 General ecology of selected microorganisms during vinification.

B. INTERACTIONS BETWEEN DIFFERENT LAB SPECIES

During vinification, antagonistic interactions occur between LAB species present in wine. For example, Edwards *et al.* (1994) reported that strains of *P. parvulus* grew well in wines that had not undergone MLF, whereas the viability of all *P. parvulus* strains declined soon after inoculation into wine that had undergone MLF catalyzed by *O. oeni* (Figure 3). The authors postulated that the inhibition of *P. parvulus* may be related to the synthesis of an unidentified inhibitory substance. Inhibition of *Pediococcus* by *L. hilgardii* was noted by Rodriguez and Manca de Nadra (1995). Lonvaud-Funel and Joyeux (1993) reported that another species of *Pediococcus, P. pentosaceus*, inhibited *O. oeni* because of the accumulation of small proteolytic-sensitive thermostable compounds. Based on these results, the authors concluded that growth of certain *Pediococcus* species in wine before MLF could lead to problems inducing the secondary fermentation.

Although interactions between LAB are frequently complicated, many LAB species produce antibacterial proteinaceous substances called *bacteriocins* that have a narrow spectrum of activity against closely related species. Many excellent reviews on bacteriocins have been published (De Vuyst and

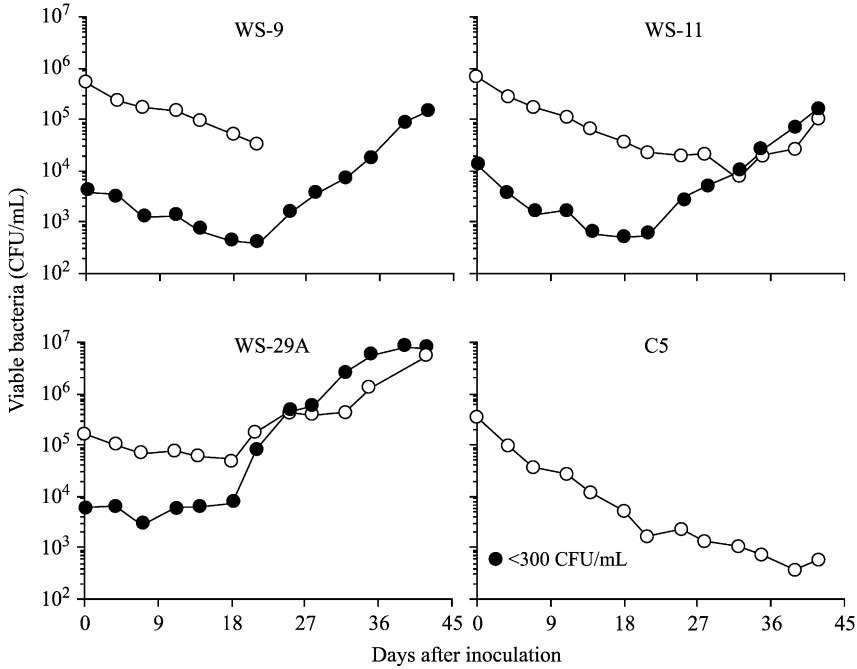

FIG. 3 Growth of *P. parvulus* WS-09, WS-11, WS-29A, and C5 inoculated into Cabernet Sauvignon MLF-negative wines (○) and MLF-positive wines (●). (Adapted from Edwards *et al.*, 1994 and with permission of the American Journal of Enology and Viticulture.)

Vandamme, 1994; Jack *et al.*, 1994; Nes *et al.*, 1996). The following discussion is restricted to bacteriocins produced by LAB present in wine.

A number of researchers have reported the production of bacteriocins by LAB species present in wine. For example, Navarro *et al.* (2000) isolated nine strains of *L. plantarum* from Rioja red wine that showed antibacterial activity, whereas Yurdugul and Bozoglu (2002) identified an isolate of *Leuconostoc mesenteroides* subspecies *cremoris* from wine that produced a bacteriocin-like inhibitory substance. Furthermore, Strasser de Saad and Manca de Nadra (1993) isolated two strains of *P. pentasaceus* that produced an inhibitory substance against strains of *Lactobacillus, Oenococcus*, and *Pediococcus.* The proteinaceous nature and narrow spectrum of activity of the inhibitory substance indicated that it was a bacteriocin.

Because of mounting consumer resistance to the excessive use of sulfur dioxide and other chemical preservatives in wine, the use of bacteriocins as preservatives has generated interest among researchers. In a study by Schoeman *et al.* (1999), bactericidal yeast strains were developed by

expressing the *pedA* pediocin gene from *P. acidilactici* in *S. cerevisiae*. The authors proposed that development of such bactericidal strains could lead to the use of *S. cerevisiae* strains capable of acting as biological control agents to inhibit the growth of spoilage bacteria. It should be noted, however, that current consumer aversion to the use of genetically modified organisms in food means the use of genetically modified wine yeast is not an option (Akada, 2002; Beringer, 2000; Boyazoglu, 2002; Dequin, 2001).

C. INTERACTIONS BETWEEN LAB AND *SACCHAROMYCES*

Winemakers have long experienced sluggish or stuck alcoholic fermentations, problems that may be attributed to insufficient nutrients to support yeast growth adequately or improper fermentation conditions (Alexandre and Charpentier, 1998; Bisson, 1999; Boulton *et al*., 1996; Groat and Ough, 1978; Ingledew and Kunkee, 1985; Kunkee, 1991; Ough, 1966; Sharf and Margalith, 1983; Tromp, 1984). In addition, the growth of unknown lactobacilli has been observed in some of these problem fermentations, leading to the speculation that uncontrolled growth of these microorganisms may also lead to sluggish fermentations (Boulton *et al*., 1996).

Evidence for the involvement of *Lactobacillus* was initially provided by Huang *et al.* (1996). Huang *et al.* (1996) observed that *Lactobacillus* spp. strain YH-15 reached a population of $>10^9$ cfu/ml two days after inoculation into a Chardonnay juice. This bacterium slowed alcoholic fermentation which eventually ceased at 5% soluble solids and was later determined to be a novel species, proposed to be *L. kunkeei* (Edwards *et al*., 1998).

Boulton *et al.* (1996) indicated that rapidly growing *Lactobacillus* species, dubbed "ferocious" lactobacilli, could produce enough acetic acid in 2–3 days to inhibit yeast metabolism. In support, *L. kunkeei* can produce between 3 and 5 g/L of acetic acid in wines undergoing stuck/sluggish fermentations (Edwards *et al*., 1999; Huang *et al*., 1996). However, Huang *et al.* (1996) noted that much lower levels of acetic acid were present in other sluggish/stuck fermentations. This indicates that acetic acid may not be the sole mechanism for the inhibition. These findings were later confirmed by Edwards *et al.* (1999), where fermentations containing *L. kunkeei* and *S. cerevisiae* were sluggish compared to the control but were not impaired with acetic acid (Figure 4). The authors concluded that acetic acid may be involved in yeast inhibition by *L. kunkeei* but that additional unidentified inhibitory mechanisms were probably involved.

The interaction between *Saccharomyces* species and *O. oeni* during the vinification process may be either stimulatory to the bacterium (Beelman *et al*., 1982; Feullat *et al*., 1985; Guilloux-Benatier *et al*., 1985; Lüthi and Vetsch, 1959) or inhibitory (Beelman *et al*., 1982; Cannon and Pilone, 1993;

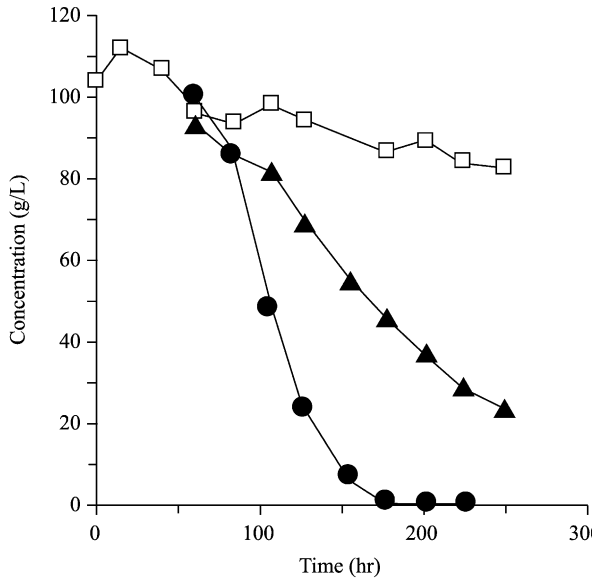

FIG. 4 Decline of glucose in a Chardonnay juice inoculated with *Saccharomyces bayanus* EC 1118 (•). *Lactobacillus kunkeei* YH-15 (□), or *S. bayarus* and *L. kunkeei* (▲). (Adapted from Edwards *et al.*, 1999 and with the permission of the American Journal of Enology and Viticulture.)

Henick-Kling and Park, 1994; King and Beelman, 1986; Larsen *et al.*, 2003). Inhibitory interactions have been reported in which the viability of *O. oeni* declined from 10^5 to 10^7 cfu/ml to undetectable populations soon after inoculation into wine (Beelman *et al.*, 1982; Fornachon, 1968; King and Beelman, 1986; Lemaresquier, 1987; Liu and Gallander, 1983; Ribéreau-Gayon, 1985; Semon *et al.*, 2001; Wibowo *et al.*, 1988). This rapid decline in bacterial viability has been commonly reported, even when *Saccharomyces* and *O. oeni* are co-inoculated at similar populations (Figure 5).

Two theories have been proposed to explain this phenomenon. First, the faster growing *Saccharomyces* may remove nutrients from a grape must (Amerine and Kunkee, 1968; Beelman *et al.*, 1982; Fornachon, 1968; Kunkee, 1967) because malolactic bacteria are nutritionally fastidious with complex needs (DuPlessis, 1963; Garvie, 1967b). Evidence for this was the rapid uptake of sterols, amino acids, and vitamins by yeast from grape must (Beelman, 1982; Beelman *et al.*, 1982; Fornachon, 1968; King and Beelman, 1986; Lafon-Lafourcade *et al.*, 1979). In support, Beelman *et al.* (1982) demonstrated that during growth in synthetic media, yeast depleted certain amino acids to concentrations that may not be sufficient for LAB growth. These authors suggest that as yeast enter stationary/death phases and lyse,

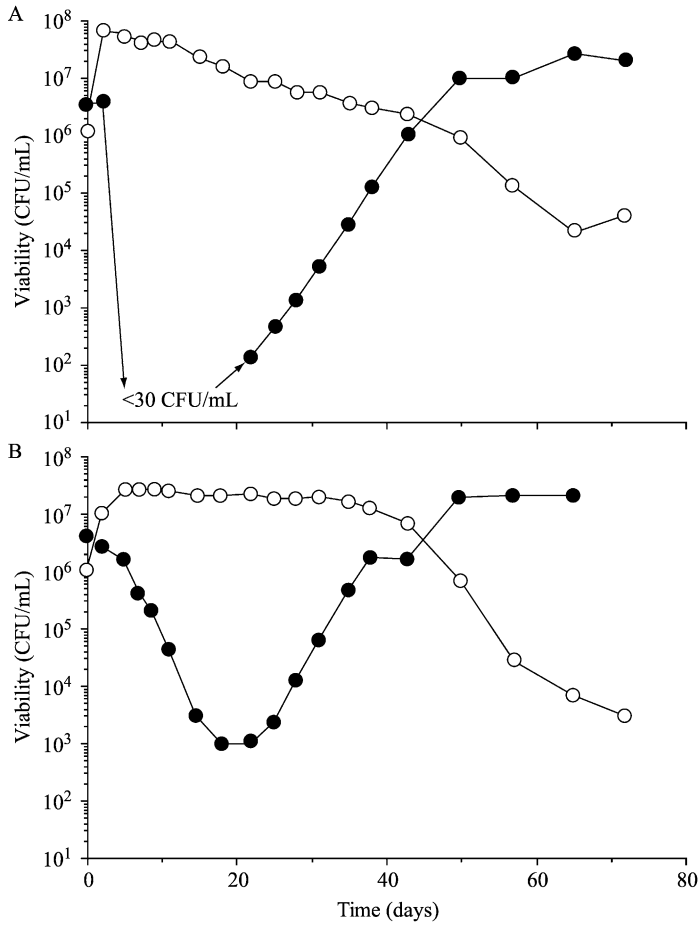

FIG. 5 Viability of *Saccharomyces cerevisiae* (○) and *Oenococcus oeni* strain EQ-54 (●) inoculated into a Chardonnay juice with the bacteria prepared using a diluted grape juice medium (A) or a lyophilized culture (B). (Adapted from Semon *et al.*, 2001 and with the permission of the Australian Journal of Grape and Wine Research.)

nutrients released into the wine would allow the recovery of *O. oeni.* Furthermore, it has been demonstrated that the autolytic activity of wine yeasts during aging on lees can affect the concentrations of amino acids, peptides, and proteins in wine (Alexandre *et al.*, 2001; Charpentier and Feuillat, 1993; Martinez-Rodriguez *et al.*, 2001).

Other studies have demonstrated that the removal of nutrients by yeast does not always explain the observed inhibition of *O. oeni*. For instance, Larsen *et al.* (2003) reported that the addition of supplemental nutrients to a

wine fermented by *S. cerevisiae* strain V1116 do not relieve the observed bacterial inhibition. Studying the impact of yeast autolysis on MLF, Patynowski *et al.* (2002) concluded that nutrient depletion by *S. cerevisiae* is not responsible for the observed bacterial inhibition. Furthermore, this research shows that the yeast produced an unidentified inhibitory factor that is progressively lost during aging. These results suggest that the second proposed theory, the production of toxic metabolites by yeast, may be responsible for the inhibition of *O. oeni*.

Saccharomyces is known to produce compounds during alcoholic fermentation that are inhibitory to *Oenococcus.* These include ethanol (Britz and Tracey, 1990; Costello *et al.*, 1983; Davis *et al.*, 1986a), SO_2 (Dott *et al.*, 1976; Eschenbruch, 1974; Eschenbruch and Bonish, 1976; Henick-Kling and Park, 1994; Romano and Suzzi, 1993; Suzzi *et al.*, 1985), medium-chain fatty acids (Capucho and San Ramao, 1994; Edwards and Beelman, 1987; Edwards *et al.*, 1990; Lonvaud-Funel *et al.*, 1988), and antibacterial proteins/peptides (Dick *et al.*, 1992). Of these compounds, SO_2 is most commonly implicated in causing bacterial inhibition (Fornachon, 1963; Henick-Kling and Park, 1994; Larsen *et al.*, 2003). SO_2 is an effective antimicrobial against wine LAB (Amerine *et al.*, 1980; Britz and Tracey, 1990; Carr *et al.*, 1976; Liu and Gallander, 1983; Ough and Crowell, 1987). When added to a must or wine, sulfur dioxide either ionizes to free SO_2 ($SO_2{\cdot}H_2O$, HSO_3^-, and/or SO_3^{2-} depending on pH level) or can become bound SO_2 by reacting with acetaldehyde, glucose, pyruvic acid, α-keto-glutaric acid, or glucose on a 1:1 molar ratio (Amerine and Ough, 1980; Burroughs and Sparks, 1973; Romano and Suzzi, 1993; Zoecklein *et al.*, 1995). Of these forms, the molecular variety of free SO_2 ($SO_2{\cdot}H_2O$) is thought to be the most antimicrobial (Edinger, 1986; King *et al.*, 1981; Macris and Markakis, 1974; Rahn and Conn, 1944; Zoecklein *et al.*, 1995).

There is conflicting information about whether some forms of bound SO_2, in particular acetaldehyde-bound SO_2, are inhibitory to wine bacteria. Early work by Fornachon (1963) reported that both *L. hilgardii* and *L. mesenteroides* were inhibited in a medium in which sulfurous acid and an excess of acetaldehyde had been added. The author determined that these bacteria could metabolize acetaldehyde-SO_2, an observation later confirmed for *O. oeni* (Osborne *et al.*, 2000). Fornachon (1963) further noted that MLF could be prevented by the presence of bound SO_2 even when the amounts of free SO_2 are negligible, possibly because of any SO_2 liberated from the metabolism of the acetaldehyde-bound SO_2. Hood (1983) provided an alternative mechanism by suggesting that any effect of bound SO_2 may be due to small amounts of free (and, therefore, molecular) SO_2 in equilibrium with the bound form. However, these results are contrary to those of Carr *et al.* (1976) who reported that acetaldehyde-bound SO_2 had no influence on the

bacterium studied (*L. plantarum*). Larsen *et al.* (2003) suggest that some forms of bound SO_2 may be more inhibitory than previously thought.

Besides being intentionally added to must/wine by winemakers, SO_2 is produced by *Saccharomyces* species during alcoholic fermentation (Dott *et al.*, 1976; Eschenbruch, 1974; Eschenbruch and Bonish, 1976; Henick-Kling and Park, 1994; Romano and Suzzi, 1993; Suzzi *et al.*, 1985). SO_2 is an intermediate produced during the assimilatory reduction of sulfate to sulfide (Amerine *et al.*, 1980; Donalies and Stahl, 2002; Eschenbruch, 1974; Rauhut, 1993; Thomas and Surdin-Kerjan, 1997). During this process, sulfate is taken up by the yeast cell via two membrane-bound permease enzymes (Rauhut, 1993) and then is reduced to sulfite via adenosine-5′-phosphosulfate and 3′-phosphadenosine-5′-phosphosulfate. Depending on needs and conditions, yeast can actively excrete sulfite via a membrane-bound sulfite pump (Avram and Bakalinsky, 1997), usually of concentrations of 10–30 mg/L, although some strains can produce amounts that exceed 100 mg/L (Eschenbruch, 1974). The presence, absence, or relative activity of genes encoding for the membrane-bound sulfite pump may explain the reported strain differences in SO_2 production by *S. cerevisiae* (Avram and Bakalinsky, 1997; Donalies and Stahl, 2002; Park and Bakalinsky, 2000).

The production of SO_2 by yeast, coupled with that added to a must/wine, has been suggested by many researchers to be the primary mechanism of bacterial inhibition during alcoholic fermentation (Fornachon, 1968; Henick-Kling, 1993; Henick-Kling and Park, 1994; Larsen *et al.*, 2003; Liu and Gallander, 1982; Lonvaud-Funel *et al.*, 1988). For instance Larsen *et al.* (2003) reported that MLF was inhibited by the high SO_2–producing yeasts *S. cerevisiae* strains V1116 and UCLM S325 (75 and 50 mg/L total SO_2, respectively), but not by a low SO_2–producing strain Saint Georges S101. Delays in entering logarithmic growth or lower peak populations of *O. oeni* were also noted in wines fermented by Zymafluor VL1, CKS 102, EC1118, and BKS 104 (Figure 6), in which total SO_2 produced ranged between 15 and 33 mg/L. However, the concentration of total SO_2 did not always correspond to the extent of bacterial growth. For example, *O. oeni* grew poorer in wines fermented by strain CKS (18 mg/L total SO_2) than it did in wines fermented by strains EC1118 and Zymafluor VL1, which contained equal or greater amounts of total SO_2. Larsen *et al.* (2003) concluded that although in some cases high SO_2–producing strains cause inhibition of MLF, other yeast strains inhibit MLF by means besides production of SO_2. In agreement, additional studies have also cast doubt over whether SO_2 produced by yeast is the sole mechanism for bacterial inhibition (Caridi and Corte, 1997; Eglinton and Henschke, 1996; King and Beelman, 1986; Lemaresquier, 1987; Wibowo *et al.*, 1988). For instance, Wibowo *et al.* (1988) found that *S. cerevisiae* inhibited the growth of *O. oeni* in wine, but that inhibition was not due to

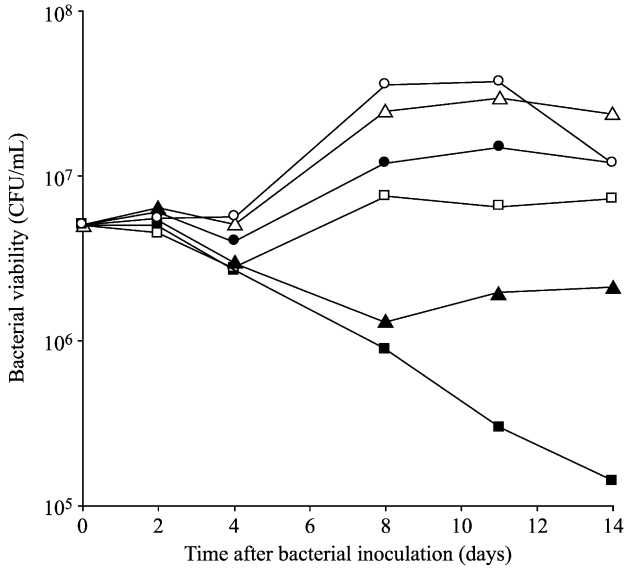

FIG. 6 Growth of *Oenococcus oeni* in Chardonnay wines prepared using yeast strains UCLM S325 (■, 50 mg/L total SO_2), Zymafluor VL1 (□, 33 mg/L total SO_2), CKS 102 (▲, 18 mg/L total SO_2), EC1118 (X[CN4], 18 mg/L total SO_2), BKS 104 (●, 15 mg/L total SO_2), or Saint Georges S101 (○, 0 mg/L total SO_2). Concentrations of total SO_2 in these wines was determined just before bacterial inoculation. (Adapted from Larsen *et al*., 2003 and with permission from the American Journal of Enology and Viticulture.)

the production of SO_2. Additionally, Eglinton and Henschke (1996) reported that production of SO_2 by S. *cerevisiae* strain AWRI 838 and related strains did not account for wines that resisted malolactic fermentation.

Wibowo *et al.* (1988) proposed that *S. cerevisiae* may inhibit *O. oeni* through the production of antibacterial proteins/peptides and not through the production of SO_2 and ethanol. In support, Dick *et al.* (1992) isolated two proteins produced by *S. cerevisiae*, which showed activity against *O. oeni.* Despite these findings, very few further studies investigating the production of antibacterial proteins/peptides by wine yeast have been undertaken.

In addition to SO_2 and antibacterial proteins/peptides, medium-chain fatty acids produced by yeast during alcoholic fermentation have also been implicated in the inhibition of malolactic bacteria (Carrete *et al*., 2002; Edwards and Beelman, 1987; Lonvaud-Funel *et al*., 1985). Inhibition of *Saccharomyces* species and some LAB by medium-chain fatty acids has been reported in grape juice and silage (Pederson *et al*., 1961; Woolford, 1975). Although this hypothesis has not been conclusively shown, Lonvaud-Funel *et al.* (1985) and

Edwards and Beelman (1987) have reported decanoic acid to be inhibitory to the growth of malolactic bacteria. Edwards and Beelman (1987) noted that decanoic acid suppressed the growth of *O. oeni* PSU-1 at a concentration of 10 mg/L, a concentration reported to be present in some wines (Houtman *et al.*, 1986). In addition, Carrete *et al.* (2002) reported that decanoic acid acted synergistically with either low pH level or ethanol to inhibit *O. oeni* ATPase, the activity of which has been linked to malolactic activity in *O. oeni* (Cox and Henick-Kling, 1995; Tourdot-Maréchal *et al.*, 1999). However, Edwards *et al.* (1990) found that MLF occurred more rapidly in wines containing 5 mg/L decanoic acid and other medium-chain fatty acids than in wines with lower levels.

IX. SUMMARY AND CONCLUSIONS

Bacteria such as *Acetobacter* and *Gluconobacter, Lactobacillus, Oenococcus*, and *Pediococcus* play important roles in determining the final quality of a wine. Several of these microorganisms can decrease wine quality. For instance, *Acetobacter* and *Gluconobacter* can cause spoilage through the production of excessive acetic acid and ethyl acetate, whereas *Lactobacillus* species may cause spoilage through increases in VA or formation of other adverse odors or flavors. *Pediococcus* species may spoil wine through the production of off-flavors such as acetoin and diacetyl and through the formation of polysaccharides. Some species of LAB can also produce biogenic amines and can contribute to ethyl carbamate formation in wine. However, not all LAB are involved in spoilage. For instance, the growth of *O. oeni* can be desirable because this species is used for MLF, a process that decreases wine acidity and contributes desirable flavors and aromas to wine.

Microbial interactions that occur in wine may be beneficial or detrimental to wine quality depending on the species involved. Examples of detrimental interactions are the inhibition of *S. cerevisiae* by *Lactobacillus* species and the inhibition of *O. oeni* by *S. cerevisiae* when MLF is desired. However, the inhibition of *O. oeni* may also be beneficial to wine quality if MLF is undesirable. Additional beneficial interactions include the stimulation of LAB growth due to yeast lysis and the inhibition of *Pediococcus* species by *O. oeni.* A better understanding of the complex interactions between LAB and *S. cerevisiae* will lead to the selection of compatible yeast and bacterial strains for the induction of alcoholic and malolactic fermentations.

In conclusion, knowledge of the bacteria involved in winemaking will allow the winemaker to minimize spoilage problems caused by AAB, lactobacilli, and pediococci and to promote or prevent MLF in a wine. Future research should include investigating the contribution of malolactic bacteria

to wine flavor, the impact of *Pediococcus* species on the sensory qualities of wine, and the mechanisms involved in the inhibition of *Saccharomyces* by *Lactobacillus* species and the inhibition of malolactic fermentation by wine yeast.

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