PCR methods for the detection of biogenic amine-producing bacteria on wine

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Abstract

Biogenic amines are low molecular weight organic bases frequently found in wine. Several toxicological problems resulting from the ingestion of wine containing biogenic amines have been described. Histamine, tyramine, phenylethylamine and putrescine are mainly produced in wine by the decarboxylation of histidine, tyrosine, phenylalanine and ornithine or arginine respectively by lactic acid bacteria action. Since the ability of microorganisms to decarboxylate amino acid is highly variable, being in most cases strain-specific, the detection of bacteria possessing amino acid decarboxylase activity is important to estimate the risk of biogenic amine content and to prevent biogenic amine accumulation in wine. Molecular methods for the early and rapid detection of these producer bacteria are becoming an alternative to traditional culture methods. PCR methods offer the advantages of speed, sensitivity, simplicity and specific detection of amino acid decarboxylase genes. Moreover, these molecular methods detect potential biogenic amine risk formation in wine before the amine is produced. Methods using quantitative PCR are efficient to enumerate biogenic amines-producing lactic acid bacteria in wine. The aim of the present review is to give a complete overview of the molecular methods proposed in the literature for the detection of biogenic amine-producing bacteria in wine. The methods can help to better control and to improve winemaking conditions in order to avoid biogenic amine production.

Keywords: wine, histamine; tyramine; phenylethylamine, putrescine; PCR methods, Real Time Quantitative PCR.

INTRODUCTION

Biogenic amines are organic bases endowed with biological activity that are frequently found in wine. They are produced mainly as a consequence of the decarboxylation of amino acids. Twenty-five different biogenic amines have been found in wines, being the putrescine the most abundant (Soufleros *et al.*, 1998).

High concentrations of biogenic amines can cause undesirable physiological effects in sensitive humans, especially when alcohol and acetaldehyde are present (Bauza *et al.*, 1995; Maynard and Schenker, 1996). More specifically, histamine is known to cause headaches, low blood pressure, heart palpitations, edema, vomiting, and diarrhea (Bauza *et al.*, 1995; Lehtonen, 1996). Tyramine and phenylethylamine can produce hypertension through the release of noradrenaline and norephedrine, respectively, which are vasoconstrictor substances (Forsythe and Redmond, 1974). Putrescine and cadaverine, although not toxic themselves, aggravate the adverse effects of histamine, tyramine, and phenylethylamine, as they interfere with the enzymes that metabolize them (ten Brink *et al.*, 1990; Straub *et al.*, 1995). Some amines, such as putrescine, may already be present in grapes (Broquedis *et al.*, 1989), whereas others can be formed and accumulated during winemaking. The main factors affecting its formation during vinification are free amino acid concentrations and the presence of microorganisms able to decarboxylate these amino acids. Amino acid concentration in grapes can be affected by fertilization treatments (Broquedis *et al.*, 1989) and in wines by winemaking treatments, such as time of maceration with skins, addition of nutrients, and racking protocols (Rivas-Gonzalo *et al.*, 1983; Zee *et al.*, 1983; Vidal-Carou *et al.*, 1990; Radler and Fäth, 1991 Lonvaud-Funel and Joyeux, 1994). The concentration of biogenic amines in wines depends on the presence and

the concentration of microorganisms with decarboxylase activity (Rivas-Gonzalo, *et al.*, 1983; Radler and Fäth, 1991; Vidal-Carou *et al.*, 1990; Zee *et al.*, 1993; Moreno-Arribas *et al.*, 2000) in addition to the precursors. The concentration of microorganisms is affected by physicochemical factors of wine such as pH, temperature, or SO_2 addition (Britz, *et al.*, 1990; Baucom, *et al.*, 1996).

Biogenic amine content in wines may be regulated in the future following the newly implemented regulations by the U.S. Food and Drug Administration (FDA) for scombroid fish (FDA). Upper limits for histamine in wine have been recommended in Germany (2 mg/L), Belgium (5-6 mg/L), and France (8 mg/ L) (Lehtonen, 1996). Switzerland has established a limit of 10 mg/L as a tolerable value for histamine in wine (Les autorités fédérales de la Confédération Suisse, 2002).

BIOGENIC AMINE PRODUCING MICROORGANISMS IN WINE

Many authors had implicated yeast and lactic acid bacteria as responsible for the formation of amines in wine (Zee *et al.*, 1983; Ough *et al.*, 1987; Vidal-Carou *et al.*, 1990; Radler and Fäth, 1991; Baucom *et al.*, 1996). However, data were complex and contradictory, which suggested that more defined studies were necessary to elucidate which kind of microorganism is the major contributor. Several researchers have demonstrated that the amine content increases with microbial growth, specifically with that of bacteria, with biogenic amine content suggested as an index of quality or of poor manufacturing practices (Zee *et al.*, 1983; Ough *et al.*, 1987; Radler and Fäth, 1991; Baucom *et al.*, 1996).

The biogenic amine production by 155 strains of lactic acid bacteria, 40 strains of acetic bacteria and 36 strains of yeast isolated from wine were analysed by Landete *et al.*, (2007a). They did not observe biogenic amine production by acetic bacteria and yeast; however, Landete et al. (2007a) found production of histamine, tyramine, phenylethylamine and putrescine by lactic acid bacteria. Moreover, a correlation of 100% was observed between biogenic amine production in synthetic medium and wine and between activity and presence of gene. With the results expose by these authors and others (Lonvaud-Funel and Joyeux, 1994; Le Jeune *et al.*, 1995; Gerrini *et al.*, 2002; Moreno-Arribas *et al.*, 2003; Landete *et al.*, 2005), we can consider than the lactic acid bacteria are the microorganisms responsible of histamine, tyramine, phenylethylamine and putrescine production in wine. The authors previously cited have showed as several wine bacterial species are capable of decarboxylating one or more amino acids, the bacterial ability to decarboxylate amino acids is highly variable and this ability seems to be strain-dependent rather than being related to species specificity. On the other hand, we can not consider that lactic acid bacteria, yeast or acetic bacteria are responsible for tryptamine and cadaverine in wine (Landete et al., 2007a). Therefore, in this work, we show molecular methods to detect producing lactic acid bacteria of histamine, tyramine, phenylethylamine and/or putrescine.

Histamine-producing lactic acid bacteria in wine

Histamine is the most important amine in food-borne intoxications, due to its strong biological activity (Cabanis, 1985). The study of histamine in wine is of particular interest as the presence of alcohol and other amines reportedly promotes its effects by inhibiting

134 human detoxification systems (Chu and Bjeldanes, 1981; Sessa *et al.*, 1984). A high
135 concentration of histamine in wine is caused by the presence of histidine decarboxylase in
136 some lactic acid bacteria (Le Jeune *et al.*, 1995; Lonvaud-Funel, 2001). There is great
137 interest in identifying and characterizing the bacteria that are able to produce histamine in
138 wine, in order to prevent its synthesis. In wines, high levels of histamine have been related
139 to spoilage by *Pediococcus* (Delfini, 1989). *Pediococcus* can be present in wine but usually
140 in a low proportion. It has been reported that some *Oenococcus oeni* strains are responsible
141 for histamine accumulation in wine (Castino, 1975; Le Jeune *et al.*, 1995; Guerrini *et al.*,
142 2002). The bacterial population in wine is a complex mixture of different species of lactic
143 acid bacteria (*Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*), with *O. oeni* as
144 the predominant species in wine during and after malolactic fermentation.
145 Landete *et al.* (2005b) showed an increase in histamine during the malolactic fermentation;
146 As the histamine concentrations found in must are very low or non- existent (Landete *et al.*,
147 2005b). So, it is normal that the concentrations of histamine must be attributed to strains of
148 lactic acid bacteria. Landete *et al.* (2005a) show that *O. oeni, Lb. hilgardii*, *Lb. mali*, *L.*
149 *mesenteroides* and *P. parvulus* can contribute to the histamine synthesis in wine, but the
150 main species responsible of high histamine production in wines seem to be *Lb. hilgardii*
151 and *P. parvulus*. Landete *et al.* (2005a) demonstrate in this work that histamine-producing
152 strains of *O. oeni* are very frequent in wine, in contrast with the paper of Moreno-Arribas *et*
153 *al.*, (2003), where no *Oenococcus* histamine producer strains were detected. However, the
154 work of Landete *et al.* (2005a) agrees with Guerrini *et al.* (2002) who found a high number
155 of *Oenococcus* histamine producers in wine, but low levels of histamine production in
156 general. Histamine-producing strains of *Lactobacillus*, *Pediococcus* and *Leuconostoc* are
157 also detected, but with lower frequencies. The results showed by Landete *et al.* (2005a) do

not disagree with the most common idea that *Pediococcus* spp. (Delfini, 1989) is the main organism responsible for histamine production, because although the percentage of *Pediococcus* histamine producers is low, some strains can produce the highest concentration of histamine. In addition, *Lb. hilgardii* is also capable of producing high levels of histamine.

More recently, a histamine producing strain (*Lactobacillus hilgardii* IOEB 0006) proved to retain or to lose the ability to produce histamine, depending on the culture conditions (Lucas *et al.*, 2005; 2008). Indeed, it was demonstrated that the *hdc*A gene in this strain was located on an unstable 80-kb plasmid, suggesting an acceptable cause for the great variability of histamine producing character among lactic acid bacteria.

Tyramine and phenylethylamine-producing lactic acid bacteria in wine

Tyrosine decarboxylase (TDC, EC 4.1.1.25) converts the amino acid tyrosine to the biogenic amine tyramine. Bacterial tyrosine decarboxylase have been only thoroughly studied and characterized in Gram-positive bacteria and, especially, in lactic acid bacteria implicated in food fermentation as cheese or wine.

The study of phenylethylamine production has received less attention, it have been demonstrated that enterococcal tyrosine decarboxylase is also able to decarboxylate phenylalanine, an amino acid structurally related to tyrosine, originating the biogenic amine phenylethylamine (Marcobal *et al.*, 2006a). Some authors such as Moreno-Arribas *et al.* (2000) and Landete *et al.* (2007) have demonstrated the simultaneous production of tyramine and phenylethylamine in lactic acid bacteria isolated from wine.

Tyramine production is not a general trait among lactic acid bacteria. Several *Lactobacillus brevis* tyramine-producing strains were isolated from wines (Moreno-Arribas *et al.*, 2000) and only 20 strains from 125 are showed to be tyramine producers (Landete *et al.*, 2007). This ability seems to be a general characteristic of *L. brevis* wine strains, however, for *L. hilgardii*, this character is strain-dependent (Landete *et al.*, 2007).

There are few reports concerning the ability of *L. plantarum* to produce tyramine in fermented food. Arena *et al.* (2007) report the identification and characterization of a tyramine-producing *L. plantarum* strain isolated from wine. These authors suggest that some *L. plantarum* strains are able to decarboxylase tyrosine in wine.

Putrescine producing lactic acid bacteria in wine

Putrescine can be synthesized either directly from ornithine by ornithine decarboxylase or indirectly from arginine via arginine decarboxylase. The arginine decarboxylase converts arginine in agmatine, thus agmatine deiminase and N-carbamoylputrescine amidohydrolase or putrescine carbamoyltransferase, biosynthetically convert agmatine to putrescine in the ADI pathway.

O. oeni strains exhibited the capability to produce putrescine by decarboxylation of ornithine (Guerrini et al., 2002). However, high concentrations of putrescine, as observed in some wines after malolactic fermentation (Soufleros et al., 1998), cannot result only from decarboxylation of free ornithine since its levels are usually low in wine. Indeed, ornithine may also be produced by microorganisms from the degradation of arginine, as above mentioned, the arginine is one of the major amino acids found in grape juice and wine.

Putrescine is the most abundant biogenic amine found in wine (Soufleros *et al.*, 1998) and agmatine is the most prevalent one in beer (Glória and Izquierdo 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* X1B, isolated from wine. Putrescine is formed from agmatine through a pathway that does not involve amino acid decarboxylase or formation of urea (Arena *et al.*, 2001).

While performing malolactic fermentation, Guerrini *et al.* (2002) demonstrated as *Oenococcus oeni* strains were very effective in forming putrescine from ornithine. The formation of putrescine from arginine by some strains has been also demonstrated by these authors. According to these authors, *O. oeni* can really and significantly contribute to the overall biogenic amine content of wines. Marcobal *et al.* (2004) identified a putrescine-producer *O. oeni* strains and sequenced its ornithine decarboxylase gene. Marcobal *et al.* (2004) have also shown that the presence of an *odc* gene is a rare event in Spanish wine *O. oeni* strains. Landete *et al.* (2008) did not find any microorganisms able to produce putrescine; however, strains of *Lb. hilgardii* and the *O. oeni* coming on from others laboratories were able to produce putrescine. Recently, Izquierdo-Cañas *et al.* (2009) found only two strains able to produce putrescine, both on synthetic medium and wine. The presence of the corresponding genes in these strains was also confirmed. According to these authors, these results suggest that *O. oeni* does not significantly contribute to the overall putrescine content of wines.

Broquedis *et al.* (1989) and Landete *et al.* (2005b) showed as the putrescine may be present in grapes. Thus, we suggest that both, microorganisms and grapes, can be the responsible of the presence of putrescine in wine.

DETECTION OF BIOGENIC AMINE PRODUCING BACTERIA IN WINE

During the last two decades, methods for the detection of biogenic amine-producing lactic acid bacteria isolated from wine have been developed. Several detection methods are based on differential growth media signalling the increase of the pH upon biogenic amine formation. Landete *et al.* (2005a) show an improved plate assay (H-MDAmod) and was compared with an enzymatic method, HPLC, and PCR of *hdc*. The conclusions drawn regarding the plate assay were: H-MDBmod is an appropriate medium to detect histamine production, because the histidine decarboxylase gene is always expressed in this medium. However, as in any plate assay the H-MDAmod medium is only suitable to detect strains of lactic acid bacteria producing histamine levels that are dangerously high for health, because its sensitivity is low, about 100 mg/L. The plate assay is simple, low cost and useful for determining lactic acid bacteria producing dangerous levels of histamine. It is possible to analyse the ability of many lactic acid bacteria to produce high amounts of histamine in a period of 2 days. Landete *et al.* (2005) suggest using H-MDAmod supplemented with natamycin and incubated under anaerobic conditions as an easy, routine system to detect the more dangerous lactic acid bacteria histamine producers in wines. Natamycin is an antibiotic that produces the death of yeast present in wine and anaerobic conditions do not allow acetic acid bacteria to grow. The lactic acid bacteria able to produce high levels of histamine are identified by a purple halo.

On the other hand, tyrosine decarboxylase activity was assayed in Tyramine Production Medium (TPM) (Landete *et al.* 2007b). Strains were streaked on TPM plates, and were considered tyramine positive if a clear zone below the grown cells developed because of

253 solubilisation of tyrosine. A correlation of 100% was observed between the results obtained
254 on TPM plates, in TPM broth, and the presence of a .positive *tdc* gene band.
255 Enzymatic methods, specific for histamine-producing bacteria, are based in the production
256 of hydrogen peroxide by the action of an oxidase enzyme on the histamine. The enzymatic
257 method improved by Landete *et al.* (2004) allow the detection of histamine concentrations
258 below 0.5 mg/L and can be employed in synthetic media and grape must and wines (white,
259 rose or red).
260 Among the different chromatographic techniques recommended for identification and
261 quantification of biogenic amine, thin layer chromatography (García-Moruno *et al.,* 2005)
262 and high performance liquid chromatography (Landete *et al.,* 2004) have been the most
263 useful. However, the detection of biogenic amine producing bacteria by conventional
264 culture techniques is often tedious and unreliable, exhibiting disadvantages such as lack of
265 speed, appearance of false positive/negative results, low sensibility, requirements for costly
266 and sophisticated equipment, as HPLC, or that only one biogenic amine is detected.
267 Early detection of biogenic amine-producing bacteria is important in the wine industry
268 because it could be a cause of wine poisoning. Therefore, the use of methods for the early
269 and rapid detection of these bacteria is important for preventing biogenic amine
270 accumulation in wine. Molecular methods for detection and identification of food-borne
271 bacteria are becoming an alternative to traditional culture methods. PCR and DNA
272 hybridization have become important methods and offer the advantages of speed, sensitive,
273 simplicity and specific detection of targeted genes. Genetic procedures accelerate getting
274 results and allow the introduction of early control measures to avoid the development of
275 these bacteria. Several studies describing loss of ability to produce biogenic amine in lactic
276 acid bacteria after prolonged storage or cultivation of isolated strains in synthetic media

277 have been reported (Lonvaud-Funel and Joyeux, 1994; Lucas *et al.*, 2005; Lucas *et al.*,,
278 2008). Molecular methods are fast, reliable and culture-independient, they are an interesting
279 alternative to solve the short comings of traditional methods. Moreover, molecular methods
280 detect potential biogenic amine risk formation in food before the amine is produced.
281 Although, an intrinsic disadvantage of PCR is the detection of non-viable cells. The ability
282 to distinguish between viable and non-viable organisms is crucial when PCR is used for
283 risk assessment of biogenic amine accumulation such as in food processing plant. Since
284 during the last decade several molecular methods have been described for the unambiguous
285 detection of bacteria capable to produce one or several biogenic amine, this article aims to
286 provide complete information about the PCR methods proposed in the literature for the
287 detection of biogenic amine producing bacteria.
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290 **Detection of histamine-producing bacteria by PCR**
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292 Histamine in wine is produced by gram-positive lactic acid bacteria during the
293 fermentation, rapid detection of histamine-producing bacteria is important for detecting and
294 preventing microbial contamination and high levels of histamine. Since histamine is the
295 decarboxylation product of histidine catalysed specifically by the enzyme histidine
296 decarboxylase (HDC; EC 4.1.1.22), it is possible to develop a molecular detection method
297 that detects the presence of the gene encoding this enzyme. Although bacterial HDC have
298 been thoroughly studied and characterized in different organisms and two enzyme families
299 have been distinguished, we talk about of Pyruvoyl-dependent HDC present in gram-

positive bacteria and especially lactic acid bacteria implicated in wine fermentation, such as *Oenococcus oeni* and *Lactobacillus hilgardii* among others.

To detect histamine-producing lactic acid bacteria, Le Jeune *et al.* (1995) designed several oligonucleotide primers (CL1, CL2, JV16HC, and JV17HC) (Table 1) based in the comparison of the nucleotide sequences of the histidine-decarboxylase genes (*hdc*) of *Lactobacillus strain* 30a and *C. perfringens*, and the amino acid sequences of these HDC and those of *L. buchneri* and *Micrococcus*. Alignment studies showed a high degree of relatedness among the *hdc* gene products of gram positive bacteria. Primer sets JV16HC/JV17HC, CL1/CL2, and CL1/JV17HC amplify by PCR internal fragments of 370, 150 or 500 pb, approximately, of the *hdc* gene, respectively. JV16HC/JV17HC primer set was shown to be suitable for the detection of all histamine-producing lactic acid bacteria analysed. The authors demonstrated that all strains identified as histamine producers gave a positive PCR result. Moreover, strains which did not exhibit HDC activity failed to give a signal in the PCR assay.

Since, the previously described PCR and colony hybridization methods (Le Jeune *et al.*, 1995) used purified DNA of isolated strains, seemed to be convenient for rapidly detecting histamine-producing bacteria, Coton *et al.* (1998b) in order to improve the rapidity of these tests to determine the frequency and distribution of histamine-producing bacteria in wines, applied them directly on wine samples. Coton *et al.* (1998b) used CL1 and JV17, a slightly modificated version of JV17HC primer (Table 1). They used CL1/JV17 primers to analyse the presence of histamine-producing bacteria directly on wine samples. Landete *et al.* (2005a) studied the ability of 136 wine lactic acid bacteria to produce histamine. They found that some lactic acid bacteria positive for histamine production were not amplified with JV16HC/JV17HC primers under the conditions originally described by Le Jeune *et al.*

(1995). By using the modified programme, histamine-producing lactobacilli, pediococci, and leuconostocs strains showed positive amplification by the JV16HC/JV17HC primers (Figure 1). Nevertheless, only 56% of the *O. oeni* histamine-producing strains showed amplification for *hdc*. Therefore, they modified the original CL1 primer sequence (Le Jeune *et al.*, 1995) and designed the CL1mod primer (Table 1). By using CL1mod/JV17HC primer set, all histamine producing *O. oeni* strains were positive in the PCR test. Constantini *et al.* (2006) used CL1/JV17HC primer set to study the potential to produce histamine in 133 lactic acid bacteria strains isolated from wines of different origins. Only one *L. hilgardii* strain was positive. Histamine production by *L. hilgardii* was confirmed trough TLC and HPLC analysis of the broth medium enriched with histidine. Since none the *O. oeni* strains analysed gave a positive PCR response, Constantini *et al. (*2006) designed a new primer set, PHDC1/PHDC2 (Table 1) based specifically on the *O. oeni hdc* sequence. The new PCR results confirmed the preceding data; none of the *O. oeni* strains analysed was able to produce histamine. Constantini *et al.* (2009) used the primer set PHDC1/PHDC2 with similar results for *Oenococcus oeni* commercial starter. These results were expected since for the starter manufacturers the absence of amino acid decarboxylase activity is now included in the selection criteria for the industrial preparation of starters. However, commercial yeast starter preparations contained lactic acid bacteria contaminants carrying *hdc* gene. These lactic acid bacteria were identified as *Lactobacillus parabuchneri* and *Lactobacillus rossiae*. Recently, the primer set JV16HC and JV17HC were used by Ruiz *et al.* (2009) to identify the presence of *hdc* gene in 8 *Oenococcus oeni* strains isolated from tempranillo wine samples in order to select those showing the highest potential as oenological starter cultures, none *Oenococcus oeni* strains were identified carrying the *hdc* gene. The primer sets JV16HC and JV17HC were also used by Izquierdo-Cañas *et al.*

(2009), they analysed the histamine production in 90 strains of *Oenococcus oeni*. Only two strains were able to produce histamine and the presence of *hdc* gene was also confirmed. The differences showed between the authors to detect the *hdc* gene can be attributed to the unstable plasmid where is located the *hdc* gene (Lucas *et al.,* 2005, 2008).

Detection of phenylethylamine and tyramine-producing bacteria by PCR

Only gram-positive bacteria have been described to produce tyramine and phenylethylamine. Lactic acid bacteria involved in wine processing can decarboxylate tyrosine to produce tyramine. These bacteria belong basically to genera *Lactobacillus*. Concerning tyrosine decarboxylases (TDC; EC 4.1.1.25), only enzymes using pyridoxal phosphate as a cofactor have been described.

It have been demonstrated that enterococcal TDC is also able to decarboxylate phenylalanine, an amino acid structurally related to tyrosine, originating the biogenic amine phenylethylamine (Marcobal *et al.,* 2006). Therefore, the oligonucleotide primers described for the detection of the *tdc* gene, are useful for the detection of phenylethylamine-producing bacteria. Landete *et al.* (2007b) demonstrates that phenylethylamine production is always associated with tyramine production in lactic acid bacteria.

Purification and microsequencing of the TDC of *Lactobacillus brevis* IOEB 9809 allowed Lucas and Lonvaud-Funel (2002) to design a degenerate primer set (P2- for/P1-rev) (Table 2) that was used to detect *tdc* gene fragments in three other *L. brevis* strains out of six screened. Marcobal *et al.,* (2005) checked the P2-for/P1-rev primer set and a new designed primer set (41/42) (Table 2) in order to choose one of them to be used in a multiplex PCR

assay. Since 41/42 set produced an unspecific fragment, the P2-for/P1-rev set was used in the multiplex PCR assay. The assay was useful by Marcobal *et al.* (2005) for the detection of tyramine-producing bacteria in control collection strains and in a wine lactic acid bacteria collection.

Constantini *et al.* (2006) also used the P2-for/P1-rev primer set to amplify the *tdc* gene of 133 strains isolated from wine and must. They also designed a new primer set, Pt3/Pt4 (Table 2), based on the *tdc L. brevis* and *E. faecalis* nucleotide sequences. The results obtained with both set of primers were the same. Only four positive strains were found, all belonging to the *L. brevis* species. The tyramine produced by these strains was quantified by HPLC, thus confirming the results observed by PCR. Similar results were observed with this primer set Pt3/Pt4 by Constantini *et al.* (2009), only *Lb. brevis* strains were found carrying the *tdc* gene. P1-rev primer was used in combination with p0303 primer (Lucas *et al.*, 2003) (Table 2, Figure 2) to analyse by PCR the presence of the *tdc* gene in 150 lactic acid bacteria strains isolated from wine (Landete *et al.*, 2007b). All the 32 strains that gave a positive PCR amplification were tyramine producers.

The non-detection of tyramine producing lactic acid bacteria in wine containing tyramine may be due to the moment of sampling. *Lb. brevis*, main responsible of tyramine concentration in wine, is present in wine during the end of alcoholic fermentation and early phases of malolactic fermentation.

Detection of putrescine-producing bacteria by PCR

Ornithine decarboxylase (ODC, EC 4.1.1.17) is a PLP dependent enzyme which catalyses the conversion of ornithine to putrescine at the beginning of the polyamine pathway.

Marcobal *et al.* (2004b) reported the identification of an ornithine decarboxylase gene (*odc*) in the putrescine-producing *O. oeni* RM83 strain by using 3/16 primer set (Table 3). These primers were designed based on two conserved domains showed by alignment of amino acid sequences of ODC proteins. The 3 and 16 primers were checked by Marcobal *et al.* (2005) to be used in a multiplex assay. In addition, they designed two new primers, 4 and 15 (Table 3), these four primers could be combined resulting in four primer sets, 3/4, 15/16, 3/16, and 4/ 15. The method was useful for the detection of putrescine-producing bacteria in control collection strains and in a wine lactic acid bacteria collection.

In a study of the ability of 133 strains of lactic acid bacteria isolated from wines to produce biogenic amine, for the detection of putrescine-producing lactic acid bacteria strains, Constantini *et al.* (2006) designed two new primers, AODC1 and AODC2 (Table 3), which were chosen by aligning nucleotide sequences of *odc* from *Lactobacillus* strain 30a and *O. oeni*. PCR assays were performed with various combinations of the four primers 3, 16, AODC1 and AODC2. Constantini *et al.* (2009) used the primer 16 and the primer AODC1 with similar results, none lactic acid bacteria were found carrying the *odc* gene. Recently, the primer set 3/16 were used by Ruiz *et al.* (2009) to identified the presence of *odc* gene in eight selected *Oenococcus oeni* strains, none *Oenococcus oeni* strains were identified carrying the *odc* gene. Izquierdo-Cañas *et al.* (2009) analysed the putrescine production in 90 strains of *Oenococcus oeni*. Only two strains were able to produce putrescine and the presence of *odc* gene was also confirmed with the primers set 3/16.

As above mentioned, the main biogenic amine associated with contamination, putrescine, can also be formed through another pathway that involves the deamination of agmatine.

Landete *et al.* (2010) demonstrated that a PCR specific method is a useful method to evidence the presence of bacteria able to form putrescine from agmatine. They show the first method to detect the genes *aguA* (agmatine deiminase) and *ptcA* (putrescine carbamoyltransferase) responsible of putrescine production from agmatine. The two gene implicated in the formation of putrescine from agmatine were detected in a *Lactobacillus hilgardii* isolated from wine using the two pairs of primers AguAF/AguAR (to detect *aguA*) and AguBF/AguBR (to detect *ptcA*) (Landete *et al.,* 2010).

Simultaneous detection of biogenic amine-producing bacteria by PCR

The multiplex PCR assay provides a technique that can be successfully used for the routine detection of strains that are potential producers of histamine, tyramine, phenylethylamine and putrescine in wine. All (two or three) target amines can be detected at one time in a multiplex PCR assay. Therefore, the multiplex PCR assays reduce reagent quantities and labor costs. Some multiplex PCR assays based on primers targeting amino acid decarboxylase gene sequences have been developed (Coton and Coton, 2005; Marcobal *et al.,* 2005, De las Rivas *et al.,* 2005; De las Rivas *et al.,* 2006). A multiplex PCR assay for the detection of histamine and tyramine and putrescine producing lactic acid bacteria from wine was developed by Marcobal *et al.* (2005). They selected three pairs of primers, the primer sets were JV16HC/ JV17HC (Table 1), P1-rev/P2-for (Table 2), and 3/16 (Table 3) for the detection of the *hdc*, *tdc* and *odc* genes, respectively.

Under the optimized conditions, the assay yielded DNA fragments of 367, 924, and 1446-bp DNA of *hdc*, *tdc*, and *odc* genes, respectively. For multiplex PCR, conditions were as

described for the uniplex reaction except that the relative concentration of the primers was optimized by checking increasing or decreasing primer concentration. When the DNA of several target organisms was included in the same reaction, two or three corresponding amplicons of different sizes were observed. This assay was useful for the detection of biogenic amine-producing bacteria in control collection strains and in a wine lactic acid bacteria collection (Marcobal *et al.,* 2005). No amplification was observed with DNA from non-biogenic amine-producing lactic acid bacteria strains.

DETECTION OF LACTIC ACID BACTERIA PRODUCING BIOGENIC AMINES IN WINE BY REAL TIME QUANTITATIVE PCR

Real-time quantitative PCR (QPCR) is an efficient technique used to detect and count microorganisms in foods (Rudi *et al.,* 2002). During the past few years, diverse methods based on QPCR were proposed to determine populations of yeasts and bacteria in wine (Phister and Mills, 2003; Delaherche *et al.,* 2004; Pinzani *et al.,* 2004; Martorell *et al.,* 2005; Neeley *et al.,* 2005). QPCR has also been used to detect and count biogenic amine producing lactic acid bacteria in food (Fernandez *et al.,* 2006; Ladero *et al.,* 2008; Torriani *et al.,* 2008). The advantages of QPCR against other methods are: determine the population of bacteria producing biogenic amines, less time-consuming than regular PCR, continuous monitoring of the PCR amplification process could be used at any point in the manufacturing process and a high number of samples might be processed simultaneously. Here, we show a review about QPCR methods to detect and count biogenic amine producing lactic acid bacteria in wine.

Detection of lactic acid bacteria carrying *hdc* gene by QPCR

A method based on QPCR was developed by Lucas *et al.* (2008) to detect and count histamine producing lactic acid bacteria in wine. Primers hdcAf and hdcAr (Table 4) were designed by Lucas *et al.*, (2008) on the basis of the sequences of *hdcA* genes from *O. oeni* IEOB 9204, *Lactobacillus hilgardii* IOEB 0006, *Lactobacillus sakei* LTH 2076, *Lactobacillus* strain 30A, *Lactobacillus buchneri* DSM 5987, and *Tetragenococcus muriaticus* LMG 18498 that were available from databases. This primer set amplifies an 84-bp internal region of *hdcA* (Table 4). Optimal QPCR conditions allowed amplification of a PCR product with a melting temperature of 80.5°C ± 0.5°C (Table 4). This method makes it possible to detect as few as 1 histamine producing cell per ml of wine, even in the presence of polyphenols or of a large excess of yeasts in wine. Although the method was based on a standard curve made with *L. hilgardii* DNA, it is assumed that it was efficient to enumerate histamine producing *O. oeni* cells. Previous QPCR methods used to enumerate lactic acid bacteria in wine were significantly less sensitive (Delaherche *et al.*, 2004; Neely *et al.,* 2005). The threshold values obtained with standard samples correlated well with populations of histamine producing lactic acid bacteria in the range of 1 to 10^7 CFU/mL. Given that the maximum population of lactic acid bacteria expected in wine is 10^6 to 10^7 cells/mL during malolactic fermentation. This method could be employed to count histamine producing lactic acid bacteria at any stage of winemaking.

Lucas *et al.,* (2008) show a analyse of 264 wines collected in numerous wineries of the Bordeaux area during malolactic fermentation revealed that almost all wines were

contaminated by histamine producing lactic acid bacteria, exceeding 10^3 CFU per ml in 70% of the samples. The QPCR assay proposed by Lucas *et al.* (2008) does not discriminate between live and dead cells nor between functional genes and pseudogenes. The results suggest that the limiting factor for histamine production in most wines is not the population of histamine producing lactic acid bacteria. Therefore, the determination of lactic acid bacteria carrying the *hdc* gene would not allow the prediction of the final concentration of histamine in wine. However, it could help to predict the risk of histamine spoilage. The results showed by Lucas *et al.* (2008) suggest that the risk of histamine production exists in almost all wines and is important when the population of histamine-producing bacteria exceeds 10^3 per ml.

Detection of lactic acid bacteria carrying *tdc* gene by QPCR.

Nannelli *et al.* (2008) develop a QPCR method allowing enumeration of lactic acid bacteria producing tyramine in wines. Primers used for QPCR were designed in conserved regions of *tdc* genes identified after aligning nucleotide sequences available in databanks. Primers tdcf and tdcr (Table 4) were based on the alignment of sequences from *Lactobacillus brevis* (AAN77279), *Lactobacillus curvatus* (BAE02560, BAE02559), *Tetragenococcus halophilus* (BAD93616), *Carnobacterium divergens* (AAQ73505), *Enterococcus faecium* (CAH04395 and EAN10106), *Enterococcus faecalis* (AAM46082 and AAO80459) and *Lactococcus lactis* (CAF33980). This primer set amplifies a 103-bp internal region of *tdc* (Table 4). Optimal QPCR conditions allowed amplification of a PCR product with a melting temperature of 82.0°C ± 0.5°C (Table 4).

515 The presence of tyramine lactic acid bacteria was investigated in 102 samples collected
516 from 2006 vintage after must obtainment or at the end of alcoholic fermentation (AF) and
517 malolactic fermentation (MLF). Bacterial populations were rather low in must ($<10^2$
518 cells/mL), while they generally increased during AF and reached their maximum levels at
519 the end of MLF. The populations of lactic acid bacteria carrying the *tdc* gene remained
520 quite low ($<10^3$ cells/mL). Nannelly *et al.* (2008) observed that only wines containing more
521 than 10^3 tyramine-producing cells ml/L contained tyramine concentrations above 1 mg/L.
522 Moreover, a linear relationship seemed to exist between the level of tyramine and the
523 population of lactic acid bacteria carrying the *tdc* gene in the range of the dataset (1–6
524 mg/L) for 10^3 to $6 \cdot 10^3$cells ml/L.
525
526
527 **Detection of lactic acid bacteria carrying *odc* and/or *agdi* gene by QPCR**
528
529 Nannelli *et al.* (2008) develop a QPCR methods allowing enumeration of lactic acid
530 bacteria producing putrescine in wines. Primers used for quantitative PCR were designed in
531 conserved regions of *odc* and *agdi* genes identified after aligning nucleotide sequences
532 available in databanks. The odcf and odcr primers (Table 4) were designed from an
533 alignment of genes coding for the well characterized ODC of *O. oeni* RM83 (CAG34069)
534 and *Lactobacillus sp*. 30a (P43099) and four putative uncharacterized ODCs of
535 *Lactobacillus acidophilus* (AAT09142), *Lactobacillus johnsonii* (NP_965822),
536 *Lactobacillus gasseri* (ZP_00047186) and *Lactobacillus salivarius* (YP_535038). This
537 primer set amplifies a 127-bp internal region of *odc* (Table 4). Optimal QPCR conditions

allowed amplification of a PCR product with a melting temperature of 81.0°C ± 0.5°C (Table 4).

Primers agdif and agdir (Table 4) derived from the alignment of *Lactobacillus brevis* (ABS19477 and ABS19479), *Lactobacillus sakei* (AAL98713 and AAL98715), *Pediococcus pentosaceus* (ZP_00322658 and ZP_00322660), *E. faecalis* (NP_814483), *L. lactis* (AAK05795), *Streptococcus mutan*s (DAA04558), and *Listeria monocytogenes* (AAT02835 and AAT02837). This primer set amplifies a 90-bp internal region of *agdi* (Table 4). Optimal QPCR conditions allowed amplification of a PCR product with a melting temperature of 85.0°C ± 0.5°C (Table 4).

The level of putrescine correlated well with the population lactic acid bacteria carrying the *odc* gene as it was above 1 mg/L when these bacteria reached the threshold value of 10^3 cells/mL and it increased quite linearly with higher lactic acid bacteria populations. In contrast, no correspondence was denoted with the populations of lactic acid bacteria carrying the *agdi* gene that were always fewer than 100 cells/mL while putrescine concentration varied from 0 to 20 mg/L.

CONCLUSIONS

Although amino acid decarboxylases are not widely distributed among bacteria, species of many genera are capable of decarboxylating one or more amino acids. However, the ability of microorganisms to decarboxylate amino acids is highly variable. It depends not only on the species, but also on the strain and the environmental conditions. The molecular techniques offer fast, easy, and reliable methods for analysing wine samples (at any step in

562 the elaboration process) for the presence of biogenic amine producing bacteria. PCR assays
563 provide methods that can be successfully used for the routine detection of bacterial strains
564 potentially producers of histamine, tyramine and putrescine in wine. These procedures are
565 highly specific method, and their results are easy to interpret compared to others
566 conventional methods.
567 Analysis of wines by means of QPCR methods showed that biogenic amine producing
568 lactic acid bacteria form significant amounts of histamine, tyramine or putrescine (above 1
569 mg/L) when their populations exceed 10^3cells/mL (Nannelli et al., 2008; Lucas *et al.*,
570 2008). In contrast, populations of biogenic amine-producing lactic acid bacteria ranging
571 from 10^3 to 10^7 cells/mL were not correlated to increasing amounts of biogenic amine. It is
572 likely that production of biogenic amine in wine depends not only on the presence of more
573 than 10^3 biogenic amine-producing lactic acid bacteria per mL, but also on other parameters
574 of wine such as the availability of amino acid precursors, pH or duration of MLF as
575 previously suggested (Martin-Alvarez *et al.,* 2006). Determination of biogenic amine-
576 producing lactic acid bacteria in wine by QPCR is an appealing approach for predicting the
577 risk of biogenic amine accumulation. However, it cannot indicate the final concentration of
578 biogenic amine that will appear in wine.
579

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REFERENCES

Arena M.E., Fiocco D., Manca de Nadra M.C., Pardo I., Spano. G. (2007). Characterization of a *Lactobacillus plantarum* Strain Able to Produce Tyramine and Partial Cloning of a Putative Tyrosine Decarboxylase Gene. Curr. Microbiol., 55:205-210.

Arena M.E., Manca de Nadra M.C. (2001). Biogenic amine production by *Lactobacillus*. J. Appl. Microbiol., 90:158-162.

Baucom T.L., Tabacchi M.H., Cottrell T.H.E., Richmond B.S. (1996). Biogenic amine content of New York state wines. J. Food Sci., 51:1376-1377.

Bauza T., Blaisse A., Teissedre P.L., Cabanis J.C., Kanny G., Moneret-Vautrin D.A. (1995). Les amines biogènes du vin, métabolisme et toxicité. Bull. O. I. V., 767-768:42-67.

Britz T.J. Tracey R.P. (1990). The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos*. J. Appl. Bacteriol., 68:23-31.

Broquedis M., Dumery B., Boucard J. (1989). Mise en èvidence de polyamines (putrescine, cadaverine, nor-spermidine, spermidine, spermin) dans les feuilles et les grappes de *Vitis* V*inifera*. L. Connaiss. Vigne Vin., 23 :1-6.

Cabanis J.C. (1985). L´histamine et sa toxicité. Bull. O.I.V., 656 :1010-1015.

Chu C.H., Bejdanes L.F. (1981). Effect of diamines, polyamines and tuna fish extracts on the binding of histamine to mucin in vitro. J. Food Sci., 47:79-88.

Constantini A., Cersosimo M., del Prete V., Garcia-Moruno E. (2006). Production of biogenic amine by lactic acid bacteria, screening by PCR, thin layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. J. Food Protect., 69:391–396.

Constantini A., Vaudano E., Del Petre V., Danei M., Garcia-Moruno E. (2009). Biogenic amine production by contaminating bacteria found in starter preparations used in winemaking. J. Agric. Food Chem., 57:10664–10669

Coton E., Coton M. (2005). Multiplex PCR for colony direct detection of gram positive histamine- and tyramine-producing bacteria. J. Microbiol. Met., 63:296–304.

Coton E., Rollan G., Bertrand A., Lonvaud-Funel A. (1998a). Histamine producing lactic acid bacteria in wines: early detection, frequency and distribution. Am. J. Enol. Vitic., 49:199–204.

Coton E., Rollan G.C., Lonvaud-Funel A. (1998b). Histidine decarboxylase of *Leuconostoc oenos* 9204: purification, kinetic properties, cloning and nucleotide sequence of the *hdc* gene. J. Appl. Microbiol. 84:143–151.

De las Rivas B., Marcobal A., Muñoz R. (2005). Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. FEMS Microbiol. Lett., 244:272–367.

De las Rivas B., Marcobal A., Carrascosa A., Muñoz R. (2006). PCR detection of food bacteria producing the biogenic amines histamine, tyramine, putrescine and cadaverine. J. Food Prot., 69:2509–2514.

Delaherche A., O Claisse., A Lonvaud-Funel. (2004). Detection and quantification of *Brettanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. J. Appl. Microbiol., 97:910–915.

Delfini C. (1989). Ability of wine malolactic bacteria to produce histamine. Scien. Aliments., 9:413-416.

FDA. Fish and Fisheries Products Hazard and Control Guide. In *Food and Drug Administration*, 1st ed.; Office of Seafood: Washington, DC, 1996; p 69.

Fernández M., del Rio, B., Linares D.M., Martín M.C., Álvarez M.A. (2006). Real-time polymerase chain reaction for quantitative detection of histamine producing bacteria: use in cheese production. J. Dairy Scien., 89:3763–3769.

Forsythe W.L., Redmond A. (1974). Two controlled trials of tyramine in children with migraine. Dev. Med. Child. Neurol. 16:794-799.

García-Moruno E., Carrascosa A., Muñoz R. (2005). A rapid and inexpensive method for the determination of biogenic amines from bacterial cultures by thin-layer chromatography. J. Food Prot. 68:625–629.

Gloria M.B, Izquierdo-Pulido M. (1999). Levels and Significance of Biogenic Amines in Brazilian Beers. J. Food Compos. Anal., 12:129-136.

Guerrini S., Mangani S., Granchi L., Vincenzini M. (2002). Biogenic amine production by *Oenococcus oeni*. Current Microbiol., 44:374-378.

Izquierdo-Cañas P.M., Gómes Alonso S., Ruiz Pérez P., Seseña Prieto S., García Romero E., Palop Herreros M.L.L. (2009). Biogenic Amine Production by *Oenococcus oeni* Isolates from Malolactic Fermentation of Tempranillo Wine. J. Food Prot., 72:907-910.

Ladero V., Linares D.M., Fernández M., Alvarez M.A. (2008). Real time quantitative PCR detection of histamine-producing lactic acid bacteria in cheese: Relation with histamine content. Food Res. Inter., 41:1015–1019.

Landete J.M., Arena M.E., Pardo I., Manca de Nadra M.C., Ferrer S. (2010). Implicated bacterial genes in putrescine production from agmatine: detection, correlation with the activity and phylogenetic analyses. Int. Microbiol., (submitted).

682 Landete J.M., Ferrer S., Pardo I. (2004). Improved enzymatic method for the rapid
683 determination of histamine in wine. Food Add. Contam., 21:1149-1154.
684
685 Landete J.M., Ferrer S., Pardo I. (2005a).Which are the lactic acid bacteria responsible for
686 histamine production in wine?. J. Appl. Microbiol., 99:580–586.
687
688 Landete J.M., Ferrer S., Polo L., Pardo I. (2005b). Biogenic amines in wines from three
689 Spanish regions. J. Agri. Food. Chem., 53:1119-1124.
690
691 Landete J.M., Ferrer S., Pardo I. (2007a) Biogenic amine production by lactic acid bacteria,
692 acetic bacteria and yeast isolated from wine. Food Control., 18:1569–1574.
693
694 Landete J.M., Pardo I., Ferrer S. (2007b). Tyramine and phenylethylamine production
695 among lactic acid bacteria isolated from wine. Int. J. Food Microbiol. 115:364–368.
696
697 Le Jeune C., Lonvaud-Funel A., Ten Brink H., Hofstra van der Vossen J.M.B.M. (1995).
698 Development of a detection system for histidine decarboxylating lactic acid bacteria
699 on DNA probes, PCR and activity test. J. Applied Bacteriol., 78:316–326.
700
701 Lehtonen P. (1996). Determination of amines and amino acids in wine A review. Am. J.
702 Enol. Vitic., 47:127-133.
703

Les autorités fédérales de la Confédération suisse. *Ordonnance sur les substrates étrangères et les composants dans les denrées alimentaires (OSEC)*; Le Département federal de l'interieur: Paris, France, 2002; Annexe (art 2, al. 6), p 1068.

Lonvaud-Funel A. (2001). Biogenic amines in wines: role of lactic acid bacteria. FEMS Microbiol. Lett., 199:9-13.

Lonvaud-Funel A., Joyeux A. (1994). Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc oenos*. J. Appl. Bacteriol. 77:401–407.

Lucas P., Claise O., Lonvaud-Funel A. (2008). High frequency of histamine-producing bacteria in the enological environment and instability of the histidine decarboxylase production phenotype. Appl. Environ. Microbiol., 74:811-817.

Lucas P., Lonvaud-Funel A. (2002). Purification and partial gene sequence of the tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809. FEMS Microbiol. Lett., 211:85–89.

Lucas P., Landete J., Coton M., Coton E., Lonvaud-Funel A. (2003). The tyrosine decarboxylase operon of *Lactobacillus brevis* IOEB 9809: characterization and conservation in tyramine-producing bacteria. FEMS Microbiol. Lett. 229:65–71.

Lucas P., Wolken W.A.M., Claisse O., Lolkema J.S., Lonvaud-Funel A. (2005). Histamine-Producing Pathway Encoded on an Unstable Plasmid in *Lactobacillus hilgardii* 0006. Appl. Environ. Microbiol., 71:1417-1424.

Maynard L.S., Schenker V.J. (1996). Monoamine-oxidase inhibition by ethanol in vitro. Nature., 196:575-57

Marcobal A., de las Rivas B., Moreno-Arribas M.V., Muñoz R. (2004b). Identification of the ornithine decarboxylase gene in the putrescine-producer *Oenococcus oeni* BIFI-83. FEMS Microbiol. Lett., 239:213–220.

Marcobal A., de las Rivas B., Moreno-Arribas M.V., Muñoz R. (2005). Multiplex PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine-producing lactic acid bacteria in foods. J. Food Prot., 68:874–878.

Marcobal A., de las Rivas B., Muñoz R. (2006). First genetic characterization of a bacterial beta-phenylethylalanine biosynthetic enzyme in Enterococcus faecium RM58. FEMS Microbiol. Lett. 258:144–149.

Martín-Alvarez P.J., Marcobal A., Polo C., Moreno-Arribas M.V. (2006). Influence of technological practices on biogenic amine contents in red wines. Eur. Food Res. Technol., 222:420-424.

Martorell P., Querol A., Fernández-Espinar M.T. (2005). Rapid identification and enumeration of *Saccharomyces cerevisiae* cells in wine by realtime PCR. Appl. Environ. Microbiol., 71:6823–6830.

Moreno-Arribas M.V., Polo M.C., Jorganes F., Muñoz R. (2003). Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. Int. J. Food Microbiol., 84:117-23.

Moreno-Arribas V., Torlois S., Joyeux A., Bertrand A., Lonvaud-Funel A. (2000). Isolation, properties and behaviour of tyramine producing lactic acid bacteria from wine. J. Appl. Microbiol., 88:584-593.

Nannelli F., Claisse O., Gindreau E., de Revel G., Lonvaud-Funel A., Lucas P. (2008). etermination of lactic acid bacteria producing biogenic amines in wine by quantitative PCR methods. Letters in Applied Microbiology. Lett. Appl. Microbiol., 47:594-599.

Neeley E.T., Phister T.G., Mills D.A. (2005). Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. Appl. Environ. Microbiol., 71**:**8954–8957.

Ough C.S., Crowell E.A., Kunke R.E., Vilas M.R.S., Lagier S. (1987). A study of histamine production by various wine bacteria in model solution and in wine. J. Food Process. Preserv., 12:63-70.

774 Phister T.G., Mills D.A. (2003). Real-time PCR assay for detection and enumeration of
775 *Dekkera bruxellensis* in wine. Appl. Environ. Microbiol., 69:7430–7434.
776
777 Pinzani P., Bonciani L., Pazzagli M.,, Orlando C., Guerrini S., Granchi L. **(**2004). Rapid
778 detection of *Oenococcus oeni* in wine by real-time quantitative PCR. Lett. Appl.
779 Microbiol., 38:118–124.
780
781 Radler F., Fäth K.P. (1991). Histamine and other biogenic amines in wines. In Proceedings
782 of the International Symposium on Nitrogen in Grapes and Wine; Rantz, J., Ed.:
783 American Society for Enology and Viticulture: Davis, CA, pp 185-195.
784
785 Rivas-Gonzalo J.C., Santos-Hernandez J.F., Mariné-Font A. (1983). Study of the evolution
786 of tyramine content during the vinification process. J. Food Sci. 48:417-418.
787
788 Rudi K., Nogva H.K., Moen B., Nissen H., Bredholt S., Møretrø T., Naterstad K., Holck A.
789 (2002). Development and application of new nucleic acid-based technologies for
790 microbial community analyses in foods. Int. J. Food Microbiol., 78:171–180.
791
792 Ruiz P., Izquierdo P.M., Seseña S., Palop M.LL. (2009). Selection of autochthonous
793 *Oenococcus oeni* strains according to their oenological properties and vinification
794 results. Int. J. Food Microbiol., doi:10.1016/j.ijfoodmicro.2009.11.027
795

796 Sessa A., Desiderio M.A. Perin, A. (1984). Effect of acute ethanol administration on
797 diamine oxidase activity on the upper gastrointestinal tract of rat. Alcohol Clin. Exp.
798 Res., 8:185-190.
799
800 Soufleros E., Marie-Lyse B. Bertrand A. (1998). Correlation between the content of
801 biogenic amines and other wine compounds. Am. J. Enol. Vitic., 49:266-277.
802
803 Straub B.W., Kicherer M., Schilcher S.M., Hammes W.P. (1995). The formation of
804 biogenic amines by fermentation organisms. Zeits. Lebens. Unter. Fors., 201:79–82.
805
806 Ten Brink B., Damink C., Joosten H.M.L.J., Huis In't Veld J.H.J. (1990). Ocurrence and
807 formation of biologically active amines in foods. Int. J. Food Microbiol., 11:73–84.
808
809 Torriani S., Gatto S., Sembeni S., Tofalo R., Suzzi G., Belletti N., Gardini F., Bover-Cid S.
810 (2008). Rapid Detection and Quantification of Tyrosine Decarboxylase Gene (*tdc*)
811 and Its Expression in Gram-Positive Bacteria Associated with Fermented Foods
812 Using PCR-Based Methods. J. Food Prot., 71:93-101.
813
814 Vidal-Carou M.C., Izquierdo-Pulido M.L., Marine-Font A. (1990). Histamine and tyramine
815 in Spanish wines: their formation during the winemaking process. Am. J. Enol. Vitic.,
816 41: 160-167.
817
818 Zee J.A., Simard R.E., L'Heureux L., Tremblay J. (1983). Biogenic amines in wines. Am.
819 J. Enol. Vitic., 34:6-9.

FIGURES

FIGURE 1. Electrophoresis of *hdc* fragment PCR amplified with primer sets JV16HC/JV17HC. Lanes 1 and 20, 1 kb ladder; lane 2, *Lactobacillus buchneri* ST2A, (lane 3) negative control *Pediococcus pentosaceus* 136, (lane 4) *Oenococcus oeni* 4042, (lane 5) *O. oeni* 4023, (lane 6) *O. oeni* 4021, (lane 7) *O. oeni* 4047, (lane 8) *O. oeni* 4010, (lane 9) *O. oeni* 3996, (lane 10) *O. oeni* 4045, (lane 11) *P. parvulus* 339, (lane 12) *P. pentosaceus* 56, (lane 13) *P. parvulus* 276, (lane 14) *Lact. hilgardii* 464, (lane 15) *Lact. plantarum* 98, (lane 16) *Lact. paracasei* 364, (lane 17) *Lact. hilgardii* 5w, (lane 18) *Leuconostoc mesenteroides* 27, (lane 19) *Leuc. Mesenteroides* 86.

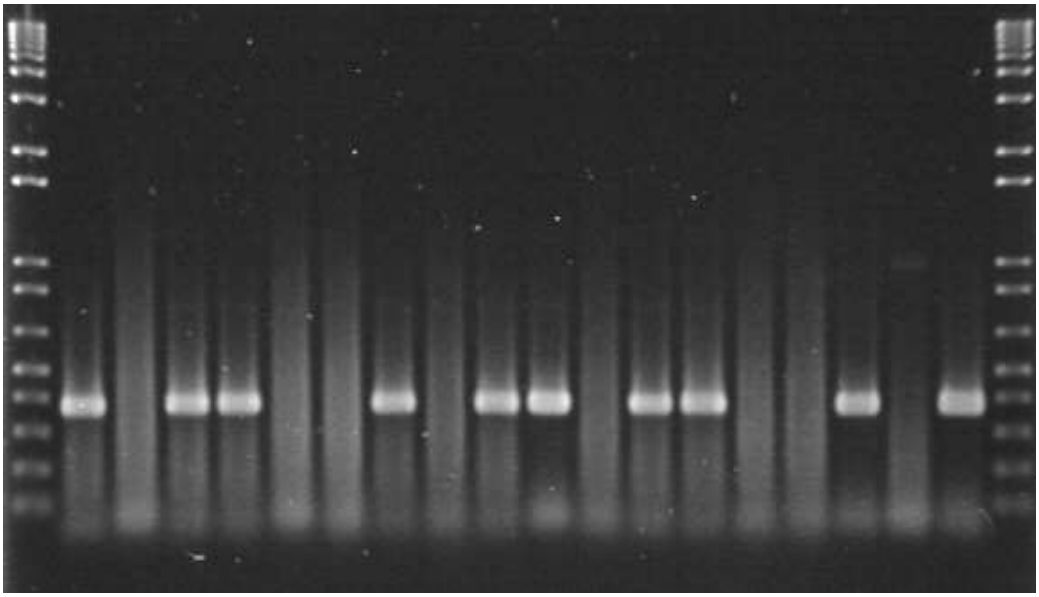
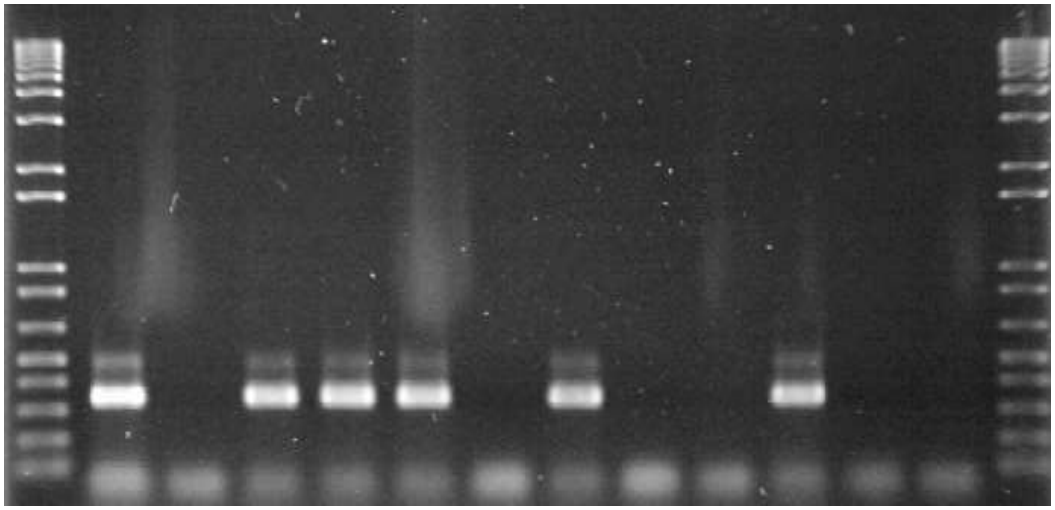


FIGURE 2. Electrophoresis of *tdc* fragment PCR amplified with primer sets p303 and P1-rev. Lanes 1 and 14: ladder; lanes 2 and 3: *Lb. hilgardii* 5w and 359; lanes 4, 5, 6, 8, 11: *Lb. brevis* J2, 9, 40, 84 and 106; lane 7: *L. curvatus*, lane 9: *Lb. casei*; lane 10: *Lb. mali*; lane 12 and 13: *Pediococcus parvulus* P339 and *Pediococcus pentosaceus* P136.



Figures 3. Electrophoresis of *odc* fragment PCR amplified with primer sets

Figures 4. Electrophoresis of multiplex PCR