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ORIGINAL ARTICLE

# Influence of ethanol, malate and arginine on histamine production of *Lactobacillus hilgardii* isolated from an Italian red wine

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**Abstract** Wine, like other fermented foods, may contain biogenic amines produced by lactic acid bacteria via amino acids decarboxylation. The most relevant amines from the toxicological standpoint are histamine and tyramine. The complexity of fermented substrates makes it difficult to suggest a priori how variables can modulate amine production. *Lactobacillus hilgardii* ISE 5211 was isolated from an Italian red wine. Besides producing lactate from malate, this strain is also able to convert arginine to ornithine and histidine to histamine. In the present investigation we studied the influence of malate, arginine and ethanol on histamine accumulation by *L. hilgardii* ISE 5211. Ethanol concentrations above 13% inhibit both histamine accumulation and bacterial growth; concentrations below 9% affect neither growth nor histamine production. However, an ethanol concentration of 11% allows a low but continuous accumulation of histamine to occur. Arginine also delays histamine accumulation, while malate appears to have no effect on histidine–histamine conversion.

**Keywords** HDC · Ethanol · MLF · ADI pathway · Histamine · Malolactic enzyme

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## Introduction

Biogenic amines (BAs) including histamine, putrescine, cadaverine and tyramine can be found in spoiled foodstuffs (fish) or in fermented foods (cheeses, sausages, cocoa and chocolate, alcoholic beverages) (Bodmer et al. 1999; Bover-Cid et al. 2001); they are produced by the microbial decarboxylation of the respective precursor amino acids (Ten Brink et al. 1990). The total content of BAs in food varies from 10–30 (alcoholic beverages, chocolate, soy products) to 200–1,500 mg/kg (sausages, cheeses) and is also related to food hygiene; high levels of BAs are usually considered an index of poor manufacturing practices (Vidal-Carou et al. 1990a, b, 1991).

Biogenic amines, possessing biological activity on the nervous and vascular systems, can cause toxic effects in humans, depending on the specific amine, its concentration and on individual sensitivity to it (Shalaby 1996; Silla Santos 1996). Among BAs, the most toxic for human health are histamine and tyramine (Moreno-Arribas 1999, 2000, 2003). Their effects on the nervous and vascular systems can generate disorders or crises in sensitive subjects (nausea, headache, palpitations, hypo- or hyper-tension, allergies, enteric histaminosis, hypertensive crises, anaphylactic shock and, sometimes, death). The amount of BAs causing crises varies with individual sensitivity; it is lower in patients treated with antidepressant monoaminooxidase inhibitor drugs (IMAO), which interact with aminooxidase enzymatic systems (MAO, DAO), since these patients have an impaired ability to oxidize amines.

Several BAs, such as histamine, putrescine, cadaverine, tyramine, and phenylethylamine, have also been found in wine in concentrations ranging from a few mg/L to tens of mg/L, (Taylor 1986; Romero et al. 2002; Sufleros et al. 1998; Anli et al. 2004; Leitao et al. 2005), most frequently in red

wines (Gloria et al. 1998); this raises some health concerns for wine consumers. The toxic effects of BAs in wine consumers may be enhanced by the presence of ethanol, which is known to be one of the most effective inhibitors of amine oxidases, the enzymes that inactivate BAs (Maynard and Schenker 1962; Sessa et al. 1984). Furthermore, volatile amines may influence the organoleptic properties of wine (Torrea and Ancìn 2002). The recommended maximum limits for histamine in wine vary from 2 to 10 mg/L in the different EU countries (Busto et al. 1996; Lehtonen 1996).

Several researches support the view that BAs are mainly formed in winemaking during the malolactic fermentation, by the action of lactic acid bacteria (LAB) causing decarboxylation of free amino acids (Vidal-Carou et al. 1990; Soufleros et al. 1998; Coton et al. 1999). This secondary fermentation, generally occurring after alcoholic fermentation, is widely appreciated for its improvement of the flavor characteristics of wine, and is usually carried out by the predominant species *Oenococcus oeni* (Lonvaud-Funel and Joyeux 1994; Guerrini et al. 2002; Gardini et al. 2005; Mangani et al. 2005) or, more occasionally, by other LAB species belonging to the genera *Lactobacillus* and *Pediococcus*, which may develop during the winemaking process (Lonvaud-Funel 1999; Arena and Manca de Nadra 2001).

In general, wine quality is closely related to the diversity and composition of the microbial species and/or strains that develop during the overall fermentative process (Romano et al. 2003a, b). The growth of each wine yeast species is characterized by a specific metabolic activity, which may contribute to the aroma and flavor characteristics of wine but that can also supply precursors for undesired amine biosynthesis by malolactic or contaminant bacteria (Lambrechts and Pretorius 2000; Brandolini et al. 2002).

The aim of the present investigation was to study the conditions that enhance or repress amine biosynthesis for the selected *Lactobacillus hilgardii* ISE 5211 strain isolated from an Italian red wine. Three parameters were considered, in view of their importance in the overall composition of wine: malate, arginine and ethanol, in order to clarify both antagonisms and synergies among energy metabolic pathways and to suggest oenological technologies to control the amount of BAs, lowering them to non-toxic levels without affecting the subtle parameters that contributes to wine quality.

## Materials and methods

### Bacterial strain

*Lactobacillus hilgardii* ISE 5211, isolated from an amine-contaminated Italian red wine and belonging to the collection of Istituto Sperimentale per l'Enologia (Asti, Italy) was kindly supplied by E. Garcia Moruno. This strain produces histamine in the presence of the precursor amino acid histidine; it also converts arginine to ornithine through the ADI pathway and ferments malate to lactate. It was grown in MRS medium (Lactobacilli MRS broth, Difco) at pH 5.5 and stored at −24°C in 0.5 mL aliquots with 0.5 mL of 40% glycerol.

### Culture conditions

All cultures (200 mL) were grown in monitored pH mode in closed 250 mL screw cap bottles, at 30°C, without shaking. Bacterial growth was monitored by O.D. measurement at 600 nm.

#### *Histamine production*

*Lactobacillus hilgardii* ISE 5211 was grown in control conditions (MRS medium pH 5.5), and in the same medium supplemented with histidine (4 g/L) to stimulate histamine production (MRS + His).

The influence of three different compounds (ethanol, malate and arginine) on histamine production was tested using MRS + His medium supplemented with:

(a) different ethanol concentrations (9, 11 and 13%);
(b) different malate concentrations (0.5, 1, 3, 5 g/L);
(c) arginine (4 g/L).

MRS + His medium was used as control.

#### *Malolactic fermentation*

The influence of histidine on malolactic fermentation was evaluated using MRS + malate (3 g/L) medium supplemented with histidine (4 g/L). The medium MRS + malate (3 g/L) was used as control.

#### *ADI pathway*

The influence of histidine on the ADI pathway was evaluated using MRS + arginine (4 g/L) medium supplemented with histidine (4 g/L). The medium MRS + arginine (4 g/L) was used as control.

### Metabolite quantification

An aliquot (1 mL) of the growth culture was harvested and centrifuged (10,000×g, 4°C, 5 min) and the supernatant (culture broth) was stored at −24°C until analyses.

Lactate and malate concentrations were determined using the K-LATE and K-LMALR enzymatic assay kits (Megazyme) respectively.

## HPLC analyses

All chemical reagents (reagent grade) were from Fluka Chemie AG. Analyses were carried out on a Shimadzu Class VP HPLC system, equipped with a temperature controller (Column Oven CTO-10AS) and a UV-VIS detector SPD-10A.

Histamine and histidine were determined without derivatization using an already-optimized ion-pair HPLC method (Coïsson et al. 2004), with a C18 reverse phase Spherisorb S5 ODS2 column (250 mm × 4.6 mm I.D.), particle size 5 μm, with a guard-column (10 mm × 4.6 mm I.D.) packed with the same phase. Pump A: Eluant 1 prepared by dissolving heptanesulphonate (8.3 mM) and $KH_2PO_4$ (9.0 mM) in ultra-pure water and adjusting the pH to 3.5 with phosphoric acid. 20 μL/L of octylamine were added as second ion-pair reagent. The solution was freshly prepared on alternate days, filtered (0.45 μm, Millipore type HA) and degassed before use. Pump B: Methyl alcohol HPLC-grade. Gradient: 100% pump A for 1 min; pump B from 0 to 26% in 5.25 min; pump B from 26 to 35% in 9 min; pump B from 35 to 42% in 1.5 min; pump B at 42% for 24 min; pump A at 100% for 9.40 min. Flow rate: 1.0 mL/min, UV-detection: 215 nm. The column was kept at 27°C during analyses. Medium samples were directly injected after 0.22 μm filtration (Millipore, HA type), volume injected: 10 μL.

Arginine and ornithine were determined after derivatization with diethylethoxymethylenemalonate (DEEMM) (Gómez-Alonso et al. 2007).

Derivatives were obtained by reaction of 1.75 mL of 1 M borate buffer (pH 9), 0.75 mL of methanol, 20 μL of medium, 0.980 mL of water, and 30 μL of DEEMM in an ultrasonic bath. The sample was then heated at 70°C for 2 h to allow degradation of excess DEEMM and reagent byproducts.

The HPLC analyses were performed using a C18 reverse phase ODS2 (Waters) column (150 mm × 4.6 mm I.D., particle size 3 μm, with a guard-column (10 mm × 4.6 mm I.D.) packed with the same phase. Pump A: 25 mM acetate buffer pH 5.8 with 0.02% sodium azide; the solution was freshly prepared on alternate days, filtered (0.45 μm, Millipore type HA) and degassed before use. Pump B: methyl alcohol and acetonitrile HPLC-grade mixture (20:80). Gradient: 10% B for 20 min; pump B from 10 to 17% in 10 min; pump B at 17% for 3 min; pump B from 17 to 40% in 21.5 min; pump B from 40 to 72% in 8 min; pump B from 72 to 82% in 5 min, pump B from 82 to 100% in 4 min; pump B at 100% B for 3 min; pump B at 10% for 10 min. Flow rate: 0.9 mL/min, UV-detection: 285 nm. The column was kept at 20°C during analyses. Sample volume injected: 10 μL.

## Preparation of protein extracts

Equivalent amounts of cells (40 g dry weight for each experiment) were treated in each protein preparation. Cells were harvested in the exponential phase, when amine accumulation and malate consumption were at maximum levels. The biomass was collected by centrifugation (3,000×*g*, 20 min, 4°C) in an ALC multispeed refrigerated centrifuge PK131R (T533 rotor) and washed twice in 40 mL 0.9% NaCl. The pellet was resuspended in 3 ml 50 mM Tris–HCl pH 7.3 containing 10 μL/mL of Nuclease mix (GE Healthcare) and sonicated in a Microsonix Sonicator Ultrasonic Liquid Processor XL2020 for a total of 30 min at 20 KHz with intervals of 20 s, keeping the cells on ice. To separate unbroken cells, the sonicated solution was centrifuged at 3,000×*g* for 20 min at 4°C in an ALC multispeed refrigerated centrifuge PK131R (T533 rotor) and subsequently the supernatant was centrifuged at 180,000×g for 1 h at 4°C in a Beckman L8–60M Ultracentrifuge (Type 60 rotor) and the supernatant was collected and dialyzed against 3 volumes of bidistilled water before determination of malolactic activity or before being analyzed by electrophoresis (SDS-PAGE). Protein concentration was evaluated by 2D-Quant kit (GE Healthcare).

## Determination of malolactic activity

The MLE activities of the cell extracts were determined in vitro by measuring the amount of L-lactate produced from the decarboxylation of L-malate at 37°C in phosphate buffer containing 0.1 mM $MnCl_2$, 0.5 mM $NAD^+$, and 37 mM L-malate (pH 5).

L-Lactate and L-malate contents were determined with the enzymatic kit K-LATE (Megazyme) and K-LMALR (Megazyme).

## SDS-PAGE

The samples were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed using 9% T acrylamide homogeneous running gels (8 cm W × 7.3 cm H) (PlusOne Acrylamide solution, GE Healthcare) and a

mini-PROTEAN 3 system (BIO-RAD). The running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 and running conditions were 200 V constant voltage, for about 45 min. *M*r markers were those of the Low *M*r Electrophoresis Calibration Kit (GE Healthcare). Gels were stained with Coomassie brilliant blue R-250 (Sigma).

### Protein identification

Protein identification was achieved through the "peptide mass fingerprinting" (PMF) strategy (Pappin et al. 1993). For MALDI-TOF analysis, the band of interest was cut from the gel and destained overnight with a solution of 25 mM ammonium bicarbonate and 50% ACN. The protein was digested in-gel with trypsin (Promega) as described by Hellmann et al. (1995). 0.5 mL of the peptide mixture were applied to a target disk and allowed to air-dry. Subsequently, 0.5 mL of matrix solution (1% w/v CHCA in 30% ACN, 0.1% TFA) were applied to the dried sample and again allowed to dry. A spectrum was obtained using a Bruker ReflexIII MALDI-TOF spectrometer. To interpret the MS spectrum of the protein digest, Mascot (http://194.42.244.117/Search_form_select.html) and MS-Fit (http://falcon.ludwig.ucl.ac.uk/MSFit.html) software packages were used.

## Results

### Influence of histidine, arginine and malate on *L. hilgardii* ISE 5211 growth curves

*L. hilgardii* ISE 5211 was grown in MRS medium pH 5.5 (MRS) and in the same medium fortified with histidine (4 g/L) (MRS + His), arginine (4 g/L) (MRS + Arg) or malate (3 g/L) (MRS + Mal).

The MRS, MRS + His and MRS + Mal cultures showed similar growth parameters: final biomass was the same, while the growth rate was slightly higher in the culture fortified with histidine and malate. On the contrary, the addition of arginine determined an increase of both growth rate and final biomass (Fig. 1)

### Histamine production of *L. hilgardii* ISE 5211 grown in MRS and MRS plus histidine medium

Histamine production of *L. hilgardii* ISE 5211 was evaluated in MRS and MRS plus histidine media (Fig. 2).

Histamine accumulation was almost undetectable when the bacteria were grown without a high concentration of the precursor amino acid (MRS). On the contrary, in the culture fortified with histidine (MRS + His), histamine accumulation began in the early exponential phase and histidine was completely converted to histamine. MRS + Arg and MRS + Mal cultures showed the same pattern as MRS alone (data not show).

### Influence of arginine on histamine production

*L. hilgardii* ISE 5211 was grown in MRS medium fortified with histidine (4 g/L) (MRS + His) to induce HDC expression and activity, and in the same medium supplemented with arginine (4 g/L) (MRS + His + Arg) in order to detect expected modulation exerted by arginine over histamine accumulation. In both cultures, histidine was completely converted to histamine, although the kinetics of histamine accumulation appeared to be slightly slowed by

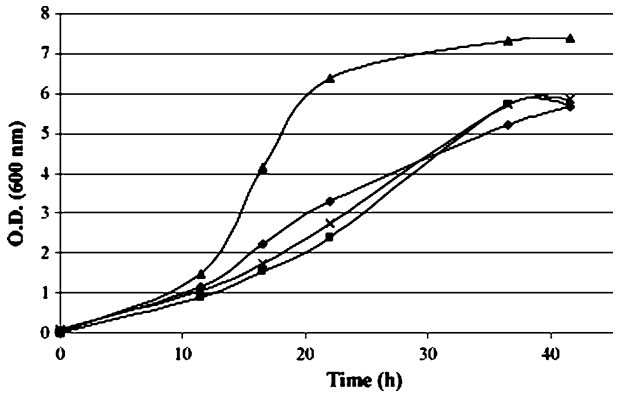


**Fig. 1** Effect of three metabolites on growth: growth curves in MRS medium (*filled square*), MRS + His (*filled diamond*), MRS + Mal (×), MRS + Arg (*filled triangle*)

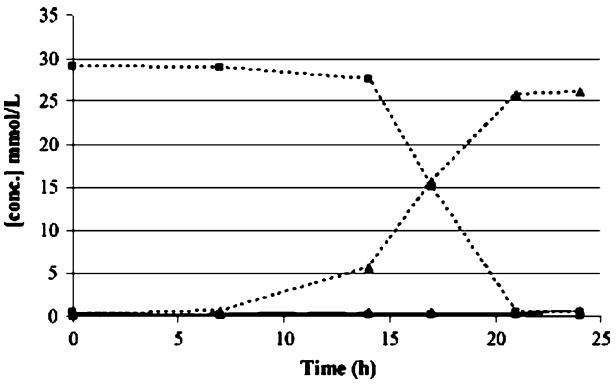


**Fig. 2** Effect of histidine supplementation on histamine production: histidine consumption (*filled square*) and histamine accumulation (*filled triangle*) in MRS + His (*hatched line*) medium. The *continuous lines* refer to not supplemented MRS

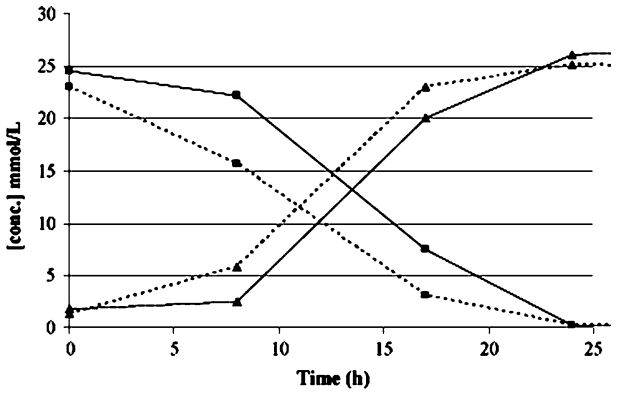


**Fig. 3** Effect of arginine supplementation on histamine production: histidine consumption (*filled square*) and histamine accumulation (*filled triangle*) during bacterial growth in MRS + His + Arg (*continuous line*) and in control medium (MRS + His) (*hatched line*)

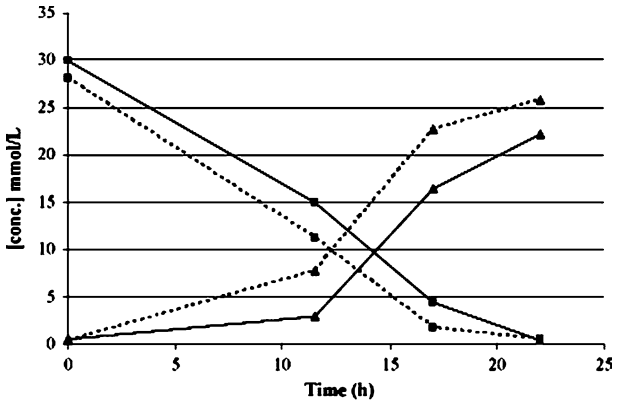


**Fig. 5** Effect of histidine supplementation on ADI pathway: arginine consumption (*filled square*) and ornithine accumulation (*filled triangle*) during bacterial growth in MRS + Arg medium (*hatched line*) and in MRS + Arg + His (*continuous line*)

the presence of arginine (Fig. 3). In order to determine whether arginine can in some way modulate HDC expression, crude extracts were analyzed by SDS-PAGE and HDC identification obtained by the PMF method (Fig. 4, inset).

The more intense band (just below 30 kDa) in (MRS + His) crude extract (lane 2) was identified by PMF as "pyruvoyl-dependent histidine decarboxylase" (HDC) from *L. hilgardii* (TrEmbl entry: Q5DLT9). The identification by PMF gave a sequence coverage of 37.3%, accounting for the most prominent signals in the MALDI-TOF spectrum (Fig. 4). When arginine was present (inset, lane 3) we observed a decreased intensity of the HDC band (similar to the control condition: lane 1) indicating that arginine represses the biosynthesis of this enzyme. This observation is therefore in accordance with the lower rate of histamine accumulation measured in this condition.

## Influence of histidine on ornithine production

*L. hilgardii* ISE 5211 was grown in MRS fortified with arginine (MRS + Arg) and in the same medium supplemented with histidine (MRS + Arg + His), in order to detect any interference of histidine in ornithine production by deimination. Histidine appeared to slow ornithine accumulation kinetics as well as arginine consumption (Fig. 5).

## Influence of malate on histamine production

*L. hilgardii* ISE 5211 was grown in MRS fortified with histidine (MRS + his) and in the same medium supplemented with malate (MRS + His + Mal), in order to evaluate expected interference between the malate and amino acid decarboxylative pathways.

No influence was detected in either the final concentration or the pattern of histamine production in the

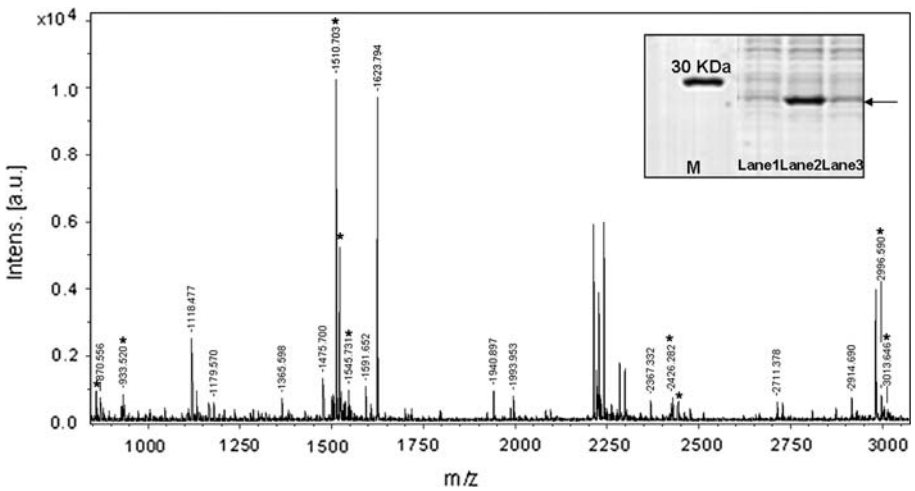


**Fig. 4** Effect of arginine supplementation on HDC biosynthesis: MALDI-TOF spectrum of the band just below 30 kDa (indicated in inset by *arrow*). *Asterisks* indicate peaks assigned by PMF method to HDC from *L. hilgardii*. Inset: SDS-PAGE of cell extracts from *L. hilgardii* ISE 5211 grown in MRS medium (lane 1), in excess of histidine (lane 2) and in excess of histidine plus arginine (lane 3). *M* molecular markers

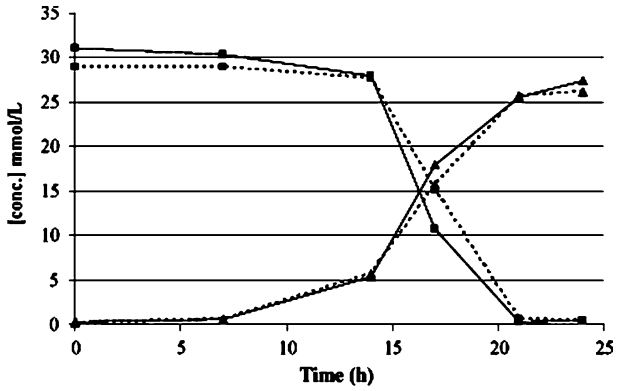


**Fig. 6** Effect of malate supplementation on histamine production: histidine consumption (*filled square*) and histamine accumulation (*filled triangle*) during bacterial growth in MRS + His medium (*hatched line*) and in MRS + His + Mal (*continuous line*)

presence of 3 g/L of malate (Fig. 6). The same results were obtained testing malate concentrations between 0.5 and 5 g/L (data not shown).

### Influence of histidine on MLF

It is difficult to study the conversion of malate to lactate through MLF by simple kinetic experiments, since malate can be catabolized through different pathways and lactate can also be originated through glycolysis. Nevertheless, the influence of histidine on MLF was investigated by measuring malate consumption in MRS + Mal and in MRS + Mal + His and the results are shown in Fig. 7: no influence of histidine on the kinetics of malate metabolization was detected.

To confirm this finding, the influence of histidine on MLF was evaluated enzymatically: *L. hilgardii* ISE 5211 was grown with and without an excess of histidine (4 g/L) and/or malate (3 g/L). The biomass was recovered, and the cytosolic proteins were cleaned and partially purified as described in the materials and methods section. The

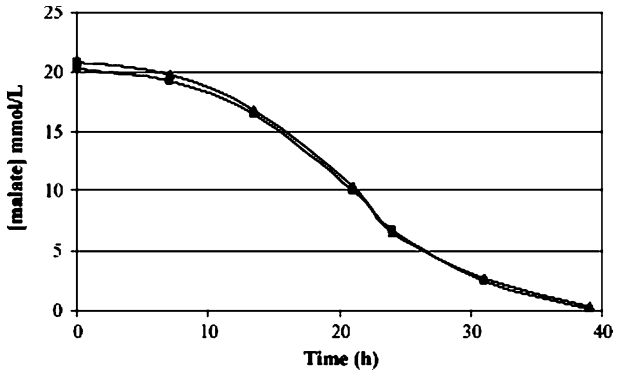


**Fig. 7** Effect of histidine supplementation on malate consumption: malate consumption during bacterial growth in MRS + Mal (*filled square*) and in MRS + Mal + His (*filled triangle*)

activity of MLE was almost totally absent in the cultures grown without malate, suggesting the inducible nature of this enzyme, as expected. On the contrary, the presence of histidine did not affect the in vitro catalytic activity of MLE, as shown in Table 1.

### Influence of ethanol on histamine production

As shown in Fig. 8a, ethanol strongly affected bacterial growth, with maximum inhibition at concentrations higher than 11%. It is not possible give a true evaluation of the inhibitory effect of 13% ethanol on histamine accumulation, because the lack of histamine appears rather to be the result of the very low content of viable cells. However, despite the poor survival rate of *L. hilgardii* ISE 5211 in 11% ethanol, a slight but continuous histamine accumulation was observed in this condition (Fig. 8b).

## Discussion

The phenomenon of BAs accumulation in wine is a complex one, since several factors are involved: availability of proteins and free aminoacids, availability of energy sources, chemical and physical conditions (pH, temperature, ethanol concentration, redox potential, etc.) that are crucial for microbial growth and activity; there may also be interactions among different bacterial strains and yeasts involved in fermentation processes (Fleet 2003).

It has been established thus far that LAB produce amines in the presence of the precursor amino acids (Landete et al. 2006) and in previous work (Pessione et al. 2005) we demonstrated the inducibility of the HDC gene by histidine in two *Lactobacillus* strains.

The possibility of finding large amounts of free amino acids in wine is low since, during alcoholic fermentation, yeasts assimilate 1,000–2,000 mg/L of the total amino acids naturally present in the grapes. At the end of this phase, only 5–12 mg/L, depending on the amino acid considered, are available in the wine, with a prevalence of arginine (16 mg/L) (Henschke and Jiranek 1993; Pozo-Bayon et al. 2005).

However, other amino acids can be found in wine as a result of yeast autolysis: when yeast cells die, once the alcoholic fermentation is completed, they release both endogenous proteins and proteases (Perrot et al. 2002). Thus different amino acids become available to malo-lactic or contaminating bacteria, of which histidine and tyrosine are the most problematic.

Arginine, present in the largest quantities, may also exert some toxicity: microbial enzymatic activity on arginine may give rise to (a) formation of the polyamine

**Table 1** Malo-lactic activity of cytosolic extracts of *L. hilgardii* ISE 5211 grown in different conditions: MRS medium alone, MRS fortified with only histidine (MRS + His), MRS fortified with only L-malate (MRS + Mal), MRS fortified with histidine plus L-malate (MRS + His + Mal)

| | MRS | | MRS + His | | MRS + Mal | | MRS + His + Mal | |
|---|---|---|---|---|---|---|---|---|
| | L-Lactate (mmol/L) | | L-Lactate (mmol/L) | | L-Lactate (mmol/L) | | L-Lactate (mmol/L) | |
| | −Malate | +Malate | −Malate | +Malate | −Malate | +Malate | −Malate | +Malate |
| 0 min | 0 | 0.07 | 0 | 0.068 | 0 | 0.16 | 0 | 0.15 |
| 50 min | 0 | 0.21 | 0 | 0.11 | 0 | 4.73 | 0 | 4.70 |
| 90 min | 0 | 0.28 | 0 | 0.25 | 0 | 6.20 | 0 | 6.16 |

The amount of L-lactate produced in vitro (mmol/L), after 50 and 90 min, has been evaluated by adding to the *L. hilgardii* ISE 5211 cytosolic extracts L-malate to trigger the decarboxylative reaction. As negative control, L-lactate has been quantified without any malate addition

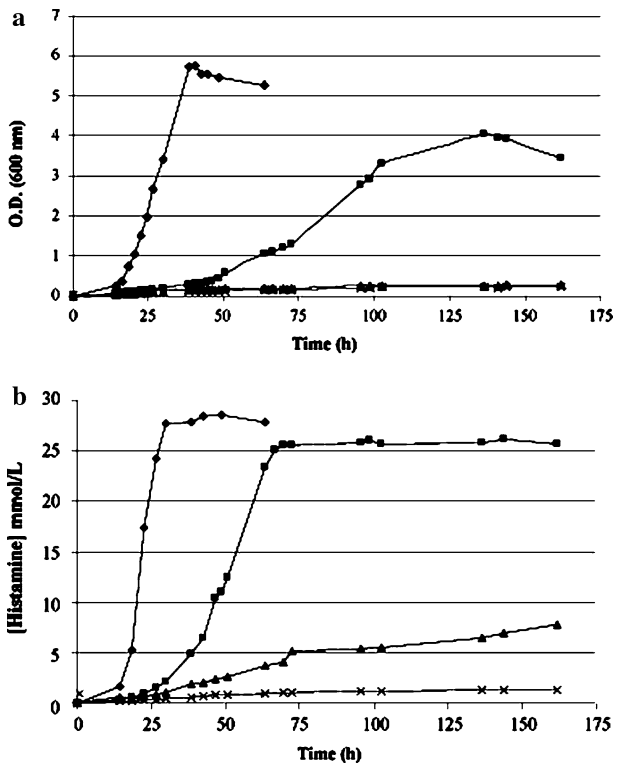


**Fig. 8** Effect of different concentrations of ethanol on histamine production: growth curves (**a**) and histamine accumulation (**b**) in *filled diamond* 0%, *filled square* 9%, *filled triangle* 11%, ×13% ethanol concentrations

agmatine by arginine decarboxylation; and/or (b) formation of $NH_3$, ornithine, carbamoyl phosphate and ATP by arginine deimination (Liu et al. 1995).

Although it enhances the toxic effect of histamine by competing with amine oxydase enzymes (Bauza and Teissedre 1995), agmatine can also have a positive effect when present in food, since it inhibits the oxidation of polyunsaturated fatty acids, α tocopherol and carotenoids (Lovaas 1991). On the contrary, all metabolites originating from arginine deimination are problematic: ammonia is toxic, ornithine can potentiate the effect of histamine (Halasz et al. 1994) and carbamoyl phosphate can combine with ethanol, in wine, originating ethyl carbamate, a carcinogenic molecule (Terrade et al. 2006). The ability to decarboxylate or deaminate arginine is strain-dependent (Landete et al. 2007).

The experimental results of the present study on the histamine-producing and arginine-deiminating strain of *L. hilgardii* ISE 5211, involved in MLF, demonstrate that there is reciprocal interference between histidine decarboxylation and arginine deimination. When arginine and histidine are present simultaneously in the culture medium, there is some delay in both histamine and ornithine accumulation (Figs. 3, 5). Furthermore, arginine appears to exert some repression on HDC biosynthesis (Fig. 4). We observed no clear prevalence of either of the two pathways and, from an applicative standpoint, this is not desirable since both routes give rise to toxic metabolites (histamine or ammonia). From the bacterial standpoint, both routes are involved in energy supply (ATP production by arginine deimination and proton-motive-force generation by histidine decarboxylation) and acidity counteracting (ammonia or histamine basification, respectively) (Pessione et al. 2005). It is possible that the higher biomass recovered in the arginine-grown cultures is due to better acidity counteraction of ammonia versus histamine (Fig. 1). This is in agreement with the observations of other authors (De Angelis et al. 2002) who found better tolerance of acidic stress in a *Lactobacillus sanfranciscensis* CB1 strain grown with arginine supplementation. In the present experiments we observed that when a single amino-acid (histidine or arginine) was supplied at a very high concentration, the entire metabolic effort of *L. hilgardii* ISE 5211 was addressed towards its energy-producing catabolism, whereas when the two amino acids were both present (at the same concentration) the metabolic flow was shared between the two pathways, resulting in a delayed accumulation of the corresponding product (histamine or ornithine) (Figs. 3, 5).

In 2002, De Angelis and co-workers reported that the production of ornithine by the ADI pathway was not affected

by glucose concentration in a *Lactobacillus sanfranciscensis* CB1 strain, but no reports concerning the reciprocal influence of histidine and arginine are currently available.

With regard to reciprocal interactions between amine production and MLF, Konings et al. (1997) reported rich growth conditions (such as media containing glucose or malate) to be unfavorable to histamine accumulation, since energy-generating mechanisms become unnecessary. Moreover, Landete et al. (2006) proved that malic acid inhibits HDC gene expression in LAB. In parallel, Lonvaud-Funel (2001) refer that the malic acid catabolite L-lactate is an inhibitor of HDC catalytic activity.

As malic acid is an abundant metabolite of wine (3–4 g/L) and since it also produces PMF and alkalinization when undergoing decarboxylation to lactate, we examined its effect on histamine accumulation. However, the expected reduction of histamine biosynthesis was not observed (Fig. 6). In parallel, being difficult to evaluate lactate accumulation and malate consumption since both metabolites can originate from (or can be addressed to) different cellular pathways, we studied the catalytic activities of the malo-lactic enzyme extracted and partially purified from cells grown on an excess of: (a) malate; (b) malate + histidine; (c) histidine. While histidine-grown cells showed no malo-lactic activity (similarly to the control medium without high concentrations of any free amino acids), the two cultures containing malate showed the same catalytic pattern despite the presence of histidine (Table 1).

Thus malate seems not to affect histidine decarboxylation, nor histidine to have any influence on MLF, in the *L. hilgardii* ISE 5211 strain under study and, from the applicative standpoint, the (low or high) malate content of a wine does not guarantee histamine safety. Scientific evidence supporting our results comes from the investigations of Mangani et al. (2005) who demonstrated that, in an *O. oeni* strain isolated from wine, amino acid decarboxylation (in this case ornithine to putrescine) continues in parallel with MLF.

Finally, ethanol was tested at three different concentrations (9, 11, and 13%) to detect any influence on the histamine content of a wine. In the *L. hilgardii* ISE 5211 strain, only the 13% ethanol cultures contained no histamine (below 0.02 mM), which was partly due to the high mortality rate of bacteria in these conditions. Thus the protective action of this ethanol concentration against the risk of histamine appears chiefly to be due to bacterial cell inhibition. On the contrary, the 9% alcohol concentration was unable to prevent cell growth or histamine production, despite the reported high sensitivity of *L. hilgardii* to ethanol (Couto et al. 1996). It is possible that some mechanisms, for example those reported by Teixeira et al. (2002), including change in membrane lipid composition and stress protein induction, may account for the peculiar ethanol resistance exhibited by this particular strain. Furthermore, the low but continuous histamine accumulation observed at the 11% ethanol concentration must be taken into account, since this accumulation continues for several days after the beginning of its biosynthesis from histidine (Fig. 8) and it can exert this effect for long time, for instance during wine storage and ageing as previously observed by Gerbaux and Monany (2000) and Gerbaux et al. (1997).

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