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Putrescine production from agmatine by *Lactobacillus hilgardii*: Effect of phenolic compounds

María R. Alberto [a,c], Mario E. Arena [b,c], María C. Manca de Nadra [a,b,c,*]

[a] *Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000 Tucumán, Argentina*
[b] *Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (UNT), Ayacucho 491, 4000 Tucumán, Argentina*
[c] *Research Career of CONICET, Argentina*

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Abstract

The influence of phenolic compounds on the growth of *Lactobacillus hilgardii* X_1B and putrescine formation was assayed at concentrations normally present in wine. Agmatine degradation increased growth and survival of the microorganism and the alkalinity of the media. Bacterial growth was stimulated by phenolic compounds, except for gallic acid and quercetin. Putrescine formation from agmatine diminished in the presence of protocatechuic, vanillic and caffeic acids, and the flavonoids catechin and rutin. The concentration of phenolic compounds decreased after five days of incubation of *L hilgardii* X_1B, except for gallic acid and quercetin. The results indicate that phenolic compounds, besides their already known beneficial properties to human health, seem to be a natural way of diminishing putrescine formation.

Keywords: Biogenic amines; Phenolic compounds; *Lactobacillus hilgardii*

1. Introduction

Biogenic amines, toxic compounds for human health, formed from the amino acid precursors by living cells are small organic compounds found in all living organisms (Janowitz, Kneifel, & Piotrowski, 2003) and they are extremely sensitive to changes in the external environment (Rodríguez, de Armas, Vicente, & Legaz, 2000). Their presence in wine has been suggested as an index of quality (i.e., bad manufacturing practices) (Lethonen, Saarimen, Vesanto, & Riekkola, 1992; Radler & Fäth, 1991). Biogenic amines have been involved in food poisoning incidences, usually from the consumption of fermented foods containing high amounts of those substances (González de Llano, Cuesta, & Rodríguez, 1998). Furthermore, biogenic amines like tyramine as well as diamines such as putrescine and cadaverine have been described as precursors of carcinogenic nitrosamines (ten Brink, Damink, Joosten, & Huis in 't Veld, 1990). Alcohol may enhance the effect of amines present in wine. In fermented foods or beverages, biogenic amines are mainly generated by bacteria that are capable to decarboxylate the amino acid precursors under favorable conditions for enzyme activity. Lactic acid bacteria are reported as the main producers of biogenic amines in alcoholic beverages. It is assumed that biogenic amines found in foods and wines are produced by specific amino acid decarboxylases from lactic acid bacteria during fermentation (Farías, Manca de Nadra, Rollan, & Strasser de Saad, 1995; Kalăc, Săvel, Křížek, Pelikánová, & Prokopová, 2002; Lucas, Landete, Coton, Coton, & Lonvaud-Funel, 2003).

Putrescine is the most abundant biogenic amine found in wine (Soufleros, Barrios, & Bertrand, 1998) and agmatine is the most prevalent one in beer (Glória & Izquierdo

* Corresponding author. Address: Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (UNT), Ayacucho 491, 4000 Tucumán, Argentina. Tel./fax: +54 381 4310465.
E-mail address: mcmanca@fbqf.unt.edu.ar (M.C. Manca de Nadra).

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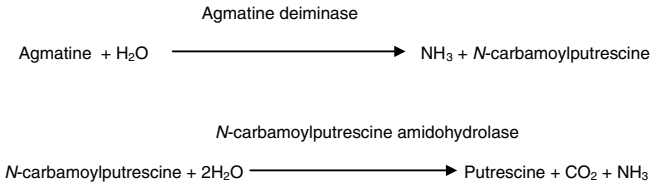
Pulido, 1999). Arena and Manca de Nadra (2001) reported that agmatine was formed as an intermediate in the formation of putrescine from arginine in *Lactobacillus hilgardii* X_1B, isolated from wine. Putrescine is formed from agmatine through a pathway that does not involve amino acid decarboxylase or formation of urea (Arena, 2001).



Production of biogenic amines under laboratory conditions does not imply similar behaviour in fermented products. Wines are complex systems with a wide number of factors influencing microbial growth and metabolism. We are not aware of information about the influence of phenolic compounds, quantitatively one of the most important substances found in wine, on putrescine formation.

Grape phenolics are the main compounds responsible for color, taste, oxidation and other chemical reactions in wine and juice. They have received considerable attention because of their potential antioxidant activity. The specific amounts and types of phenolics present in grapes and wines depend on a number of factors, including variety and maturity of the grape, seasonal conditions, storage and the vinification process (phenol carboxylic acids, 100–200 mg/l, catechin, 10–400 mg/l, quercetin, 5–20 mg/l).

Phenolic compounds may affect growth and metabolism of lactic acid bacteria (Alberto, Farías, & Manca de Nadra, 2001, 2002). In addition, Alberto, Gómez-Cordovés, and Manca de Nadra (2004) demonstrated the degradation of gallic acid and catechin by *L. hilgardii* 5w.

We have no knowledge that studies have been conducted to ascertain if phenolic compounds, natural components of red wine, can affect biogenic amine production by wine lactic acid bacteria.

This paper reports on the influence of phenolic compounds on growth survival and agmatine metabolism of *L. hilgardii* X_1B, a bacterium from wine able to produce important levels of putrescine.

2. Materials and methods

2.1. Microorganism

L. hilgardii X_1B was isolated from Argentine wine (Strasser de Saad & Manca de Nadra, 1987).

2.2. Chemicals

Gallic acid was obtained from Merck, catechin and agmatine were obtained from Sigma, and vanillic acid, quercetin, protocatechuic acid, rutin and caffeic acid were purchased from ICN.

2.3. Media, growth conditions and culture procedures

The basal medium (BM) contained, in g/l peptone (Britania), 5; yeast extract (Britania), 3; glucose (Cicarelli), 1; pyridoxal-5-phosphate (Sigma), 0.005 and 10% tomato juice. Putrescine formation was tested by adding 0.5 g/l of agmatine to the BM. The media were adjusted to pH 4.5 and sterilized at 121 °C for 15 min. Phenolic compound solutions were prepared in ethanol. All media were adjusted to a final ethanol concentration of 7% (v/v). The filter-sterilized phenolic compounds were added to the autoclaved media to reach the final concentration normally present in red wine (100 mg/l for gallic acid, vanillic acid and protocatechuic acid, 10 mg/l for caffeic acid, 50 mg/l for rutin and quercetin and 200 mg/l for catechin). Each experiment was carried out in triplicate.

The strain was pre-cultured in BM and BM + agmatine and then experimental media were inoculated at a concentration of 2.5×10^8 cells/ml. All cultures were incubated in darkness without shaking at 30 °C for five days under microaerobic conditions. Culture samples were taken at different times for growth and pH measurements and then centrifuged (4000*g* for 10 min) and frozen (−20 °C) for subsequent chemical analyses.

2.4. Growth measurement

Bacterial growth was monitored by periodic measurement of optical density at 560 nm in a tunable microplate reader (Versamax, Molecular Devices) and by direct counting of colony-forming units (CFUs/ml) determined by plating 0.1 ml of inoculated media on MRS pH 6.0 (De Man, Rogosa, & Sharpe, 1960) with 10% tomato juice.

2.5. Analytical determinations

Putrescine and agmatine concentrations were determined by HPLC analysis of the amine derivatives formed by orthophtaldialdehyde (OPA) and mercaptoethanol, according to the method described by Alberto, Arena, and Manca de Nadra (2002). Ammonia was determined by the method proposed by Russel (1944). Colorimetric determination of total phenolics was based on the procedure of Singleton and Rossi (1965) modified by Alberto et al. (2001).

The pH was determined with a pH-meter equipped with a glass electrode, which was calibrated against standard buffer solutions (Anedra) at pH 4.0 and 7.0.

2.6. Statistical analysis

All determinations were carried out in triplicate and the standard deviation was calculated using Excel-MS software.

3. Results

3.1. Effect of phenolic compounds on bacterial growth

Fig. 1 shows the influence of agmatine and different phenolic compounds on *L. hilgardii* growth. After 50 h of incubation at 30 °C, maximal growth (OD 560 nm) after addition of agmatine increased from 0.390 to 0.430 with respect to control media. In the presence of phenolic acids (Fig. 1a and c) the OD_{560} increased from 0.365 to 0.412 for gallic acid, from 0.432 to 0.488 for vanillic acid, from 0.381 to 0.554 for protocatechuic acid and from 0.534 to 0.541 for caffeic acid due to the addition of agmatine. In the presence of flavonoid compounds (Fig. 1b and d) growth increased from 0.538 to 0.570 for rutin, from 0.416 to 0.457 for quercetin, and from 0.541 to 0.582 for catechin after addition of agmatine.

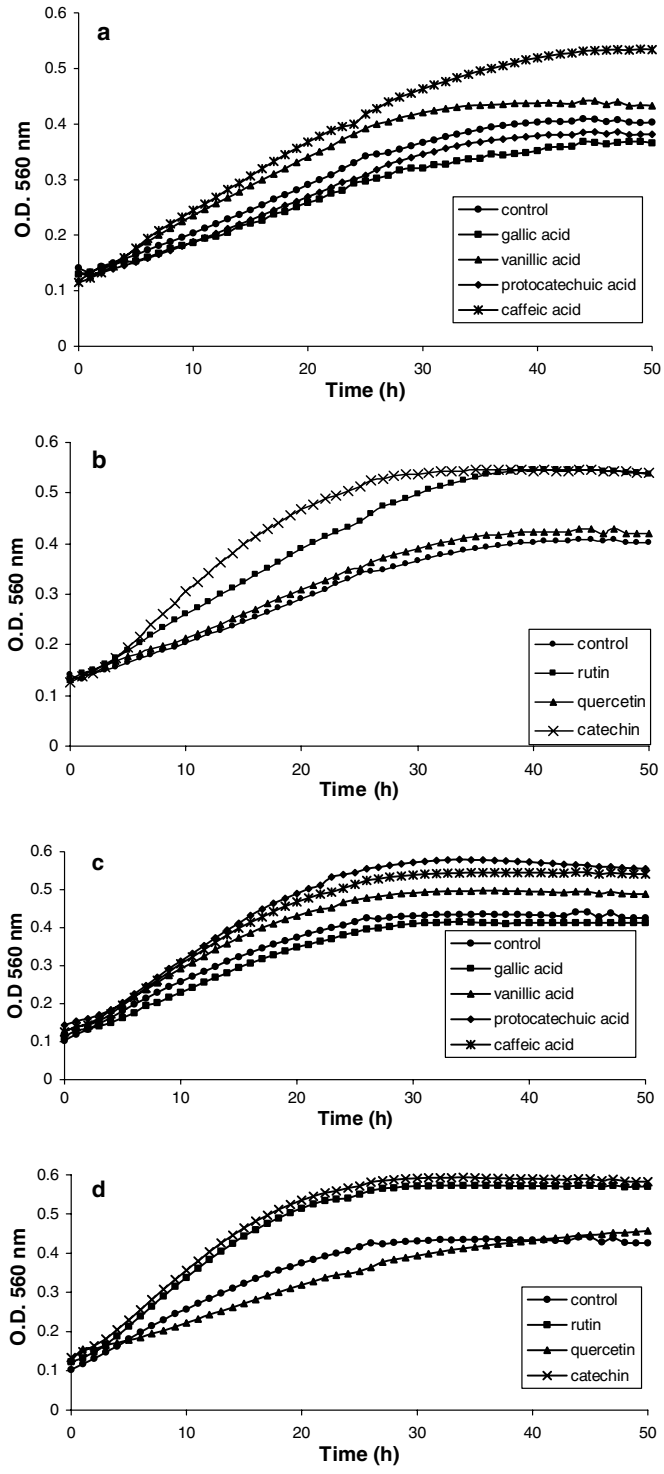


Fig. 1. Growth curves of *Lactobacillus hilgardii* X_1B in: (a) BM plus non-flavonoid compounds; (b) BM plus flavonoid compounds; (c) BM + agmatine plus non-flavonoid compounds and (d) BM + agmatine plus flavonoid compounds. RSD (relative standard deviation) ⩽3%.

With the addition of agmatine to BM growth of *L. hilgardii* increased 45% in the presence of protocatechuic acid and between 8% and 13% in the presence of the other phenolic compounds, except for caffeic acid when no modification was observed. Furthermore, addition of agmatine to the basal media increased the maximal growth rate under all conditions assayed except for the media supplemented with quercetin when no stimulatory effect was observed.

In the media without agmatine (Fig. 1a and b) caffeic acid, rutin and catechin showed an increase of 37%, 38% and 39%, respectively, on bacterial growth. A lower stimulatory effect (11%) was observed with vanillic acid. Gallic acid, protocatechuic acid and quercetin did not modify bacterial growth. Addition of phenolic compounds to the basal media increased the maximal growth rate with the exception of the phenolic acids gallic and protocatechuic.

In the presence of agmatine (Fig. 1c and b) the phenolic compounds stimulated bacterial growth, except for gallic acid and quercetin. Highest growth was observed in the presence of the flavonoid compounds rutin (33%) and catechin (35%). Despite the different assay concentrations, the non-flavonoids caffeic and protocatechuic acids showed a similar stimulatory effect (26% and 29%, respectively). Vanillic acid showed a lower stimulatory effect (13%).

3.2. Influence of phenolic compounds on bacterial survival

The effect of agmatine and phenolic compounds on *L. hilgardii* survival was assessed after five days of incubation in BM at 30 °C (Fig. 2).

The stimulatory effect of agmatine on bacterial survival showed in this figure coincides with the effect observed for maximal growth after 50 h of incubation (Fig. 1). In the presence of agmatine the non-flavonoid, protocatechuic acid, showed the highest positive effect on survival (124%) followed by caffeic acid (90%) and vanillic acid (72%). Among the flavonoid compounds rutin and catechin showed the highest effect, 121% and 207%, respectively. Gallic acid and quercetin neither modified maximal growth nor affected the survival of the lactic acid bacterium. Of the seven phenolic compounds assessed for their effect on *L. hilgardii* growth and survival catechin showed the highest stimulatory effect.

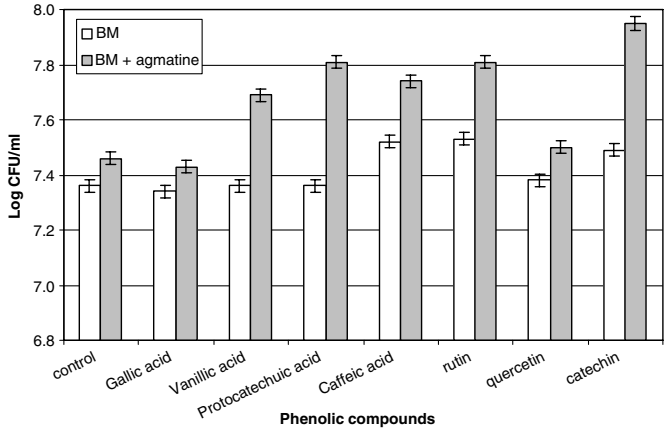


Fig. 2. Survival of *Lactobacillus hilgardii* in BM and BM plus agmatine in presence of different wine phenolic compounds after five days of incubation at 30 °C.

3.3. Influence of phenolic compounds on putrescine formation

The effect of phenolic compounds on putrescine production from agmatine by *L. hilgardii* X_1B after five days of incubation at 30 °C is shown in Fig. 3.

Compared to control media (no phenolic compounds added) conversion of agmatine into putrescine was lower in the presence of phenolic compounds, with the exception of gallic acid and quercetin. The putrescine formed was 29%, 40%, 23%, 15% and 22% lower in the presence of vanillic, protocatechuic and caffeic acids and rutin and catechin, respectively.

Less agmatine was consumed by *L. hilgardii* in the presence of vanillic acid (16%), protocatechuic acid (14%), caffeic acid (23%), rutin (7%) and catechin (9%) when compared to control media. The fact that putrescine formation could be inhibited by phenolic compounds is desirable from a hygienical-sanitary point of view.

The theoretical recovery of two moles of ammonia per mole of putrescine formed from agmatine, is well correlated with the production of 1.7, 1.4, 2.2, 2.5, 2.1, 2.1, 1.8 and 1.5 mmol/l of ammonia per mmol/l of putrescine found in BM and in BM supplemented with gallic acid, vanillic acid, protocatechuic acid, caffeic acid, rutin, quercetin and catechin, respectively.

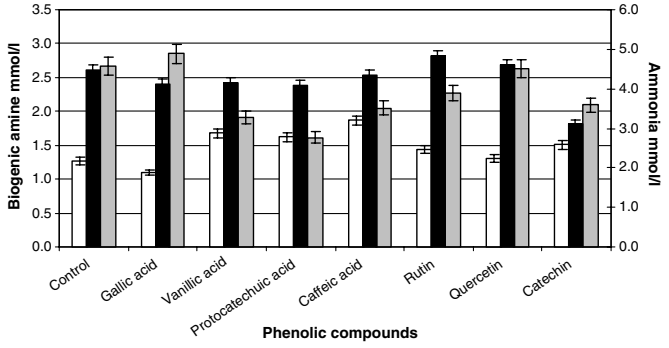


Fig. 3. Effect of phenolic compounds on the bioconversion of agmatine into putrescine by *Lactobacillus hilgardii* after five days of incubation at 30 °C. Initial agmatine concentration was 3.87 mmol/l. Residual agmatine (□), prutrescine production (▒) and ammonia production (■).

3.4. Final pH Values in the different media

The initial pH for all media was 4.50. After five days of incubation at 30 °C media inoculated with *L. hilgardii* showed different pH values in the presence or absence of agmatine. In the absence of agmatine pH was between 4.10 and 4.12 in the different culture supernatants. In all media supplemented with agmatine the final pH was higher (between 4.41 and 4.51) than that observed in media without agmatine.

In BM + agmatine supplemented with gallic, vanillic and protocatechuic acid, the final pH was significantly higher (4.51, 4.49 and 4.50, respectively) than that in the control medium without phenolics (4.41). In the presence of caffeic acid, rutin, quercetin and catechin the pH was about 4.46. Only in the media with agmatine the presence of phenolic compounds modified the final pH.

3.5. Modification of the concentration of phenolic compounds after growth of *L. hilgardii* X_1B

Fig. 4 shows the decrease in phenolic compounds concentration after five days of incubation with or without agmatine. Only caffeic acid, at an initial concentration of 10 mg/l, disappeared completely after five days of incubation with *L. hilgardii* X_1B so much in presence or absence of agmatine. In both cases survival increased (see Fig. 2). In the presence of agmatine diminution in vanillic acid (5.8%), protocatechuic acid (6.2%) and catechin (12.6%) was higher than in its absence. This fact was correlated with the higher cell number reached in the medium supplemented with agmatine (Fig. 2).

In the media supplemented with gallic acid or quercetin no increase in either growth or survival was observed. The

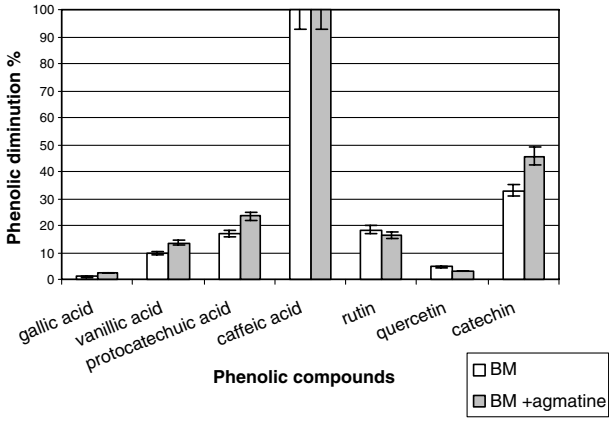


Fig. 4. Diminution (%) in the phenolic compound concentrations after incubation of *Lactobacillus hilgardii* in BM and BM + agmatine. Initial concentration for gallic, vanillic and protocatechuic acids 100 mg/l; caffeic acid 10 mg/l; rutin and quercetin 50 mg/l and catechin 200 mg/l.

modifications observed in gallic acid or quercetin concentrations in the media were negligible.

4. Discussion

L. hilgardii represents an important organism in fermented beverage spoilage and is usually involved in wine making and other food processes (Tonon & Lonvaud-Funel, 2002). The presence of agmatine in the media increased growth of *L. hilgardii* X_1B. It is well known that decarboxylation may be used to generate metabolic energy via a general mechanism common to a variety of decarboxylases (Christensen, Dudley, Pederson, & Steele, 1999). This fact could explain the increase in growth in the presence of agmatine.

The different results observed in bacterial growth in the presence or absence of agmatine suggest that there is an interaction between agmatine metabolism by *L. hilgardii* X_1B and some of the phenolic compounds. With respect to the effect of phenolic compounds on bacterial growth, the only phenolic acid that did not modify *L. hilgardii* growth was gallic acid; the other phenolics had a stimulatory effect. Of the three flavonoids, quercetin did not affect the growth, whereas the two others showed a stimulatory effect, which was similar. Campos, Couto, and Hogg (2003) reported that *p*-coumaric, caffeic, ferulic, protocatechuic, gallic and vanillic acids present at 100 mg/l did not significantly affect growth of *L. hilgardii*. Stead (1993) informed that caffeic acid at 100 mg/l stimulated the growth of *L. collinoides* strains. Reguant, Bordons, Arola, and Rozés (2000) reported that phenolic compounds affected the growth of *Oenococcus oeni* in different ways, depending on their type and concentration. In previous work, Alberto et al. (2001) reported that *L. hilgardii* 5w from wine showed a growth stimulatory effect in the presence of gallic acid and catechin at concentrations normally present in wine. Rodriguez Vaquero, Alberto, and Manca de Nadra (2004) reported the effect of non-flavonoid phenolic compounds on *L. hilgardii* 5w growth. At 100 mg/l gallic acid increased growth, protocatechuic acid decreased growth, whereas vanillic acid did not show any modification. At 10 mg/l caffeic acid did not affect the growth of *L. hilgardii*. The different effect of phenolic compounds on bacterial growth observed among different species and strains of lactic acid bacteria indicate that their effect is strain dependent.

Tkachenko, Pshenichnov, and Nesterova (2001) reported that addition of putrescine to *Escherichia coli* cultures previously exposed to oxidative stress led to an increase in cell survival and that putrescine produced a protective effect on the DNA damaged by reactive oxygen species. Tkachenko and Nesterova (2003) and Tkachenko (2004) reported the ability of putrescine to decrease the mutation rate under oxidative stress in *E. coli*. In our results the presence of phenolic compounds, recognized antioxidants, decreased the formation of putrescine, because the phenolics themselves could protect the cells against oxidative stress. Almost all the agmatine degraded was recovered as putrescine, between 88% and 103%, with the exception of protocatechuic acid with a recovery of 72%. Gallic acid and quercetin did not inhibit putrescine formation and neither increased bacterial growth nor survival in the presence of agmatine. Therefore, these results suggest that there are no interactions between gallic acid and quercetin and agmatine metabolism.

As a consequence of the ammonia production, agmatine degradation could be considered favorable to strains adaptation to acid environments. The lower quantity of agmatine recovered as putrescine and the smaller amount of ammonia produced in the media supplemented with protocatechuic acid suggest that this phenolic acid could interfere in the conversion of *N*-carbamoylputrescine into putrescine.

Previous studies have showed that some hydroxybenzoic acids could inhibit bacterial metabolism (Vivas, Lonvaud-Funel, & Glories, 1997). A possible explanation for this inhibitory effect is that some polyphenols can interact with cell enzymes (Campos et al., 2003; McManus, Davis, Beart, Gaffney, & Lilley, 1985).

On the other hand, seven *Lactobacillus* strains isolated from malt whisky fermentations degraded the hydroxycinnamic acid (*p*-coumaric acid) by decarboxylation (Van Beek & Priest, 2000). The conversion of agmatine into putrescine involves *N*-carbamoylputrescine decarboxylation with release of CO_2. The phenolic decarboxylation could compete with *N*-carbamoylputrescine decarboxylation causing inhibition of putrescine formation.

In the present work we have demonstrated the ability of *L. hilgardii* X_1B to modify the concentration of wine phenolic compounds in culture media. Previously, Alberto et al. (2004) demonstrated that *L. hilgardii* 5w was able to consume and degrade gallic acid and catechin. Cavin, Andioc, Etievant, and Divies (1993) demonstrated that *Lactobacillus* and *Pediococcus* strains were able to metabolize phenol carboxylic acids (ferulic and *p*-coumaric) to produce vinyl derivatives. Whiting and Carr (1959) reported that *L. collinoides* has the capacity to metabolize hydroxycinnamic acids by reduction of their side chain. Reguant et al. (2000) reported that *O. oeni* did not utilize *p*-coumaric acid, caffeic acid, gallic acid and catechin, whereas *L plantarum* metabolized *p*-coumaric acid.

In conclusion, as the potential production of biogenic amines is of great toxicological significance, the inhibitory effect on putrescine formation by natural compounds present in wine is important. Our results indicate that phenolic compounds that have well-known beneficial properties to human health appear to be a natural means of diminishing putrescine formation from agmatine.

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