RESEARCH LETTER

Arginine and citrulline do not stimulate growth of two *Oenococcus oeni* strains in wine

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Abstract

Arginine metabolism by wine lactic acid bacteria (LAB) may lead to wine quality degradation. While arginine is essential for growth of the wine relevant LAB *Oenococcus oeni*, it remains unclear whether it also stimulates its growth. This study evaluated the effect of arginine and citrulline, the partially metabolized intermediate of the arginine deiminase pathway, on the growth of two commercial *O. oeni* strains in comparison with a *Lactobacillus buchneri* strain in wine and at wine pH values. Neither arginine nor citrulline increased growth of both *O. oeni* strains in comparison with the *L. buchneri* strain. However, arginine and citrulline were partially degraded in all incubations. The extent of citrulline degradation correlated with lower pH values in oenococcal cultivations but with higher pH values in those of the *L. buchneri* strain. The degradation kinetics of *O. oeni* and *L. buchneri* for malic acid and arginine differed and the latter grew in sterile filtered post-malolactic fermentation wine. This study shows that arginine and citrulline did not stimulate growth of the two *O. oeni* strains studied, and that their physiological role differed among the wine LAB considered. While arginine may play a role in wine microbiological stability, other nutrients should be investigated for their suitability to create a selective ecological advantage for *O. oeni* strains in wine.

Introduction

In the production of many red and a few white wines the involvement of lactic acid bacteria (LAB) is considered essential. In a process called malolactic fermentation (MLF), wine LAB reduce the acidity of wines by transforming malic to lactic acid and also carry out a number of other wine aroma relevant transformations (Lonvaud-Funel, 1999). Among the latter, there are transformations, which may be considered positive, such as the release of aroma compounds from precursor molecules by bacterial glycosidases (D'Incecco *et al.*, 2004; Bloem *et al.*, 2008). The desirability of others, such as the formation of diacetyl (Bartowsky & Henschke, 2004) or the reduction of acetaldehyde (Osborne *et al.*, 2006) may depend on the wine style. Clearly, the formation of large concentrations of acetic acid as well as biogenic amines (Coton *et al.*, 1999) is undesirable and can lead to unmarketable wines. Formation of biogenic amines requires decarboxylation of amino acids among which arginine stands out because it is quantitatively the most important amino acid in musts and wines of many grape varieties (Sponholz, 1991; Henschke & Jiranek, 1993). In addition to several biogenic amines (agmatine, putrescine, spermidine and spermine), arginine may also be responsible for the formation of citrulline, a direct precursor of carcinogenic ethyl carbamate (Liu & Pilone, 1998). Complete degradation of arginine by heterofermentative wine LAB occurs by the arginine deiminase (ADI) pathway (Liu *et al.*, 1996), and leads to the formation of ornithine, CO_2 and ammonia according to reactions (1)–(3). During arginine degradation, small amounts of the partially metabolized intermediate citrulline are excreted, as well (Mira de Orduña *et al.*, 2000):

$$\text{L-arginine} + H_2O \xrightarrow[\text{ADI (EC 3.5.3.6)}]{\text{arginine deiminase}} \text{L-citrulline} + NH_4^+ \qquad (1)$$

$$\text{L-citrulline} + P_i \overset{\text{ornithine transcarbamylase (OTC (EC 2.1.3.3))}}{\longleftrightarrow} \text{L-ornithine} + \text{carbamoyl-P} \quad (2)$$

$$\text{carbamoyl-P} + \text{ADP} \overset{\text{carbamate kinase (CK (EC 2.7.2.2))}}{\longleftrightarrow} \text{ATP} + CO_2 + NH_4^+ \quad (3)$$

Because of its relevance for wine quality degradation, the practical and microbiological implications of arginine metabolism by wine LAB has been studied extensively (Liu *et al.*, 1994; Mira de Orduña *et al.*, 2000, 2001; Arena & de Nadra, 2002; Tonon & Lonvaud-Funel, 2002; Arena & Manca de Nadra, 2005; Terrade & Mira de Orduña, 2006). One study, which investigated the kinetics of bacterial arginine metabolism with the aim of reducing citrulline formation, indicated that in contrast to non-oenococci, growth of *Oenococcus oeni* may not be stimulated by arginine (Mira de Orduña *et al.*, 2001). However, other studies have argued that degradation of arginine increases growth of wine LAB including lactobacilli and oenococci (Tonon & Lonvaud-Funel, 2000; Tonon *et al.*, 2001) on the basis of growth experiments and intracellular ATP measurements. The ability of *O. oeni* to link arginine degradation to growth and hence, to compete with the non-*Oenococcus* native microbiota in the multiseptic wine environment is of practical relevance because of the significance of this species for quality winemaking.

The objective of this investigation was to clarify the effect of arginine on the growth of *O. oeni* under physiological conditions, i.e. in wine combining different arginine concentrations with various pH levels. For this, two commercial *O. oeni* strains were compared with a non-*Oenococcus* isolated from wine. Citrulline was also considered because the excretion of this partially metabolized intermediate by arginine-degrading LAB (Mira de Orduña *et al.*, 2000) could provide a potentially energetic substrate to other LAB. Finally, the effect of arginine on the microbial stability of finished wines was studied by reinoculating sterile filtered post-MLF wines with LAB.

Materials and methods

Microorganisms

The LAB used were from the Wine Culture Collection at the Department of Food Science & Technology, Cornell University, USA. *Lactobacillus buchneri* CUC-3 was originally isolated from a wine undergoing spontaneous MLF. The *O. oeni* strains were pure isolates taken from the commercial cultures EQ54 (Lallemand Inc., Canada) and VFO (Chr. Hansen, Denmark).

Media and cultivations

Base wine was produced from a pure white *Vitis vinifera* juice without preservatives (Grapetise, Pacific Beverages, Australia) with a total acidity of 7.5 g L^{-1} as tartaric acid. It was adjusted with sucrose to a total soluble content of 16 °Bx (1.0652 specific gravity) at 20 °C, and was fermented at 18 °C after inoculation with 2% (v/v) of *Saccharomyces cerevisiae* var. *bayanus* strain Première Cuvée (PC), pregrown in the same must with 5 g L^{-1} yeast extract added. After completion of alcoholic fermentation, wines were racked-off the yeast sediment and allowed to further sediment at 4 °C overnight before filtration through cellulose pads (Pall SeitzSchenk EKS, Scott Laboratories, Pickering, Canada). The resulting wine had 8.9% (v/v) ethanol and no free SO_2 was detected. Glucose, fructose and malic acid concentrations were 20 mg L^{-1}, 900 mg L^{-1} and 1.2 g L^{-1}, respectively. Ammonia, urea and arginine were present only in trace amounts, i.e. < 1 mg L^{-1}. The pH after degassing was 3.16. The base wine was adjusted to 3 g L^{-1} malic acid and 1.0 g L^{-1} glucose.

For the study of the effect of arginine on growth, 450-mL batches of the base wine were adjusted to give 0, 300, 600 and 900 mg L^{-1} of arginine. The batches were subsequently subdivided into 150-mL batches, which were adjusted to pH 3.1, 3.3 and 3.5 for each arginine concentration with 5 M HCl or 5 M NaOH. After sterile filtration (0.22-μm nylon filters, Millipore), 10-mL aliquots were added to sterile screw-cap spectrophotometer glass tubes. Samples were taken at the time of inoculation and during the death phase of the cultures to determine whether arginine degradation had taken place. For the study of the effect of citrulline on growth, wines were prepared as described above, but arginine was replaced by citrulline and the wines were adjusted to pH 3.3, 3.5 and 3.7. Samples were taken at the time of inoculation and during the death phase of the cultures for citrulline quantification.

The LAB strains were pregrown in MRS medium (de Man *et al.*, 1960) containing 52 g MRS broth (Oxoid, Hampshire, UK) L^{-1} of water at pH 4.5. The final inocula were prepared by separating the cells from the pregrowth medium by centrifugation (5000 ***g***, 10 min) to avoid carryover of media constituents and metabolic products, and were then washed twice with sterile water before inoculation to give an initial $OD_{600\,nm}$ of 0.05.

For the twofold incubations in wine (Figs 3 and 4), the same wine adjusted to 3 g L^{-1} of malic acid, 1 g L^{-1} of arginine and pH 3.5 with 5 M NaOH was used. Incubations were carried in 500-mL glass flasks and the LAB were prepared as described above. Wines were incubated with either *L. buchneri* CUC-3 or *O. oeni* EQ54, and, after reaching stationary phase, the microorganisms were removed by sterile filtration followed by incubation with the other strain.

Bacterial growth in all experiments was measured by $OD_{600\,nm}$ and subtracting the values of a noninoculated control. All cultivations were done in duplicate at 20 °C.

Analysis

Qualitative arginine degradation was carried out colorimetrically by the Staron–Allard method as described by Micklus & Stein (1973). Quantitative arginine and citrulline analysis was carried out on a Shimadzu Class VP system by RP-HPLC after precolumn derivatization with *o*-phthaldialdehyde/3-mercaptopropionic acid according to the method of Bartók *et al.* (1994). Chromatographic separations were performed with a 100 mm × 4.6 mm ID column (Supelco) filled with 3 µm Hypersil ODS (Shandon, Cheshire, UK). A cartridge type zero-dead-volume guard column (Phenomenex Securityguard with two 4 mm × 3 mm ID ODS cartridges) was attached directly to the analytical column, preceded by a 0.5-µm in-line filter (Upchurch, Oak Harbour, WA). Glucose, fructose and ethanol were analyzed with enzymatic test kits (Roche, now r-biopharm, Germany). Total acidity and free SO_2 were measured by acid–base titration with standardized 0.1 M NaOH and by the method of Ripper as described by Amerine & Ough (1974), respectively. Ammonia and urea were analyzed enzymatically according to Mira de Orduña (2001). Malic acid was analyzed with formic acid as internal standard by ion exchange HPLC (Dionex Summit HPLC system, Sunnyvale). Samples were filtered through 0.2-µm nylon filters (Millipore) and 10 µL were directly injected. Separations took place on a 250 mm × 4.6 mm ID Supelcogel H column preceded by a 50 mm × 4.6 mm ID Supelguard C610H (Supelco) precolumn with the same filling and a 0.5-µm in-line filter (Upchurch) under isocratic conditions with a 0.1% H_3PO_4 mobile phase (HPLC grade, Sigma) at a 0.2 mL min^{-1} flow rate. UV detection of malic acid occurred at 210 nm.

Multiple statistical comparisons were carried out with SPSS v14 (SPSS Inc., Chicago, IL) using one-way analysis of variance with Tukey's honestly significant difference test at a confidence level of $P = 0.05$.

Results

Figure 1 shows specific maximum growth yield (Y_{max}) values obtained in wine cultivations with selected LAB where different initial pH values and arginine concentrations were combined. In this study, the pH had a marked effect on the growth of all LAB, and *L. buchneri* CUC-3 was growth inhibited ($Y_{max} < 20\%$ of maximum value reached) at pH 3.3 and 3.1 (Fig. 1c). Increasing arginine concentrations did not lead to higher Y_{max} values for *O. oeni* EQ54 and VFO (Fig. 1a and b). Instead, statistically significant growth inhibition of *O. oeni* EQ54 was observed at pH 3.5 and arginine concentrations of 600 and 900 mg L^{-1} compared with lower arginine concentrations (Fig. 1a). In contrast, arginine clearly stimulated growth of *L. buchneri* CUC-3 at pH 3.5 in a statistically significant manner (Fig. 1c). A qualitative arginine determination in culture

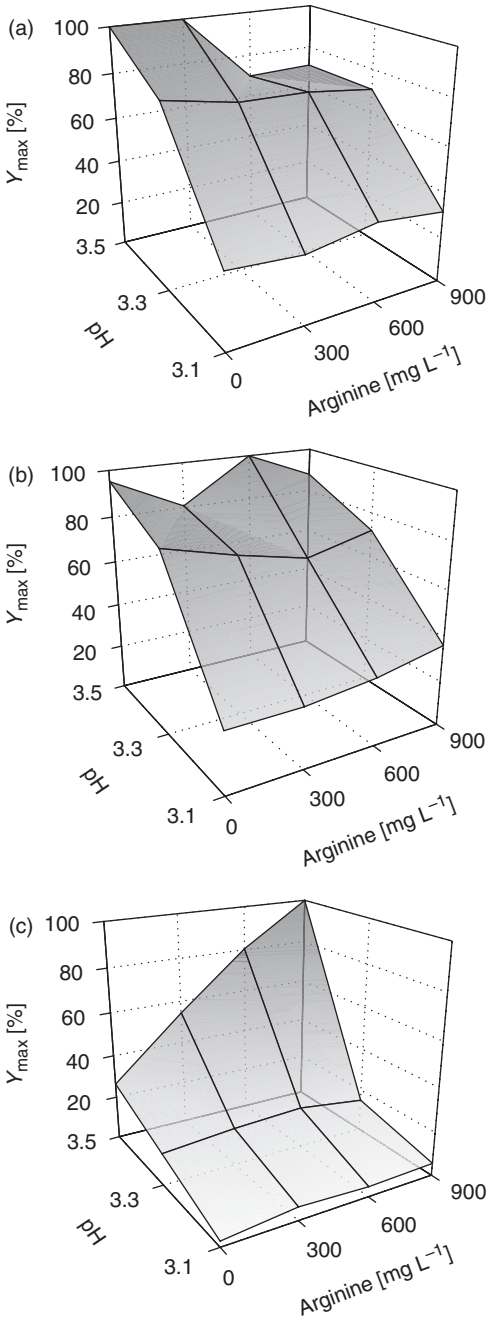

Fig. 1. Effect of initial arginine concentrations on maximum growth yields (Y_{max}) of selected LAB in wine at different pH values. (a) *Oenococcus oeni* EQ54; (b) *O. oeni* VFO; (c) *Lactobacillus buchneri* CUC-3. Values are displayed in percent of the highest Y_{max} value reached for each strain. 100% growth corresponding to 62 mg L^{-1} dry weight for strain EQ54, 56 mg L^{-1} for VFO and 144 mg L^{-1} for CUC-3. Data points represent averages from duplicate incubations.

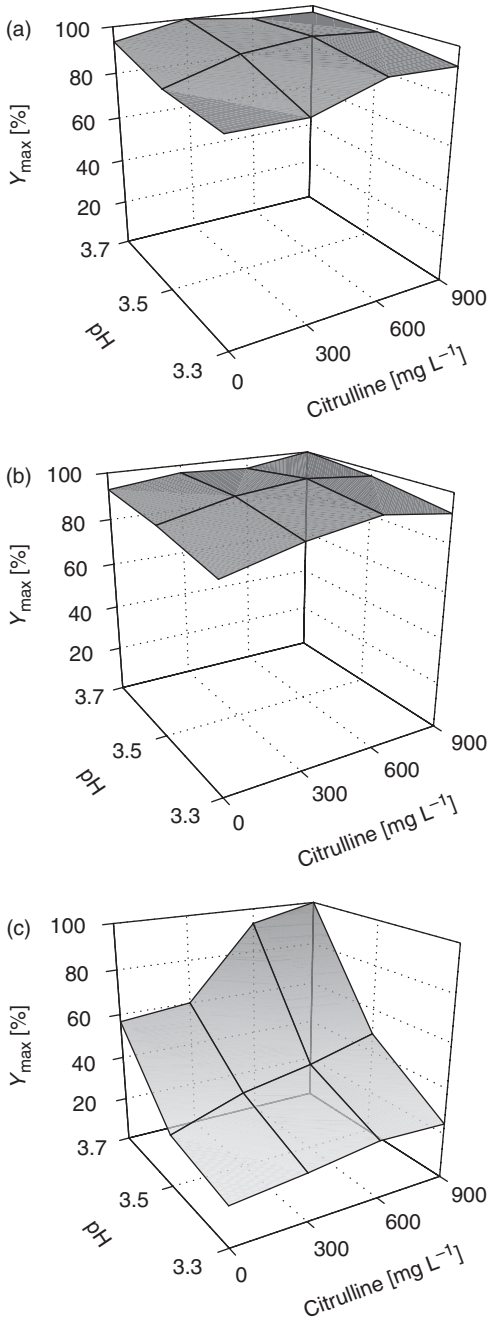

Fig. 2. Effect of initial citrulline concentrations on maximum growth yields (Y_{max}) of selected LAB in wine at different pH values. (a) *Oenococcus oeni* EQ54; (b) *O. oeni* VFO; (c) *Lactobacillus buchneri* CUC-3. Values are displayed in percent of the highest Y_{max} value reached for each strain. 100% growth corresponding to 151 mg L^{-1} of dry weight for strain EQ54, 158 mg L^{-1} for VFO and 68 mg L^{-1} for CUC-3. Data points represent averages from duplicate incubations.

supernatants of stationary phase cultures revealed that arginine degradation occurred in all treatments.

Results from the study of the effect of citrulline on bacterial growth are shown in Fig. 2. A statistically significant stimulatory effect of citrulline on growth of *L. buchneri* CUC-3 was found at pH 3.5 and 3.7 (Fig. 2c) although this stimulation was less marked than for arginine. Except for a slight growth increase for the comparison of Y_{max} values obtained for strain VFO at 0 and 600 mg L^{-1} of citrulline at pH 3.3, no stimulation of growth by citrulline could be demonstrated for the two *O. oeni* strains (Fig. 2b).

Quantitative determination of residual citrulline concentrations in supernatants of stationary phase cultures and comparison with the initial citrulline levels showed that some citrulline had been degraded in all treatments (Table 1). In all cultures, higher initial citrulline concentrations resulted in higher total citrulline degradation. However, differences existed with regard to the effect of the pH value on citrulline degradation. The extent of citrulline degradation was positively correlated with Y_{max} values in *L. buchneri* CUC-3. Accordingly, more citrulline was degraded at higher pH values (Table 1 and Fig. 2c). Both *O. oeni* cultures displayed an opposite behaviour. Namely, more citrulline was degraded at lower pH values (Table 1 and Fig. 2a and b).

Figures 3 and 4 show data from the study of microbial stability in wines that had supported growth by wine LAB. Inoculation of *O. oeni* EQ54 into wine after alcoholic fermentation led to complete malic acid degradation followed by arginine degradation in late exponential/early stationary phase, which was interrupted by sterile filtration (Fig. 3). After reinoculation with *L. buchneri* CUC-3, growth occurred in the absence of malic acid and arginine was completely degraded (Fig. 3).

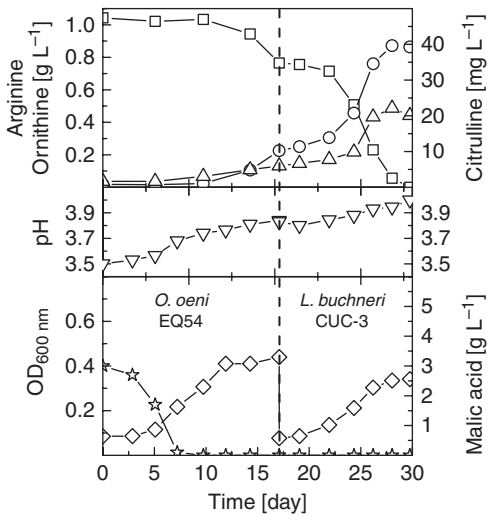

Fig. 3. Growth and metabolic activity of *Lactobacillus buchneri* CUC-3 in sterile filtered wine that previously supported growth of *Oenococcus oeni* EQ54. Wine was sterile filtered and re-inoculated with strain CUC-3 at the time indicated by the dashed line. □, arginine; ○, ornithine; △, citrulline; ▽, pH; ◇, OD; ☆, malic acid. Data points represent averages from duplicate incubations.

Inoculation of *L. buchneri* CUC-3 into post-AF wine also led to a biphasic degradation pattern with arginine being degraded first to completion followed by malic acid degradation, which was interrupted by sterile filtration. After reinoculation, growth of *O. oeni* EQ54 was supported in the absence of arginine, and malic acid was depleted. In all incubations, arginine and malic acid degradation was accompanied by a pH increase.

Discussion

In wines, arginine is a prevalent amino acid, whose microbial degradation may lead to wine quality depreciation. It is also important from a microbial physiology point of view.

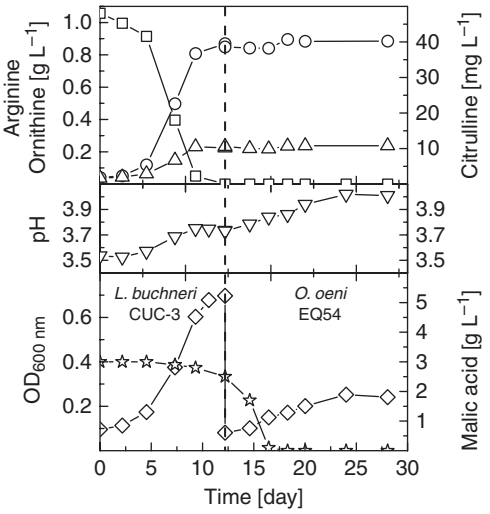

Fig. 4. Growth and metabolic activity of *Oenococcus oeni* EQ54 in sterile filtered wine that previously supported growth of *Lactobacillus buchneri* CUC-3. Wine was sterile filtered and re-inoculated with strain EQ54 at the time indicated by the dashed line. □, arginine; ○, ornithine; △, citrulline; ▽, pH; ◇, OD; ☆, malic acid. Data points represent averages from duplicate incubations.

Its degradation by wine LAB in the ADI pathway allows for ATP formation by substrate level phosphorylation (Liu & Pilone, 1998) and does not produce reduced dinucleotides or potentially growth inhibiting organic acids.

Oenococcus oeni is generally preferred for carrying out MLF and its ability to use energy sources thus may affect its ability to prevail in wines. Arginine is essential for growth of wine LAB (Garvie, 1967; Fourcassie *et al.*, 1992; Remize *et al.*, 2006) but its ability to serve as energy source in *O. oeni* has been disputed. Tonon & Lonvaud-Funel (2000) showed that ATP was produced after arginine addition to resting cells of *O. oeni* in a buffer. However, the ability of growing cells of *O. oeni* and other wine LAB to generate energy from arginine degradation in wine and at wine pH values may depend on other factors, including the growth phase association of arginine degradation, and the possible presence of futile cycles leading to energy dissipation as described for other LAB (Cook & Russell, 1994).

In this work, increasing arginine and citrulline concentrations stimulated growth of *L. buchneri* CUC-3 at pH values where significant growth occurred but this was not found for two *O. oeni* strains. A previous study (Tonon & Lonvaud-Funel, 2000) had suggested that arginine increased the growth of wine LAB including *O. oeni*, and a molar growth yield for arginine (Y_{arg}) was presented. However, this study had been carried out at high and nonphysiological pH values (4.5 and above), and the contributions of nonglucose carbon sources present in the complex laboratory medium applied were not considered (Tonon & Lonvaud-Funel, 2000). In contrast, the current study demonstrates that two commercial strains of *O. oeni* were not able to increase growth from arginine or citrulline degradation in wine and across wine pH values. These results are noteworthy because arginine and citrulline had been degraded in all cultivations. An analysis of the citrulline degradation during incubations revealed differences between the two *O. oeni* strains and the *L. buchneri* strain. Higher citrulline degradation was

Table 1. Total citrulline degradation over the course of cultivations of LAB in wine adjusted to different initial pH values and citrulline concentrations

		Citrulline degradation (mg L^{-1}) at initial citrulline concentration (g L^{-1}) of:		
Strain	pH	0.3	0.6	0.9
CUC-3	3.3	4.8 ± 0.8 a	46.8 ± 0.7 a	100.4 ± 1.2 a
	3.5	19.3 ± 1.2 b	85.3 ± 0.7 b	134.7 ± 0.8 b
	3.7	20.7 ± 0.6 b	167.1 ± 1.9 c	362.4 ± 3.6 c
VFO	3.3	38.2 ± 0.9 a	150.1 ± 1.9 a	269.7 ± 2.7 a
	3.5	32.2 ± 1.1 b	123.4 ± 0.8 b	220.2 ± 0.8 b
	3.7	15.4 ± 0.4 c	79.6 ± 1.5 c	207.8 ± 1.8 c
EQ54	3.3	43.2 ± 1.3 a	160.4 ± 0.2 a	303.4 ± 0.7 a
	3.5	34.8 ± 1.1 b	125.0 ± 1.0 b	247.9 ± 1.9 b
	3.7	26.3 ± 0.8 c	164.2 ± 0.9 a	238.8 ± 0.9 c

Different letters within columns indicate statistically significant differences among the averages of duplicate incubations of the respective strains at one initial citrulline concentration.

correlated with growth in *L. buchneri* CUC-3, and thus with high pH values, but it was correlated with low pH values in the case of both *O. oeni* suggesting that the physiological role of the ADI pathway in this species may lie exclusively in enhanced adaptation of cells to acidic environments rather than energy conservation. This would agree with the observation that viable but nonculturable cells of *O. oeni* were able to recover the ability of cell division upon addition of arginine (Tonon *et al.*, 2001).

It is widely accepted that MLF by wine LAB leads to increased microbial stability (Margalit, 1990; Henick-Kling, 1993; Boulton *et al.*, 1996; Lonvaud-Funel, 1999; Volschenk *et al.*, 2006). This effect is ascribed to the consumption of nutrients, specifically malic acid, which has been shown to stimulate growth of *O. oeni* (Henick-Kling, 1993; Cox & Henick-Kling, 1995). Yet, MLF increases wine pH, which reduces microbial stability, and energetic substrates other than malic acid may remain to support potential microbial contaminants. Hence, this work also evaluated the role of arginine in simulated contaminations of sterile filtered post-MLF wines.

The results show that even after successful MLF by *O. oeni* EQ54 with complete malic acid degradation, the wine was still able to support growth of *L. buchneri* CUC-3 that led to complete arginine degradation and a further pH increase. This illustrates the potential of post-MLF spoilage with wine LAB having substrate preferences that differ from those of *O. oeni*. The biphasic degradation pattern of *O. oeni* EQ54 with regard to malic acid and arginine also provides a possible reason for the failure of arginine to increase growth of *O. oeni*. Specifically, arginine degradation by *O. oeni* EQ54 only occurred in late exponential phase where an effect on growth is limited. In contrast *L. buchneri* CUC-3 also showed a biphasic degradation pattern, but malic acid was only metabolized after degradation of arginine, which was growth associated.

These results clearly demonstrate that energetic substrates other than sugars and malic acid, namely arginine, have to be considered for the evaluation of microbiological stability of post-MLF wines. This aspect may increase in relevance because of the overall attempts in the wine industry to reduce the concentration of the wine preservative SO_2, the effect of climate change on increasing must and wine pH values (Coombe, 1987; Jones *et al.*, 2005), and the widespread use of yeast and bacterial nutrients in modern winemaking, which may lead to higher residues of energetic nutrients.

It has been suggested to carry out MLF with pure *O. oeni* strains and to inactivate the biomass after malic acid depletion in order to prevent other metabolic transformations that could lead to wine quality degradation (Terrade & Mira de Orduña, 2006). However, this technique may only be beneficial if, at the same time, the activity of other wine LAB with different substrate utilization pattern, such as *L. buchneri* CUC-3, is prevented effectively over time.

Based on the degradation kinetics of arginine, as well as the growth studies presented here, arginine or citrulline cannot be considered as stimulating for the growth of *O. oeni*. Other nutrients should be investigated for their suitability to create a selective ecological advantage for this species in wine.

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