Molecular Identification of Lactic Acid Bacteria Occurring in Must and Wine

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INTRODUCTION

Lactic acid bacteria (LAB) are a major bacterial group occurring in fermenting grape must and wine. Some LAB are well adapted to the milieu of must and wine, because of their relatively high acid and ethanol tolerance. Up to now, 25 different species of LAB have been identified in wine (König & Fröhlich, 2009; Mañes-Lázaro et al., 2008a, 2008b, 2009). Recently Mesas et al. (2011) reported the occurrence of new species of LAB isolated from Spanish wines. Beside several species of the genus Lactobacillus, some pediococci and leuconostocs as well as Oenococcus oeni and Weissella paramesenteroides could be found (Dicks & Endo, 2009). Other groups of the wine microbiota are acetic acid bacteria (AAB) (Guillamón & Mas, 2009; Wirth et al., 2011) and the eukaryotic yeasts (Bisson & Joseph, 2009). AAB are mostly undesirable and indicative of wine spoilage (Bartowsky & Pfund, 2009; Wirth et al., 2011).

Oenococcus oeni is the main bacterial starter culture which is responsible for the malolactic fermentation (MLF) in wine, facilitating decarboxylation of malic acid to lactic acid. This reaction leads to a microbial stabilisation, as only a few of these species are able to form unwanted metabolites in wine (Bartowsky & Pfund, 2009). One example is the formation of exopolysaccharides by Pediococcus damnosus or Leuconostoc mesenteroides (Monterosino et al., 2008). Due to high viscosity, these ropy wines are difficult to filter and need specific enzyme treatment (Blättel et al., 2011). However, another by-product of LAB, the biogenic amines (Landete et al., 2005; Garai et al., 2007; Kaschak et al., 2009; Vincenzini et al., 2009) are of more concern to consumers.

These low molecular nitrogen compounds are mainly formed by four enzymatic reactions: (a) decarboxylation, (b) transamination (c) reductive amination and (d) degradation of certain precursor amino compounds. Biogenic amines appear in different fermented foods and beverages such as fish, meat, cheese, wine and milk (Askar & Treptow, 1986). Usually, they are an indication of microbiological activity. Studies have shown that biogenic amines can be responsible for several health problems. For example histamine can induce headaches, hypertension and digestive problems while tyramine is often associated with migraine (Smits et al., 2008). These effects are strongly enhanced in wine, because of the presence of high levels of ethanol and occurrence of biogenic amines other than histamine. Ethanol inhibits monoamine oxidase (MAO), an enzyme which is responsible for the degradation of biogenic amines in humans (Smits et al., 2008). Thus, even much smaller concentrations of biogenic amines than those observed in cheese or fish; bear the potential for health problems in highly susceptible individuals.

Today, the most common biogenic amines in European wines are tyramine, histamine, phenylethylamine and putrescine (Leitão et al., 2005; Kaschak et al., 2009). Besides

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300
the production of biogenic amines by microorganisms such as LAB or yeast, some amines such as ethanolamine, ethylamine and putresine are already found in grapes (Del Prete et al., 2009). Various viticultural and oenological factors such as geographic region, grape variety, anti-fungal treatment of grapes, juice quality, must pH, fermentation activators, use of MLF starter cultures and storage on lees have a substantial impact on the content of biogenic amines in wine (Marques et al., 2008; Bach et al., 2011).

LAB species from the genera Lactobacillus, Leuconostoc and Pediococcus as well as Oenococcus are potential biogenic amine producers in wine (Guerrini et al., 2002; Moreno-Arribas et al., 2003; Landete et al., 2005). Most of these species can spoil wine and therefore can be indicative of poor winemaking and bad sanitisation practice. Strains of Oenococcus oeni which are used for commercial MLF starter cultures are generally selected for their inability to form biogenic amines.

For the quantitative determination of the biogenic amines in must and wine or culture supernatants, several HPLC-methods have been described (Onal, 2007; Garcia-Villar et al., 2009; Kaschak et al., 2009; Peña-Gallego et al., 2009). A quick, less tedious and less costly method for qualitative analysis is thin-layer chromatography (TLC) (Latorre-Moratalla et al., 2009).

An updated report on the concentration and distribution of biogenic amines in German wines from discounters was recently published (Kaschak et al., 2009). Because of the problems they may cause in wine, rapid identification of lactic acid bacteria is required.

MATERIALS AND METHODS

Isolation of bacteria from wine samples

Fifty wine samples (6 Chardonnay, 20 Spatburgunder and 24 Weißburgunder) from experimental winemaking by the Dienstleistungszentrum Ländlicher Raum-Rheinpfalz (DLR), Neustadt, Germany, were analyzed for the occurrence of biogenic amines forming lactic acid bacteria strains. The winemaking varied in the type of alcoholic fermentation (addition of yeasts or spontaneous fermentation), the type of malolactic fermentation (none, spontaneous or inoculated), addition of lysozyme or sulfite and fermentation under different pH values. Isolation of total LAB was achieved by cultivation on Man Rogosa Sharpe agar (MRS; De Man et al., 1960) and tomato juice agar (TJM). TJM consisted of peptone 2.5%, yeast extract 0.5%, glucose 0.5%, ammonium hydrogen carbonate 0.35%, K2HPO4 0.2%, Tween 80 0.1% (v/v), MgSO4 0.02%, MnSO4 0.005% and tomato juice (25%, v/v; Neu’s, Freinsheim, Germany). For inhibition of yeast growth, cycloheximide (0.002%, w/w; Sigma-Aldrich, Steinheim, Germany) and potassium sorbate (0.067%, w/w; Merck, Darmstadt, Germany) were added. Serial dilutions in sterile saline solution (0.9% NaCl, w/v; Roth, Karlsruhe, Germany) of each must sample were performed and 1 ml of each sample was mixed with 15 ml media. The mixture was poured into Petri dishes. The samples were incubated at 30°C until colonies appeared. Single colonies were reaped and transferred into the respective culture broths (MRS or TJM). The purification of the cultures was obtained by repeated streakings on either MRS or TJM agar. Pure bacterial isolates were cultured at 30°C in MRS or TJM broth till an optical density at 600 nm from 0.8 was reached. They were then used for the screening of biogenic amines production. The cultures were also stored in MRS or TJM media containing glycerol (20%, v/v; Roth, Karlsruhe, Germany) at -75°C.

Extraction of DNA from bacterial isolates

The cells from a bacterial culture (1 ml) were harvested by centrifugation (10 min, 13,000 x g). The pellet was then suspended in 1 ml sterile saline solution (0.9% NaCl, w/v) to remove the residual media compounds from the cells and centrifuged twice more for a further 10 min at 13,000 x g. For the extraction of total DNA the DNeasy® blood & tissue kit (Qiagen, Hilden, Germany) was used according to manufacturer’s instructions for gram-positive bacteria with minor modifications. The cells were incubated with lysozyme (20 mg/ml, Sigma-Aldrich, Steinheim, Germany) at 37°C for 2 h. The treatment with proteinase K was performed at 72°C for 30 min. Finally, the DNA was dissolved in 100 μl AE-buffer (DNeasy® blood & tissue kit; Qiagen, Hilden, Germany) and used immediately or stored at -20°C.

Identification of bacterial isolates

The identification of the bacterial isolates was performed by specifically amplified polymorphic DNA-PCR (SAPD-PCR; Pfannebecker & Fröhlich, 2008). The primer C-Not, including the NotI recognition sequence (5'-GCGGCCGC-3') with an additional adenine desoxyribonucleotide at 5' and a cytosine desoxyribonucleotide at the 3'end, was used. Twenty five strains of wine relevant LAB were used as references (Table 1). The reference strains were obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ, Brunswick, Germany). Amplification reactions and conditions were described by Pfannebecker & Fröhlich (2008). The SAPD-PCR was performed in a MJ Mini™ Thermocycler (Biorad, Munich, Germany).

After amplification, the samples were analysed by gel electrophoresis, using agarose gels containing 1.5% agarose (w/v) and 0.0001% sodium silicate (Na2SiO3, v/v) in 1 x TBE buffer. Electrophoresis was performed in a horizontal chamber (BioRad, Munich, Germany) at 65 V for approximately 3 h, using GeneRuler™ DNA Ladder Mix SM0331 (Fermentas, St. Leon-Rot, Germany) as molecular size marker. Gels were stained in an ethidium bromide solution (0.002 mg/ml; Roth, Karlsruhe, Germany) for 30 min and finally, gel bands were viewed under UV light in a Bio Vision CN 3000 darkroom and analyzed with Vision Capt 14.1 software (Vilber Lourmat, Eberhardzell, Germany). Comparing the banding patterns of the samples with the reference strains allowed the identification of the isolates (Table 1). Organisms with unknown banding patterns were indentified via 16S rDNA-sequencing after PCR carried out by Eurofins MWG Operon, Ebersberg, Germany.

Determination of biogenic amine formation

To obtain cell-free culture supernatants, 1 ml cell suspensions of the bacterial cultures were centrifuged twice for 10 min at 13,000 x g. Qualitative detection of the biogenic amines was achieved by high-performance thin-layer chromatography (hp-TLC) after derivatisation of the samples with dansyl...
chloride. A dansyl chloride solution in acetone (400 µl, 5 mg/ml, w/v; Sigma-Aldrich, Steinheim, Germany) was added to a mixture of 200 µl of cell-free culture supernatant and 200 µl of a saturated sodium hydrogen carbonate solution (NaHCO₃, Roth, Karlsruhe, Germany) and mixed thoroughly. After incubation in the dark for 24 h, the samples were mixed with 100 µl of a proline solution (100 mg/ml; Roth, Karlsruhe, Germany) to bind the remaining free dansyl chloride. To separate the amine derivates from the residual compounds, the sample was precipitated with 500 µl toluel (Merck, Darmstadt, Germany) for 30 min at -20°C. For further analysis, the toluel extract, including the dansyl derivates, was transferred to separate reaction tubes. For separation of biogenic amines, 5 µl of each sample were spotted onto hp-TLC plates (Silica 60 F₂₅₄, 10 cm x 20 cm, Merck, Darmstadt, Germany) at 10 mm intervals and within 10 mm distance from the lower edge of the plate. Before use, the silica TLC plates were activated for 1 h at 100°C. As a reference for the identification of biogenic amines in the samples, standard solutions of 11 biogenic amines (cadaverine, ethanolamine, ethylamine, hexylamine, histamine, isoamylamine, phenylethylamine, putrescine, serotonine, tryptamine and tyramine) were derivatised with dansyl chloride. Negative control water was added instead of the sample. For the chromatographic separation of the derivates two different solvent systems were used: (a) toluol, triethylamine and chloroform (10:7:6, v/v/v) for the separation of ethylamine, hexylamine, histamine, isoamylamine, serotonine and tyramine; and (b) chloroform, diethylether and triethylamine (6:4:1, v/v/v) for the separation of cadaverin, ethanolamine, putrescine and tryptamine. The solvents were loaded into a horizontal TLC chamber (Camag, Muttenz, Switzerland). Tween 80 (1%, v/v; Merck, Darmstadt, Germany) was added to the solvent in order to enhance the fluorescence signal (Linares et al., 1998). After chromatographic separation, the TLC plates were air-dried in the dark and the dansyl-derivates of the biogenic amine were viewed at 312 nm. To identify the biogenic amines in the samples, the spots were compared with Rf values of the standard biogenic amines. In addition, hue and colour intensity of the spots yielded a semi-quantitative estimation of several biogenic amines in the samples.

Culture supernatants of some strains were selected for high-performance liquid chromatography analyses to check the TLC results. A recently published HPLC method by Kaschak et al. (2009) was applied. A solid-phase extraction using Strata®SCX cation exchange cartridges was followed by pre-column derivatisation with ortho-phthaldialdehyd (OPA), gradient elution from an ODS-2 column (Bischoff, Leonberg, Germany) and fluorimetric detection. The culture media (MRS) were sterile-filtered through a 0.2 µm cellulose acetate membrane (Fischer, Schwerte, Germany) and analysed by HPLC. Samples were measured in duplicates with heptylamine as internal standard. Concentrations were calculated by the LC Solution Software (Shimadzu, Kyoto, Japan) using an external calibration for each biogenic amine (Kaschak et al., 2009).

RESULTS AND DISCUSSION

Identification of wine related lactic acid bacteria by SAPD-PCR

DNA isolated from the corresponding standard type strains of wine relevant LAB was used. Banding patterns of the amplified oligonucleotide fragments of standard type strains after SAPD-PCR are shown in Figure 1.

Combined with the length of the fragments given in Table 1, the SAPD-PCR is an excellent method for the identification of all wine relevant LAB in pure cultures.

With the results from Figure 1 and Table 1 it was possible to identify the wine related LAB by their fragment patterns. For a practical approval of the SAPD-PCR, the bacterial strains isolated from the wine samples were distinguished by this method. Figure 2 shows the fragment patterns of the different wine related LAB and certain bacterial isolates. It could be observed that strains from the same species showed an equally fragmented pattern. This pattern was similar to the type strains. To establish whether the same banding pattern could be used for the identification of the bacterial isolates, the 16S rDNA was amplified and sequenced. The results of this sequencing confirmed that isolates with the same fragment pattern belonged to the same species.

By comparison of the fragment pattern of the bacterial isolates with those of the wine related type strains of LAB, it was possible to identify the LAB isolates. Results of the identification of the bacterial isolates by SAPD-PCR are shown in Table 2. The majority of the strains were identified as Lactobacillus brevis (57 strains), followed by Oenococcus oeni (36 strains). Other species of the genus Lactobacillus such as Lactobacillus casei or Lactobacillus paracasei were present at a lower titer. Only one strain of Lactobacillus delbrueckii was found. Two species of the genus Pediococcus, namely Pediococcus damnosus and Pediococcus parvulus, could also be identified. A total of 121 LAB could be isolated from wine samples and identified by SAPD-PCR.

Bacterial strains with unknown fragment pattern were identified by 16S rDNA sequencing. By using this method, four strains of acetic acid bacteria could be distinguished. Landete et al. (2011) reviewed different molecular methods for the direct detection of biogenic amine-producing bacteria on wine. These methods were based mainly on the detection of certain decarboxylase genes. The bacteria could not be identified. The formation of biogenic amines of the LAB identified by SAPD-PCR in this study was verified by thin-layer chromatography.

Biogenic amine production by the bacterial isolates

Separation of a standard mixture of biogenic amines, after the derivatisation with dansyl chloride, using TLC is shown in Figure 3. In contrast to a TLC method for detection of biogenic amines described by Latorre-Moratalle et al. (2009) which uses a solvent system consisting of chloroform-diethyl ether-triethylamine (4:1:1, v/v/v) the separation of 11 wine relevant LAB could be determined by using two solvent systems as described above (see Figure 3). In addition, lower amounts of samples and derivatisation reagents were sufficient for the detection of biogenic amines (Lapa-Guimarães & Pickova, 2004). A solid phase extraction as mentioned by Meseguer Lloret et al. (2004) was not required...
to purify the amines for determination.

The biogenic amines formed by the isolates were identified by comparing them to the RF values of the reference compounds. Table 3 shows the distribution of biogenic amines produced by the bacterial isolates. In total, 71 strains, corresponding to 59% of all LAB-isolates were able to produce biogenic amines. The most prevalent amines were tyramine, histamine and ethylamine which were formed by 90%, 31% and 15% of the biogenic amines producers respectively. Phenylethylamine could only be detected in 6 isolates.

A formation of biogenic amines was observed for all 57 strains of Lactobacillus brevis (Fig. 4). The dominant biogenic amine found in the culture supernatant of Lactobacillus brevis was tyramine, which was formed by 96% of the strains. Histamine was produced by 19% of the Lactobacillus brevis strains. Furthermore, 19 isolates were able to produce several biogenic amines. For example, three strains produced ethylamine, phenylethylamine and tyramine. In the other species of the LAB-isolates a formation of biogenic amines was observed by half of these strains. Tyramine was produced by all the bacterial isolates except Lactobacillus paracasei which only formed histamine and ethylamine. Species such as Lactobacillus delbrueckii and most important Oenococcus oeni did not show any production of biogenic amines in the tested experimental wines (see Table 2).

For quantitative determination, several strains of biogenic amine producers were selected for analysis by HPLC. All biogenic amines detected by TLC could also be detected by HPLC. Tyramine was produced by all tested strains in concentrations from 1.22 to 8.89 mg/L. Histamine was found in higher concentrations (3.02 to 11.96 mg/l) in the culture media of all strains. The biogenic amine ethylamine was produced in the range from 4.61 to 6.72 mg/l. Phenylethylamine was found in lower concentrations (0.23 to 0.53 mg/l).

Besides the biogenic amines which were found by TLC,
Molecular Identification of LAB

### TABLE 1
Identification scheme for the type strains of wine relevant lactic acid bacteria based on SAPD-PCR fragment pattern.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Collection No.</th>
<th>SAPD-PCR fragment length [bp]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus bobalius</em></td>
<td>DSM 19674</td>
<td>3465, 1785, 1026, 847, 740, 652, 576, 535, 492</td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em></td>
<td>DSM 20054</td>
<td>2060, 1652, 1378, 914, 840, 740, 705, 597, 525, 479, 417, 378, 296</td>
</tr>
<tr>
<td><em>Lactobacillus buchneri</em></td>
<td>DSM 20057</td>
<td>2374, 2099, 1673, 1535, 1338, 1107, 987, 914, 862, 775, 694, 640, 573, 535, 492, 440, 394</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>DSM 20011</td>
<td>3290, 3000, 2459, 2171, 1695, 1464, 1310, 1089, 961, 885, 796, 740, 625, 471, 440, 333</td>
</tr>
<tr>
<td><em>Lactobacillus curvatus</em></td>
<td>DSM 20019</td>
<td>2844, 2237, 1416, 1107, 921, 792, 676, 579, 558, 475, 338</td>
</tr>
<tr>
<td><em>Lactobacillus delbrueckii</em></td>
<td>DSM 20074</td>
<td>2135, 1903, 1695, 1428, 1107, 914, 832, 771, 694, 647, 548, 471, 335</td>
</tr>
<tr>
<td><em>Lactobacillus diolivorans</em></td>
<td>DSM 14421</td>
<td>2403, 2269, 1739, 1452, 1249, 974, 869, 806, 735, 670, 551, 507, 488</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em></td>
<td>DSM 20052</td>
<td>2330, 1611, 1365, 1265, 1072, 892, 783, 744, 700, 630, 573, 535, 492, 440, 394</td>
</tr>
<tr>
<td><em>Lactobacillus fructivorans</em></td>
<td>DSM 20203</td>
<td>2844, 2188, 2060, 1500, 1217, 914, 711, 522, 414</td>
</tr>
<tr>
<td><em>Lactobacillus hilgardii</em></td>
<td>DSM 20176</td>
<td>2221, 2041, 1717, 1265, 1143, 921, 749, 658, 564, 522, 432</td>
</tr>
<tr>
<td><em>Lactobacillus jensenii</em></td>
<td>DSM 20557</td>
<td>1808, 1500, 914, 840, 753, 688, 647, 573, 488, 410</td>
</tr>
<tr>
<td><em>Lactobacillus kunkeei</em></td>
<td>DSM 12361</td>
<td>1855, 1488, 1324, 1072, 948, 869, 753, 688, 475, 338</td>
</tr>
<tr>
<td><em>Lactobacillus mali</em></td>
<td>DSM 20444</td>
<td>1808, 1517, 1351, 1162, 854, 806, 664, 579, 542, 410, 338</td>
</tr>
<tr>
<td><em>Lactobacillus nagelii</em></td>
<td>DSM 13675</td>
<td>2445, 2285, 2000, 1832, 1553, 1365, 1026, 885, 716, 532</td>
</tr>
<tr>
<td><em>Lactobacillus oeni</em></td>
<td>DSM 19972</td>
<td>2254, 2000, 1265, 1026, 900, 792, 721, 558, 352</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em></td>
<td>DSM 5622</td>
<td>3147, 2345, 2153, 1717, 1416, 1000, 832, 749, 711, 658, 579, 542, 475</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>DSM 20174</td>
<td>1378, 1233, 1040, 921, 847, 762, 744, 664, 597, 447</td>
</tr>
<tr>
<td><em>Lactobacillus svarum</em></td>
<td>DSM 19971</td>
<td>2625, 2459, 2345, 2221, 1338, 1200, 935, 819, 740, 630, 579, 400</td>
</tr>
<tr>
<td><em>Lactobacillus vini</em></td>
<td>DSM 20605</td>
<td>2768, 1652, 1440, 1310, 869, 792, 735, 597, 535, 376</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em></td>
<td>DSM 20343</td>
<td>1350, 1149, 1105, 880, 825, 576, 400, 332</td>
</tr>
<tr>
<td><em>Oenococcus oeni</em></td>
<td>DSM 20252</td>
<td>1315, 1200, 1124, 980, 920, 815, 704, 607, 421, 355</td>
</tr>
<tr>
<td><em>Pediococcus damnosus</em></td>
<td>DSM 20331</td>
<td>2255, 2106, 1850, 1520, 1406, 1297, 1000, 865, 800, 726, 435, 385, 353</td>
</tr>
<tr>
<td><em>Pediococcus parvulus</em></td>
<td>DSM 20332</td>
<td>980, 795, 678, 600, 554, 482, 444, 397, 353, 229</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em></td>
<td>DSM 20336</td>
<td>1263, 1022, 975, 930, 805, 685, 607, 558, 488, 424, 387</td>
</tr>
<tr>
<td><em>Weisella paramesenteroides</em></td>
<td>DSM 20288</td>
<td>2075, 1626, 1472, 1255, 1099, 935, 910, 805, 721</td>
</tr>
</tbody>
</table>

### TABLE 2
Identity of bacterial strains (LAB) isolated from wine.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Isolates</th>
<th>Biogenic amine producers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus brevis</em></td>
<td>57 (47.11)</td>
<td>57 (80.28)</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em></td>
<td>5 (4.13)</td>
<td>4 (5.63)</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>3 (2.45)</td>
<td>2 (2.82)</td>
</tr>
<tr>
<td><em>Lactobacillus delbrueckii</em></td>
<td>1 (0.83)</td>
<td>0</td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em></td>
<td>5 (4.13)</td>
<td>2 (2.82)</td>
</tr>
<tr>
<td><em>Pediococcus damnosus</em></td>
<td>9 (7.44)</td>
<td>4 (5.63)</td>
</tr>
<tr>
<td><em>Pediococcus parvulus</em></td>
<td>5 (4.13)</td>
<td>2 (2.82)</td>
</tr>
<tr>
<td><em>Oenococcus oeni</em></td>
<td>36 (29.75)</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Percentage of the total number of isolates (121 strains) are given in brackets.

*b* Percentage of the total number of biogenic amine producers (71 strains) are given in brackets.

### TABLE 3
Distribution of biogenic amines found by the TLC screening of the bacterial isolates for the production of biogenic amines.

<table>
<thead>
<tr>
<th>Biogenic amine</th>
<th>Total isolates</th>
<th><em>Lactobacillus brevis</em></th>
<th><em>Lactobacillus casei</em></th>
<th><em>Lactobacillus paracasei</em></th>
<th><em>Leuconostoc mesenteroides</em></th>
<th><em>Pediococcus damnosus</em></th>
<th><em>Pediococcus parvulus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyramine</td>
<td>68</td>
<td>55</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Histamine</td>
<td>16</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Ethylamine</td>
<td>11</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Total number of biogenic amines producing strains.

five additional amines were detected by HPLC: cadaverine, ethanolamine, isoamylamine, putrescine and tryptamine. Cadaverine, putrescine and tryptamine were detected in lower concentration (<1 mg/l), while ethanolamine and isoamylamine could be detected up to 4.01 mg/l.

The results of this study show that more than half of the LAB isolated from wine samples are able to form biogenic amines in two synthetic media (MRS or TJM). These media were selected to determine the potential capability of the bacterial isolates to produce biogenic amines under laboratory conditions. Eight different species could be identified by SAPD-PCR. This method allowed identification of all tested wine relevant LAB in pure culture due to their specific banding patterns. Over one third of the 121 strains were contributed to Lactobacillus brevis. A formation of biogenic amines by these LAB species was also observed by Landete et al. (2007). Other biogenic amine producers, such as Lactobacillus casei and Lactobacillus paracasei in biologically aged wines were described by Moreno-Arribas & Polo (2008). Although some strains of Oenococcus oeni can have the genetic capability for biogenic amine production (Izquierdo Canas et al., 2009), none of the 36 isolated strains showed a formation of biogenic amines in the tested growth media.

The TLC results could be confirmed by HPLC analysis. Beside ethylamine, histamine, phenylethylamine and tyramine, which were detected by TLC, ethanolamine and isoamylamine were found in higher concentrations. Ethanolamine was also found in musts from sterilised grapes and it is therefore not necessarily produced by microbial activities (Cecchini & Morassut, 2010). The HPLC results showed that wine bacteria have the capability to produce ethanolamine under laboratory conditions. According to the recent work of Kaschak et al. (2009) ethanolamine (7.7 mg/L in white wine, 10.1 mg/L in red wine) and isoamylamine (1.5 mg/l in white wine; 10.3 mg/l in red wine) were found in 57 German wines. Histamine and tyramine were the most abundant biogenic amines produced by the bacterial isolates from experimental wines, in contrast to the lower amounts found by Kaschak et al. (2009) in commercial wines of medium quality.

FIGURE 2
SAPD-PCR profiles (1.5% agarose and 0.0001% sodium silicate gel, negative image) of amplified DNA fragments from different bacterial strains isolated from wine samples (A. Pediococcus damnosus, B. Lactobacillus brevis; C. Lactobacillus paracasei and D. Oenococcus oeni). The DSMZ collection numbers and DNA fragment lengths of LAB type strains are given in Table 1. M = marker; - = negative control (water).

Molecular Identification of LAB

Separation of 11 wine relevant biogenic amines (10 mg/l; after derivatisation with dansyl chloride) by hp-TLC (Silica 60 F254 plate, negative image). The used solvent systems were (A) chloroform, diethylether, triethylamine (6:2:1, v/v/v) and (B) toluole, triethylamine, chloroform (10:7:6, v/v/v). Rf values are given in brackets (Running distance: 8 cm).

Separation of biogenic amines in the culture supernatant from different bacterial strains isolated from wine by hp-TLC (Silica 60 F254 plate) after derivatisation with dansyl chloride (negative image). The used solvent system was toluole, triethylamine, chloroform (10:7:6, v/v/v). - = negative control (water).

Molecular Identification of LAB

FIGURE 5
Separation of biogenic amines in two different white wines – with or without the addition of biogenic amines – by hp-TLC (Silica 60 F254 plate) after derivatisation with dansyl chloride (negative image). Some wine samples were diluted (1:10, with water) and added by different concentrations of biogenic amine standard (1% or 10%; the concentration of the standard was 100 mg/l). The used solvent system was toluene, triethylamine, chloroform (10:7:6, v/v/v). BA = biogenic amines; Ch = Chardonnay; WB = Weiβburgunder. -= negative control (water).

FIGURE 6
Separation of biogenic amines in two different red wines – with or without the addition of biogenic amines – by hp-TLC (Silica 60 F254 plate) after derivatisation with dansyl chloride (negative image). Some wine samples were diluted (1:10, with water) and added by different concentrations of biogenic amine standard (1% or 10%; the concentration of the standard was 100 mg/l). The used solvent system was toluene, triethylamine, chloroform (10:7:6, v/v/v). BA = biogenic amines; DF = Dornfelder; SB = Spätburgunder. -= negative control (water).
**Detection of biogenic amines in wine by thin layer chromatography**

It is important for a winemaker to respond to the occurrence of biogenic amines as soon as possible. Thus the applicability of a detection method during winemaking is of special interest. To this end, the thin layer chromatography is a cheap and relatively quick method for the determination of these undesired wine compounds. Figure 5 and 6 show that detection of biogenic amines in four different wines (Chardonnay, Dornfelder, Spätburgunder and Weiβburgunder) could be achieved by the development of a thin layer chromatography system.

The detection limit of the analysed biogenic amine standard was 1 mg/l for cadaverine, ethanolamine and putrescine and 10 mg/l for the rest of the tested amines. It could be observed that in all tested wines ethanolamine and/or putrescine was detected in higher concentration, according to the fluorescence intensity of the signal. Also a slighter signal of ethylamine was present in Chardonnay, Dornfelder and Spätburgunder. The presence of these amines in grapes was described by Del Prete et al. (2009).

**CONCLUSIONS**

This study indicated that LAB pose a possible risk for wine quality, due to their presence in many wine samples and their ability to produce biogenic amines and other undesired flavours. It could also pose a health risk, since tyramine, which is linked to the appearance of migraine, was the most prevalent biogenic amine under the test conditions. Contrary to other reports (Silla-Santos, 1996; Leitão et al., 2005; Landete et al., 2007) histamine does not seem to be the predominant biogenic amine in recent German discount or experimental wines. Cheap and quick detection of 11 biogenic amines was performed by thin-layer chromatography with two solvent systems. For these reasons TLC presents a suitable tool for winemakers to obtain a first qualitative overview of production of biogenic amines during must fermentation.

For bacterial identification, the SAPD-PCR (Fröhlich & Pfannebecker, Patent application WO/2007/131776) should be established as the best method for reproducible results. This method enabled the identification of the 25 tested wine relevant LAB in pure culture due to their specific banding patterns after PCR, which may allow prompt measures by winemakers.

**LITERATURE CITED**


Molecular Identification of LAB


