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Putrescine production from different amino acid precursors by lactic acid bacteria from wine and cider

Antonella Costantini[a], Roberta Pietroniro[a, b], Francesca Doria[a], Enrica Pessione[b], Emilia Garcia-Moruno[a]

a Consiglio per la Ricerca e la sperimentazione in Agricoltura (Centro di ricerca per l'Enologia), Via P. Micca 35, 14100 Asti, Italy
b Department of Life Sciences and System Biology, University of Turin, Via Accademia Albertina 13, 10123 Torino, Italy

Highlights

- This is a study of the amino acid precursors related with putrescine production TLC is a useful method for BA detection. Putrescine production from agmatine is a feature of most *Lactobacillus brevis* strains. Tyramine and putrescine production is correlated in *Lactobacillus brevis*. One *Pediococcus* strain possessed both pathways, ADI and AgDI, for putrescine production.

Abstract

The aim of this work was to study the production of biogenic amines and particularly putrescine in lactic acid bacteria (LAB) related to wine and cider. We applied an analytical protocol that involves the use of PCR and TLC techniques to determine the production of putrescine from different precursors. Moreover, we also studied the ability of the *Lactobacillus* and *Pediococcus* tested to produce histamine and tyramine. The results showed that the majority of the *Lactobacillus brevis* analyzed harbour both *AgDI* and *tdc* genes and are tyramine and putrescine producers. Conversely, among the other LAB tested, only one *Lactobacillus hilgardii* and one *Pediococcus pentosaceus* produced putrescine. The *AgDI* gene was also detected in two other LAB (*Lactobacillus mali* and *Pediococcus parvulus*), but no putrescine production was observed. Finally, *hdc* gene and histamine production were found in strains (*L. hilgardii* 5211, isolated from wine, and *Lactobacillus casei* 18, isolated from cider) that were not putrescine producers.

Keywords

Biogenic amines; Arginine; Putrescine; Agmatine; Lactic acid bacteria

1. Introduction

During the production of wine and cider, two types of microorganism play a role: yeasts, which perform the alcoholic fermentation, and lactic acid bacteria (LAB), which are responsible for the malolactic fermentation (MLF). In wine, the LAB present belong mainly to four genera, *Oenococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, with *Oenococcus oeni* being the main species responsible of MLF in most wines (Van Vuuren and Dicks, 1993). During the cider-making process, the most abundant bacterial species is also *O. oeni* together with other LAB belonging chiefly to *Lactobacillus* species (Dueñas et al., 1994).

MLF is a process with positive effects on the organoleptic properties of wine and cider, but some LAB strains can also produce undesirable metabolites, such as biogenic amines (BA). BA are organic bases frequently found in fermented food and beverages. Some BA (such as histamine, tyramine, beta phenylethyl amine, and triptamine) are bioactive molecules acting at the central nervous system or at vascular level (Medina et al., 2003). At high concentrations, BA are risk factors for food intoxication, while moderate levels may lead to food intolerance. In any case, they are undesirable in all foods and beverages, as they can induce headaches, respiratory distress, hyper/hypotension, and several allergic disorders (Ladero et al., 2010 and Ladero et al., 2011). Among BA, histamine is the most dangerous due to the toxicological effects derived from its vasoactive and psychoactive properties (Halasz et al., 1994). Furthermore, tyramine and diamines, such as putrescine and cadaverine, have been described as precursors of carcinogenic nitrosamines (ten Brink et al., 1990).

BA in fermented foods are principally produced by LAB by decarboxylation of amino acid precursors. Decarboxylation of amino acids, such as histidine, tyrosine, and ornithine, results in formation of the corresponding BA, histamine, tyramine, and putrescine, respectively, which are the most frequently encountered in wine and cider (Garai et al., 2006, Garai et al., 2007, Vidal-Carou et al., 1989 and Zee et al., 1983). Among these, putrescine is the most abundant BA found both in wine (Soufleros et al., 1998) and cider (Garai et al., 2006).

The production of putrescine by LAB is related to arginine catabolism, and different metabolic pathways have been reported in the literature for arginine degradation by LAB. Kuensch et al. (1974) proposed the urea cycle (Fig. 1) as the main arginine-degrading pathway used by some strains of formerly *Leuconostoc oenos* (now classified as *O. oeni*), i.e., arginine is first converted to urea and ornithine, followed by the transformation of urea to ammonia and carbon dioxide and the conversion of ornithine to citrulline. This route was later extended to other wine LAB capable of breaking down arginine (Sponholz et al., 1991 and Sponholz, 1992). At that time, and although the urea cycle was not enzymatically demonstrated in LAB, it was also suggested that wine LAB are able to degrade arginine through the arginine deiminase (ADI) pathway (Pilone et al., 1991). Taking into consideration the existence of the ADI pathway enzymes, the absence of arginase and urease, and the stoichiometry of arginine conversion to ornithine and ammonia, Liu et al. (1996) concluded that the ADI pathway (Fig. 1) is the exclusive route for arginine catabolism in wine LAB able to degrade arginine. According to this pathway, one mole of arginine is converted to two moles of ammonia and one mole of ornithine, ATP, and carbon dioxide. In this regard, the presence of this pathway in wine and cider LAB, Arena et al. (2002) reported that arginine is metabolized via arginine deiminase pathway by *Lactobacillus hilgardii* X1B, isolated from wine, with the formation of *N*-carbamoyl-P as intermediate. Arginine degrading pathway via the urea cycle or via the ADI pathway will produce ornithine, the direct precursor of putrescine, by the action of ornithine decarboxylase enzyme (ODC).

Fig. 1.

Schematic representation of the metabolic pathways involved in putrescine formation. ADI: arginine deiminase pathway; AgDI: agmatine deiminase pathway. The urea cycle is also represented even though it has not been enzymatically demonstrated in LAB.

Figure options

ODC is a PLP-dependent enzyme that catalyzes the conversion of ornithine to putrescine. Many bacteria contain two forms of ODC, a biosynthetic or constitutive and a biodegradative or inducible form (Marcobal et al., 2004). Among LAB, a biodegradative ODC had only been described in *Lactobacillus* 30a (Gale, 1946). In 2004, Marcobal et al. reported the identification of an *odc* gene in the putrescine-producer *O. oeni* BIFI-83 strain. Later, the *odc* gene was also identified and sequenced in three *O. oeni* wine strains and in two *O. oeni* cider strains (Bonnin-Jusserand et al., 2011).

It has also been reported that putrescine can be generated by the decarboxylation of arginine into agmatine, which is then directly converted into putrescine (Cunin et al., 1986) or via carbamoylputrescine (AgDI pathway; Fig. 1). The formation of putrescine through the AgDI pathway has been described in *Pseudomonas* spp. (Stalon and Mercenier, 1984), *Pseudomonas aeruginosa* (Nakada and Itoh, 2003), *Streptococcus faecalis* (Simon and Stalon, 1982), *Bacillus cereus* (Ivanova et al., 2003), and *Bacillus subtilis* (Sekowska et al., 1998).

Lucas et al. (2007) found that AgDI pathway genes are linked to the tyrosine decarboxylase operon in a strain of *Lactobacillus brevis* (*L. brevis* IOEB 9809). These authors found that *L. brevis* IOEB 9809 produced putrescine from agmatine but not from arginine, indicating the lack of a pathway converting arginine into agmatine.

The aim of this work was to investigate the ability of different strains belonging to *Lactobacillus* and *Pediococcus* genera to biosynthesize putrescine and to determine the metabolic pathway utilized. The production of putrescine from intermediate metabolites was determined by thin layer chromatography (TLC) and PCR detection of ornithine decarboxylase and agmatine deiminase

genes. The possible relationship between the production of putrescine and the presence of BA that can be found in cider and wine, such as tyramine and histamine, was also addressed.

2. Materials and methods

2.1. Microorganisms and growth conditions

A total of 21 *Lactobacillus* spp. strains and two *Pediococcus* spp. strains were studied. Some strains belong to the collection of the Centro di Ricerca per l'Enologia (CRA-ENO), and others have been isolated from wine, cider, or as contaminants of commercial yeast starter (Costantini et al., 2009). Bacterial species and their origin are reported in Table 1. LAB from cider were kindly provided by Dr. Moreno-Arribas. Bacteria were cultured in deMan Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany), pH 6.3, and incubated at 30 °C.

Table 1.

Origin of LAB species analyzed.

Strain	Species	Isolation source/collection
5197	*L. brevis*	CRA-ENO collection
5198	*L. brevis*	CRA-ENO collection
5033	*L. brevis*	CRA-ENO collection
5202	*L. brevis*	CRA-ENO collection
CEGi/9	*L. brevis*	ADY contaminant
DV10l8	*L. brevis*	ADY contaminant
ALB1	*L. brevis*	ADY contaminant
ALB24	*L. brevis*	ADY contaminant
MCS/i	*L. brevis*	ADY contaminant
ALBTQ4	*L. brevis*	ADY contaminant
43/b	*Lactobacillus buchneri*	ADY contaminant
CM40	*Lactobacillus parabuchneri*	ADY contaminant
5206	*Lactobacillus hilgardii*	CRA-ENO collection
5215	*L. hilgardii*	CRA-ENO collection
5211	*L. hilgardii*	CRA-ENO collection
38/4	*Lactobacillus plantarum*	Cider
13	*Lactobacillus collinoides*	Cider
22	*Lactobacillus mali*	Cider
18	*L. casei*	Cider
50	*L. zeae*	Cider
54	*Lactobacillus diolivorans*	Cider
CM34	*Pediococcus parvulus*	ADY contaminant
CM3/29	*Pediococcus pentosaceus*	ADY contaminant
Lactobacillus 30a	*Lactobacillus* spp.	ATCC33222

CRA-ENO: CRA-Centro di ricerca per l'enologia, Asti, Italy; ADY: active dry yeasts; ATCC: American Type Culture Collection.

Table options

2.2. Detection of BA decarboxylase genes: DNA extraction and PCR amplification

DNA was extracted from bacterial culture as previously described (Arena et al., 2008). The presence of the agmatine deiminase (*AgDI*), ornithine decarboxylase (*odc*), histidine decarboxylase (*hdc*), and tyrosine decarboxylase (*tdc*) genes in bacterial cultures was assessed by PCR. Reactions were performed using a MyCycler instrument (Biorad) in a 20-μL volume containing 2 × Taq polymerase buffer, 2 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, and 1 U of Taq DNA polymerase (5′Prime; Eppendorf, Milan, Italy).

To detect the *odc* gene, the primers listed in Table 2 were used. PCR conditions were as follows: 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min for 30 cycles.

Table 2.

Primers used for the detection of *odc* gene.

Primer	Sequence	Reference
3	GTNTTYAAYGCNGAYAARACNTAYTTYGT	Marcobal et al. (2004)
4	ATNGARTTNAGTTCRCAYTTYTCNGG	Marcobal et al. (2004)
15	GGTAYTGTTYGAYCGGAAWAAWCAYAA	Marcobal et al. (2004)
16	TACRCARAATACTCCNGGNGGRTANGG	Marcobal et al. (2004)
odcf	TGCACTTCCATATCCTCCAG	Nannelli et al. (2008)
odcr	GAATTTCTGGAGCAAATCCA	Nannelli et al. (2008)
Aodc1	GMTCGTGAAATYAARCKG	Costantini et al. (2006)
Aodc3	TTDGAYCGDAATCAYAARTCC	This study
Aodc4	AGCYARTCKRAATGGACGCTT	This study
Aodc5	CTGGACACCAAGGTGGACAA	This study
Aodc6	CTGGTGCAAATCCTGGGAA	This study

Table options

To detect the *AgDI* gene, PCR were performed with the primer set AGDIfor (GAACGACTAGCAGCTAGTTAT) AGDIrev (CCAATAGCCGATACTACCTTG) as described by Lucas et al. (2007). The presence of *tdc* and *hdc* genes was investigated using primers Pt3/Pt4 and PHDC1/PHDC2, respectively, as described by Costantini et al. (2006).

The PCR products were separated on agarose gel in 0.5 × TAE (Tris-acetate/EDTA), stained with ethidium bromide and visualized under a UV lamp (GelDoc 2000, Biorad).

The 16S rDNA gene was amplified by PCR as described by Marchesi et al. (1998), and the PCR products were sequenced (BMR Genomics, Padua, Italy). BLAST analysis was conducted to confirm the bacterial species.

2.3. Thin layer chromatography (TLC)

Lactobacilli and *Pediococci* were grown at 30 °C in MRS broth supplemented with the biogenic amine precursor amino acids histidine (5 mg/mL), ornithine (5 mg/mL), and tyrosine (2 mg/mL) according to Garcia-Moruno et al. (2005). To study the putrescine production pathway, the broths were supplemented with 5 mg/mL of arginine, citrulline, or agmatine. Samples taken at 9 and 12 days of growth were analyzed according to Garcia-Moruno et al. (2005). The amines were fractionated on silica gel plates (silica gel 60 F254s, Merck) with a mobile phase solution of chloroform-triethylamine (4:1), and the spots were visualized under UV light.

2.4. Phylogenetic analysis of ODC

The amino acid sequences of ODC from different LAB species were retrieved from GenBank: *Lb. 30*a (accession no. P43099); *O. oeni* (accession no. CAG34069); *Lactobacillus acidophilus* (accession no. AAT09142); *Lactobacillus johnsonii* (accession no. NP_965822); *Lactobacillus gasseri* (accession no. ZP_00047186); *L. brevis* (accession no. AFC60624); *Lactobacillus casei* (accession no. B3WEZ8), *Lactobacillus rhamnosus* (accession no. YP_003171444.1), and *Lactobacillus zeae* (accession no. ZP_09452258.1). These sequences were aligned by ClustalW and used to obtain the phylogenetic tree by using TreeTop (GeneBee, http://www.genebee.msu.su).

3. Results and discussion

The aim of this work was to study putrescine production from different precursors to determine the metabolic pathway (ADI or AgDi) used by LAB generally present in wine and cider. The studied strains were isolated from wine (CRA-ENO collection) and cider, as well as from active dried yeast preparations normally utilized as starters for alcoholic fermentation. The biosynthetic routes for putrescine production in 23 LAB strains were explored by genetic analysis and by means of metabolite detection in media having the precursor substrate. Furthermore, additional information about the considered strains was obtained by searching the production of two others bio active amines: histamine and tyramine.

3.1. BA production by *Lactobacillus* and *Pediococcus* strains

Except *L. brevis* MCS/I (a not amine producer) and *L. brevis* ALBTQ4, which produces putrescine but not tyramine, all the remaining strains biosynthesized both putrescine and tyramine (Table 3), suggesting that the parallel production of these two amines can be considered a feature of most *L. brevis* strains. These findings partially agreed with previous reports (Coton et al., 2010) describing the frequent detection of both *agdi* and *tdc* genes in LAB and were simultaneously carried by both *L. brevis* and *L. hilgardii*. However, in our analysis, no such feature was found in any of the *L. hilgardii* strains screened, but only in *L. brevis* strains. Analysis of 13 *L. brevis* from different sources (only one coming from wine) showed that only two strains were unable to produce tyramine (Coton and Coton, 2009).

Table 3.

Biogenic amines produced by the tested LAB species.

LAB species	Putrescine from Arg	Putrescine from Agm	Putrescine from Orn	Putrescine from Cit	Other produced amines	Genes amplified by PCR
5197 (*Lactobacillus brevis*)	−	+	−	−	Tyramine	*tdc*, *agDI*
5198 (*L. brevis*)	−	+	−	−	Tyramine	*tdc*, *agDI*
5202 (*L. brevis*)	−	+	−	−	Tyramine	*tdc*, *agDI*
5033 (*L. brevis*)	−	+	−	−	Tyramine	*tdc*, *agDI*
CEGi/9 (*L. brevis*)	–	+	−	−	Tyramine	*tdc*, *agDI*
DV10L8 (*L. brevis*)	–	+	−	−	Tyramine	*tdc*, *agDI*
ALB1 (*L. brevis*)	–	+	−	−	Tyramine	*tdc*, *agDI*
ALB24 (*L. brevis*)	–	+	−	−	Tyramine	*tdc*, *agDI*
ALBTQ4 (*L. brevis*)	–	–	+	−	–	*odc*
MCS/i (*L. brevis*)	–	–	–	–	–	
5206 (*Lactobacillus hilgardii*)	−	+	−	−	–	*agDI*
5215 (*L. hilgardii*)	–	–	–	–	–	
5211 (*L. hilgardii*)	–	–	–	–	Histamine	*hdc*
13 (*Lactobacillus collinoides*)	−	–	–	–	–	
22 (*Lactobacillus mali*)	−	–	–	–	–	*agDI*
18 (*Lactobacillus casei*)	−	–	–	–	Histamine	*hdc*
50 (*Lactobacillus zeae*)	−	–	–	–	–	
54 (*Lactobacillus diolivorans*)	−	–	–	–	–	
38/4 (*Lactobacillus plantarum*)	−	–	–	–	–	

LAB species	Putrescine from Arg	Putrescine from Agm	Putrescine from Orn	Putrescine from Cit	Other produced amines	Genes amplified by PCR
43/b (*Lactobacillus buchneri*)	–	–	–	–	–	
CM40 (*Lactobacillus parabuchneri*)	–	–	–	–	–	
CM34 (*Pediococcus parvulus*)	–	–	–	–	–	*agDI*
CM3/29 (*Pediococcus pentosaceus*)	+	+	+	+	–	*agDI*

Table options

Our data show that putrescine was produced from agmatine, as determined by TLC analysis, in all *L. brevis* strains analyzed except in ALBTQ4, thus indicating the presence of the agmatine deiminase (AgDI) pathway (Fig. 1). Fig. 2A shows the TLC patterns, in the presence of different precursor amino acids, of the only *L. brevis* strain (ALBTQ4) unable to produce putrescine from agmatine, as putrescine production by ALBTQ4 occurs only when ornithine is used as precursor.

Fig. 2.

TLC of (A) ALBTQ4 (*Lactobacillus brevis*); lane 1: standard putrescine, tyramine, histamine; lane 2: standard of putrescine; lane 3: ALBTQ4 with arginine: lane 4: ALBTQ4 with ornithine; lane 5: ALBTQ4 with agmatine; lane 6: ALBTQ4 with citrulline; lane 7: negative control.

(B) CM 3/29 (*P. pentosaceus*); lane 1: standard putrescine, tyramine, histamine; lane 2: standard of putrescine; lane 3: CM 3/29 with arginine: lane 4: CM 3/29 with ornithine; lane

5: CM 3/29 with agmatine; lane 6: with citrulline. Arrow indicates putrescine (PUT), histamine (HISTA), and tyramine (TYRA).

Figure options

Regarding the other LAB species analysed (Table 3), putrescine is only produced by a *L. hilgardii* (strain 5206) and by a *Pediococcus pentosaceus* (strain CM3/29). In the case of *L. hilgardii* 5206, TLC analysis showed that putrescine is produced only from the agmatine precursor, while *P. pentosaceus* CM3/29 produced putrescine from arginine, agmatine, citrulline, and ornithine (Fig. 2B), indicative of the use of both the AgDI and the ADI pathways. Furthermore, two strains, *L. hilgardii* 5211, isolated from wine, and *L. casei* 18, isolated from cider, display the ability to biosynthesize histamine.

3.2. Genetic determinants for AgDI, ADC, and ODC

Almost all the *L. brevis* tested (except MCS/I and ALBTQ4 strains) harbour the *AgDI* gene in their genome (Table 3), confirming the results obtained by TLC analysis. These results are in agreement with the data previously reported by Lucas et al. (2007) for *L. brevis* IOEB 9809 and suggest that this could be a specific feature of most *L. brevis*. Apart from these, AgDI gene was present only in other four strains: *L. hilgardii* 5206, *Lactobacillus mali* 22, *Pediococcus parvulus* CM 34, and *P. pentosaceus* CM3/29 (Table 3). As far as we know, this is the first report describing the presence of *AgDI* gene in *L. mali*, although this gene was recently found in a *L. hilgardii* strain (Landete et al., 2010). Nevertheless, and even though the *AgDI* gene is present in *P. parvulus* CM 34 and *L. mali* 22, this fact does not support the production of putrescine by them under the conditions used (Table 3). One possible explanation could be that, in these strains, the enzyme encoded by this gene possesses a very low catalytic activity generating not enough amount of carbamoyl-putrescine and, consequently, not sufficient amount of putrescine to be detected with the standard techniques, or either that agmatine is not the unique inducer required; thus, more complex systems are needed for gene induction and/or transcriptional/translational regulation. However, it should be noted that AgDI cluster inactivation, due to the presence of an insertion sequence element in some strains of *Lactococcus lactis*, has been recently reported (Ladero et al., 2011).

The picture is more complex when considering the *odc* gene. In the ornithine producer *L. brevis* ALBTQ4, this gene could not be amplified using the previously described primers available in the literature (Table 2), although it was correctly amplified in *Lb*. 30a and *O. oeni* BIFI-83 strains used as controls. Therefore, a new set of degenerated primers Aodc3/Aodc4 was designed by alignment of the protein sequences of *L. gasseri* (ZP000471861), *L. acidophilus* (AAT09142.1), *L. johnsonii* (NP965822), *Lb*. 30a (P430992), and *O. oeni* BIFI-83 (CAG340691), and again, only the control *odc* from *O. oeni* and *Lb*. 30a were amplified.

It has been proposed (Marcobal et al., 2004) that the distribution of ODC proteins did not follow the phylogeny of their hosts and that, for example, *Lb*. 30a ODC showed lower identity with ODC proteins found in other members of the *Lactobacillus* genus (44% with *L. johnsonii* and *L. gasseri*, respectively) than with enzymes from unrelated microorganisms such as *Haemophilus influenzae* (62%), *Pasteurella multocida* (59%), *Shewanella oneidensis* (59%), and also from *E. coli* (57%), being the highest sequence identity (67%) found between *Lb*. 30a and *O. oeni*.

More recently, the sequencing of the complete *odc* gene from *O. oeni* and *L. brevis* showed an 83% identity (Romano et al., 2012). The alignment of the sequences of *odc* gene from *O. oeni* (AJ746165.3) and *L. brevis* IOEB 9906 (JN120479.1) strains allowed the design of two new primers aodc5/aodc6 (Table 2) that amplify *odc* gene from *L. brevis* ALBTQ4 and also from the

positive control (*O. oeni* Bifi-83). These results are supported by phylogenetic analysis of the ODC sequences from different LAB species. *O. oeni* and *L. brevis* clustered together and very close to *Lb*. 30a, while *L. zeae*, *L. casei*, and *L. rhamnosus* clustered in a separate branch (Fig. 3). A third branch included *L. gasseri*, *L. johnsonii*, and *L. acidophilus*, but they should be considered putative ODC, as their function has not yet been biochemically demonstrated.

Fig. 3.

Phylogenetic tree constructed by using TreeTop from the GeneBee of ODC sequences from different LAB species. The amino acid sequences were obtained from GenBank with the following accession number: *Lb. 30*a (P43099); *Oenococcus oeni* (CAG34069); *Lactobacillus acidophilus* (AAT09142); *Lactobacillus johnsonii* (NP_965822); *Lactobacillus gasseri* (ZP_00047186); *Lactobacillus brevis* (AFC60624); *Lactobacillus casei* (B3WEZ8), *Lactobacillus rhamnosus* (YP_003171444.1), and *Lactobacillus zeae* (ZP_09452258.1).

Figure options

None of the primers used in the PCR assays amplified the *odc* gene in *P. pentosaceus* CM 3/29 which, as shown by TLC, produces putrescine from ornithine, suggesting that its ODC evolved separately from the other LAB ODC proteins. Nowadays, only a 69 amino acids long ODC sequence (CCC15150) from *Pediococcus* is available and its function has not yet been demonstrated.

PCR assays on *adc* gene were not performed because only Arginine/lysine/ornithine decarboxylase from *L. casei str. Zhang* (UniProtKB/TrEMBL access number D8GHB7) and from *L. rhamnosus* HN001 (UniProtKB/TrEMBL accession number B5QJZ6) are available for the design of specific primers, as the other available *adc* gene sequences are from species, such as *Salmonella typhimurium*, not related to wine (Álvarez-Ordóñez et al., 2010).

3.3. Genetic determinants for TDC and HDC

Because amine-producing LAB often harbours determinants for different amino acid decarboxylases, we investigated the presence of *tdc* and *hdc* genes in the studied strains. PCR analyses demonstrated the presence of the *tdc* gene in the majority of *L. brevis* strains tested, with the only exception of the ALBTQ4 and MCS/I strains, which also lack the *AgDI* gene.

Contrary to the description of the *AgDI* and *tdc* genes in two close operons in a strain of *P. pentosaceus* (Lucas et al., 2007), none of the strains tested here, other than those of the species *L. brevis*, harbour the genetic determinants for TDC.

In agreement with a previous report (Costantini et al., 2006), the presence of *hdc* gene in *L. hilgardii* 5211 strain isolated from wine was confirmed. The *hdc* gene was also detected in *L. casei* 18 isolated from cider. These two strains were not putrescine producers. Tyramine and histamine are the most represented BA in wine and cider. Tyramine has toxicological properties and is presumed to play an active role in the development of migraine, hypertension, psychological depression, schizophrenia, and Parkinson disorder (Branchek and Blackburn, 2003). Histamine has been associated with headache, allergies, and hypotension (Maintz and Novak, 2007). In the present investigation, most tyramine production observed is ascribable to *L. brevis* (Table 3). Furthermore, our results indicate (Table 3) that two strains belonging to different species of

Lactobacillus, *L. hilgardii* 5211, isolated from wine, and *L. casei* 18, isolated from cider, also biosynthesize histamine, in agreement with their genetic pattern. Histamine production by *L. casei* has been previously demonstrated (Garai et al., 2007).

3.4. Conclusion: risk assessment and metabolic considerations

From an oenological standpoint, the presence of *AgDI* gene in the considered *L. brevis* strains does not constitute a risk for putrescine generation since the amino acid agmatine, the precursor of the pathway, is present in very limited concentrations in wine (Buňka et al., 2012). On the contrary, arginine concentration is very high during and after alcoholic fermentation, ranging from a few hundred mg/L to 2.4 g/L (Spayd and Andersen-Bagge, 1996). Although in must arginine is mostly metabolized by yeasts during vinification, significant amounts are still present at the end of the alcoholic fermentation (Liu and Pilone, 1998). In addition, Manca de Nadra et al. (1999) reported that arginine is quantitatively one of the most important amino acids obtained by the proteolytic activity of *O. oeni* on the nitrogenous macromolecular fraction of wines, and consequently, arginine is generally available for wine lactobacilli metabolism.

As mentioned previously, two possible pathways can play a role in the conversion of arginine to putrescine (Fig. 1): (a) ADI pathway followed by ornithine decarboxylation (ADI + ODC) and (b) arginine decarboxylation plus AgDI route (ADC + AgDI).

In the ADI–ODC pathway, most ornithine produced is exchanged with new arginine in the ARG/ORN antiport system. This membrane-bound protein ensures the continuity of the ADI route and therefore of ATP generation via the formation of carbamoyl-phosphate, thus solving energy problems for the bacterial cells. Furthermore, it ensures pH control as well, due to the deimination of arginine to citrulline generating ammonia. This constitutes a very important homeostatic factor in LAB whose energy metabolism is chiefly based upon lactic fermentation and hence is acid generating. Moreover, this strategy can also usefully allow a better survival of lactobacilli (not as acid tolerant as oenococci) in the very acidic wine environment.

In the ADC + AgDI route, a proton gradient is created due to arginine/agmatine antiport systems. This gradient constitutes a proton motive force to fulfill cellular works such as the uptake of nutrients against concentration gradient; furthermore, 2 mol of ammonia are released ensuring pH buffering, but in this pathway, there is an ATP mole less than in the ADI pathway (the ATP derived from carbamoyl phosphate).

In the present investigation, only *P. pentosaceus* CM3/29 showed to be able to produce putrescine from arginine, suggesting that this strain, like *Weissella halotolerans* (Pereira et al., 2009), possesses both ADI and ODC enzymes, as indicated by its ability to biosynthesize putrescine from arginine, citrulline, and ornithine. The AgDI pathway, as showed by the conversion of agmatine to putrescine, seems also to be present. However, it should be noted that the presence of the *adc* gene could not be determined. Further investigations of this bacterial strain are needed to both definitively assess its potential risk in oenology and to elucidate its interesting genetic and physiological features.

It is also important to consider that the toxic effect of most BA (especially tyramine and histamine) can be potentiated by putrescine (Al Bulushi et al., 2009). Since in this study most of the *L. brevis* strains analyzed were also able to produce tyramine, putrescine should be regarded not only as a molecule altering the wine organoleptic properties but also as a factor enhancing the risks of health effects exerted by tyramine on humans (headache and hypertension). Therefore, there is a need for an accurate screening of the strains that should be used as starters for MLF, especially when dealing

with *L. brevis* and with ADC-possessing strains. On the contrary, the majority of the other *Lactobacillus* species does not biosynthesize BA, except two histamine-producing strains (*L. hilgardii* and *L. casei*), although none of them produce putrescine.

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