Molecular Identification of Lactic Acid Bacteria Occurring in Must and Wine

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A specifically amplified polymorphic DNA-polymerase chain reaction (SAPD-PCR), a molecular fingerprinting method based on the amplification of specific gene sequences, was applied in order to allow a rapid identification of lactic acid bacteria (LAB) occurring in must and wine. The applicability of this method was confirmed with isolated strains from different wine samples from the German wine growing region Palatinate. In addition, the formation of biogenic amines by the isolated strains was studied. More than half of the bacterial isolates from 50 red and white wine samples were able to produce biogenic amines. General health concerns related to biogenic amines in must and wine underline the need for an identification of these species. The majority of the isolated strains were assigned to the species *Lactobacillus brevis*. The major biogenic amines in the investigated wines which were detected by thin-layer chromatography and HPLC were tyramine, histamine and ethylamine.

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 *Weisella 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 & Henschke, 2008; 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 low concentrations of or no substrates for further MLF remain in the wine. Beside the positive aspects of LAB, they are also able to form unwanted metabolites in wine (Bartowsky, 2009). One example is the formation of exopolysaccharides by *Pediococcus damnosus* or *Leuconostoc mesenteroides* (Montersino *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 (Smit *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 (Smit *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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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 (Önal, 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 Spätburgunder 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 citrate 0.35%, K_2HPO_4 0.2%, Tween 80 0.1% (v/w), $MgSO_4$ 0.02%, $MnSO_4$ 0.005% and tomato juice (25%, v/w; 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/w; 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/w) 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 manufactor´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 (Na_2SiO_3, 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 ($NaHCO_3$, 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 toluol (Merck, Darmstadt, Germany) for 30 min at -20°C. For further analysis, the toluol 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 F_{254}, 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 fluorimetic 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,

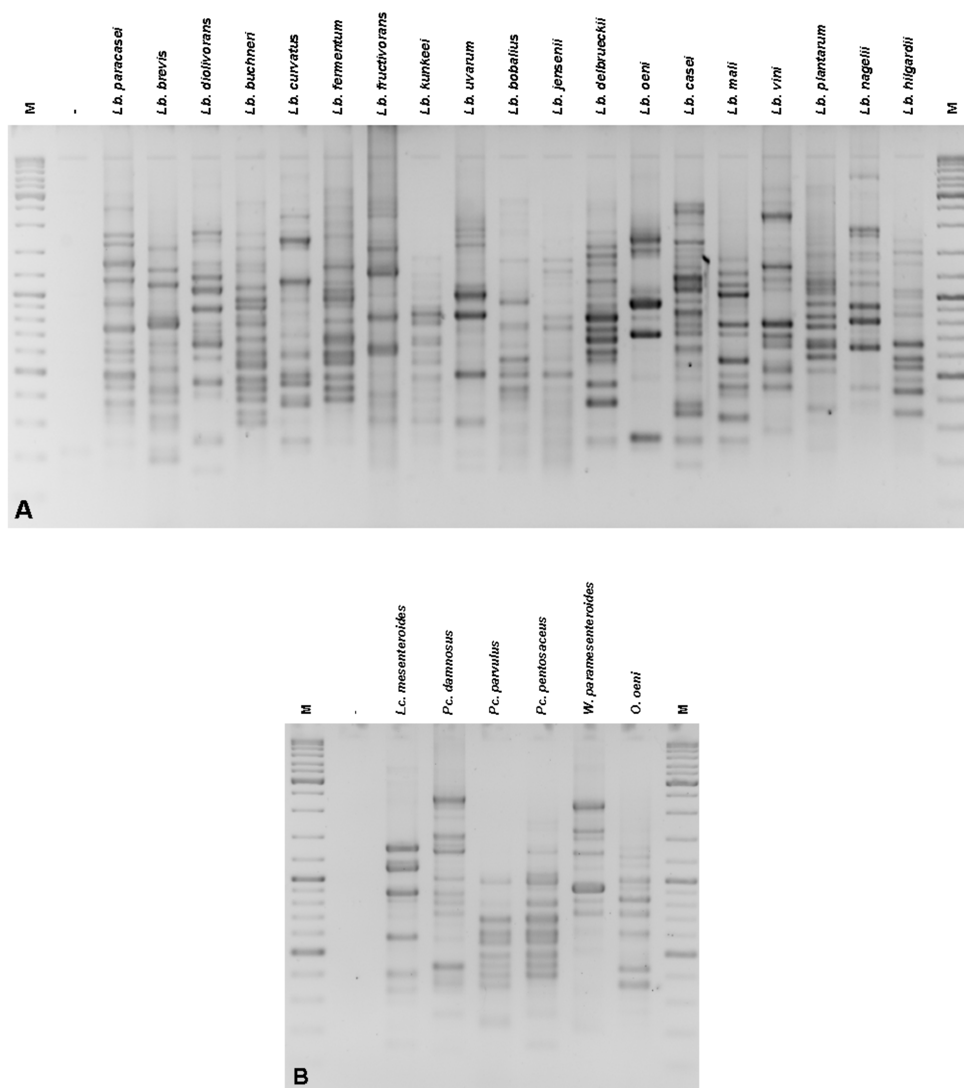

FIGURE 1

SAPD-PCR profiles (1.5% agarose and 0.0001% sodium silicate gel, negative image) of amplified DNA fragments from the 25 wine relevant lactic acid reference bacteria (A. the genus *Lactobacillus*, B. other LAB species). The DSMZ collection numbers and DNA fragment lengths of LAB species are given in Table 1. M = marker; - = negative control (water).

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

Bacterial species	Collection No.	SAPD-PCR fragment length [bp]
Lactobacillus bobalius	DSM 19674	3465, 1785, 1026, 847, 740, 652, 576, 535, 492
Lactobacillus brevis	DSM 20054	2060, 1652, 1378, 914, 840, 740, 705, 597, 525, 479, 417, 378, 296
Lactobacillus buchneri	DSM 20057	2374, 2099, 1673, 1535, 1338, 1107, 987, 914, 862, 775, 694, 609, 573, 535, 492, 440, 394
Lactobacillus casei	DSM 20011	3290, 3000, 2459, 2171, 1695, 1464, 1310, 1089, 961, 885, 796, 740, 625, 471, 440, 333
Lactobacillus curvatus	DSM 20019	2844, 2237, 1416, 1107, 921, 792, 676, 579, 558, 475, 338
Lactobacillus delbrueckii	DSM 20074	2135, 1903, 1695, 1428, 1107, 914, 832, 771, 694, 647, 548, 471, 335
Lactobacillus diolivorans	DSM 14421	2403, 2269, 1739, 1452, 1249, 974, 869, 806, 735, 670, 551, 507, 338
Lactobacillus fermentum	DSM 20052	2330, 1611, 1365, 1265, 1072, 892, 783, 744, 700, 630, 573, 535, 488
Lactobacillus fructivorans	DSM 20203	2844, 2188, 2060, 1500, 1217, 914, 711, 522, 414
Lactobacillus hilgardii	DSM 20176	2221, 2041, 1717, 1265, 1143, 921, 749, 658, 600, 564, 522, 432
Lactobacillus jensenii	DSM 20557	1808, 1500, 914, 840, 576, 525
Lactobacillus kunkeei	DSM 12361	1855, 1488, 1324, 1072, 948, 869, 753, 688, 647, 573, 488, 410
Lactobacillus mali	DSM 20444	1808, 1517, 1351, 1162, 854, 806, 664, 579, 542, 410, 338
Lactobacillus nagelii	DSM 13675	2445, 2285, 2000, 1832, 1553, 1365, 1026, 885, 716, 532
Lactobacillus oeni	DSM 19972	2254, 2000, 1265, 1026, 900, 792, 721, 558, 352
Lactobacillus paracasei	DSM 5622	3147, 2345, 2153, 1717, 1416, 1000, 832, 749, 711, 658, 579, 542, 475
Lactobacillus plantarum	DSM 20174	1378, 1233, 1040, 921, 847, 762, 744, 664, 597, 447
Lactobacillus uvarum	DSM 19971	2625, 2459, 2345, 2221, 1338, 1200, 935, 819, 740, 630, 579, 400
Lactobacillus vini	DSM 20605	2768, 1652, 1440, 1310, 869, 792, 735, 597, 535, 376
Leuconostoc mesenteroides	DSM 20343	1350, 1149, 1105, 880, 825, 576, 400, 332
Oenococcus oeni	DSM 20252	1315, 1200, 1124, 980, 920, 815, 704, 607, 421, 355
Pediococcus damnosus	DSM 20331	2255, 2106, 1850, 1520, 1406, 1297, 1000, 865, 800, 726, 435, 385, 353
Pediococcus parvulus	DSM 20332	980, 795, 678, 600, 554, 482, 444, 397, 353, 229
Pediococcus pentosaceus	DSM 20336	1263, 1022, 975, 930, 805, 685, 607, 558, 488, 424, 387
Weisella paramesenteroides	DSM 20288	2075, 1626, 1472, 1255, 1099, 935, 910, 805, 721

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

Bacterial species	Isolates[a]	Biogenic amine producers[b]
Lactobacillus brevis	57 (47.11)	57 (80.28)
Lactobacillus paracasei	5 (4.13)	4 (5.63)
Lactobacillus casei	3 (2.45)	2 (2.82)
Lactobacillus delbrueckii	1 (0.83)	0
Leuconostoc mesenteroides	5 (4.13)	2 (2.82)
Pediococcus damnosus	9 (7.44)	4 (5.63)
Pediococcus parvulus	5 (4.13)	2 (2.82)
Oenococcus oeni	36 (29.75)	0

[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.

Biogenic amine	Total isolates[a]	*Lactobacillus brevis*[a]	*Lactobacillus casei*[a]	*Lactobacillus paracasei*[a]	*Leuconostoc mesenteroides*[a]	*Pediococcus damnosus*[a]	*Pediococcus parvulus*[a]
Tyramine	68	55	2	4	2	3	2
Histamine	16	11	1	0	0	2	2
Ethylamine	11	8	0	2	0	0	1
Phenylethylamine	6	6	0	0	0	0	0

[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 off 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.

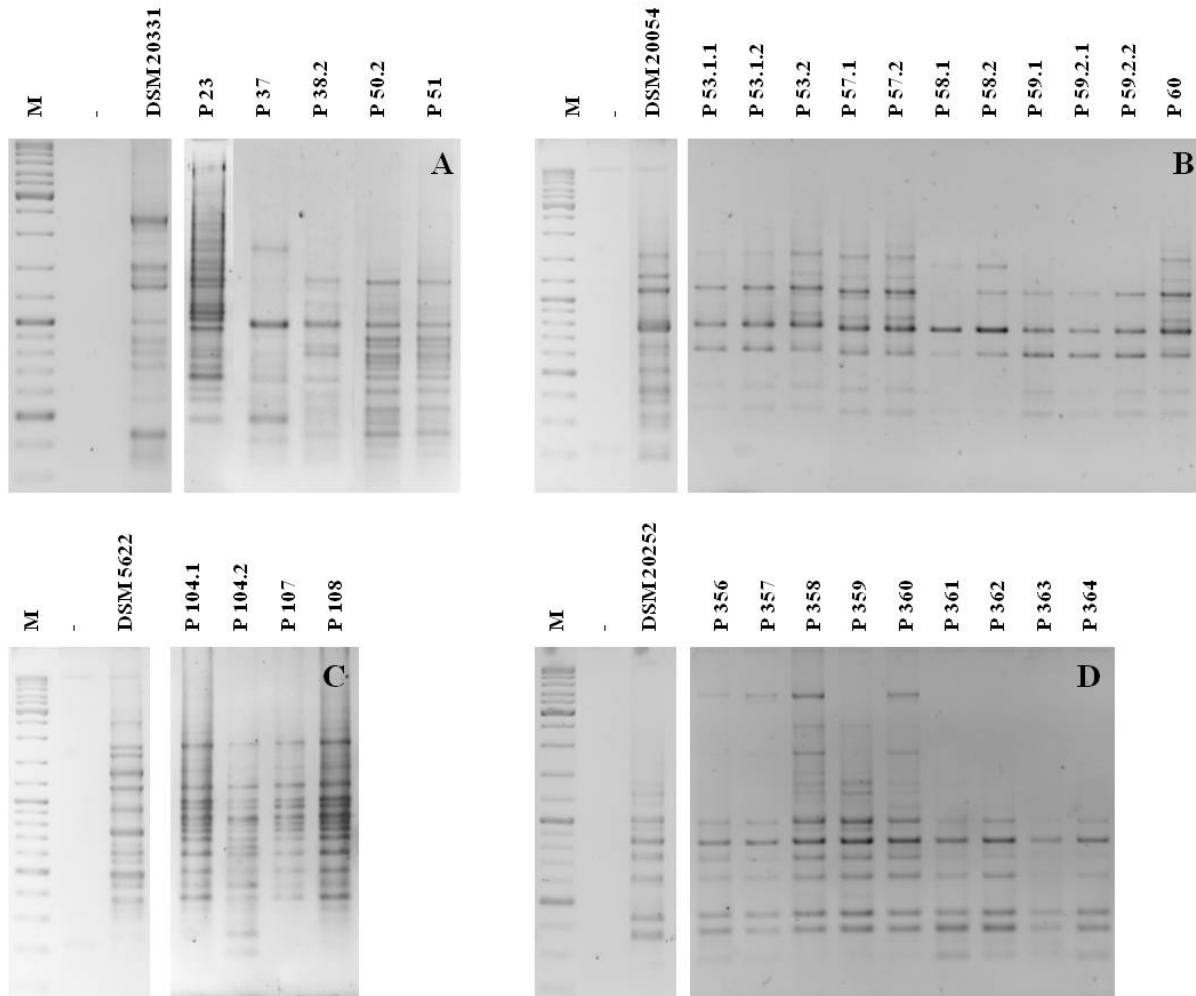

FIGURE 2

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).

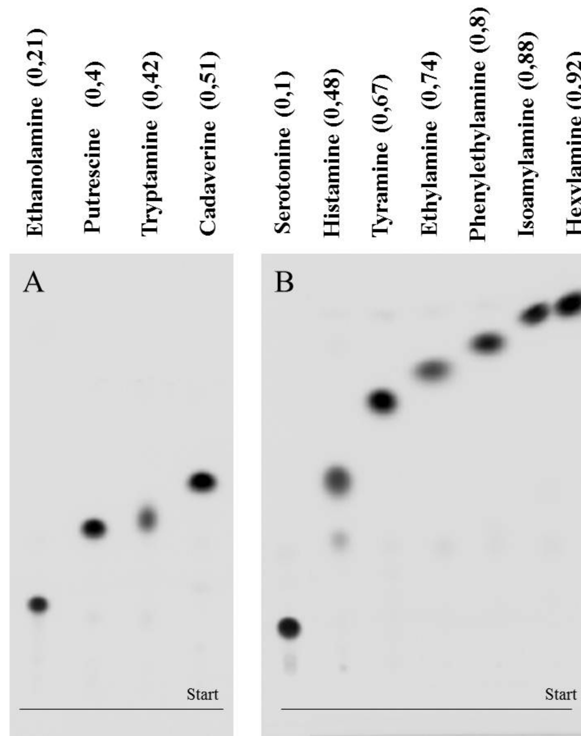

FIGURE 3
Separation of 11 wine relevant biogenic amines (10 mg/l; after derivatisation with dansyl chloride) by hp-TLC (Silica 60 F254 plate, negative image). The used solvent systems were (A) chloroform, diethylether, triethylamine (6:2:1, v/v/v) and (B) toluole, triethylamine, chloroform (10:7:6, v/v/v). Rf values are given in brackets (Running distance: 8 cm).

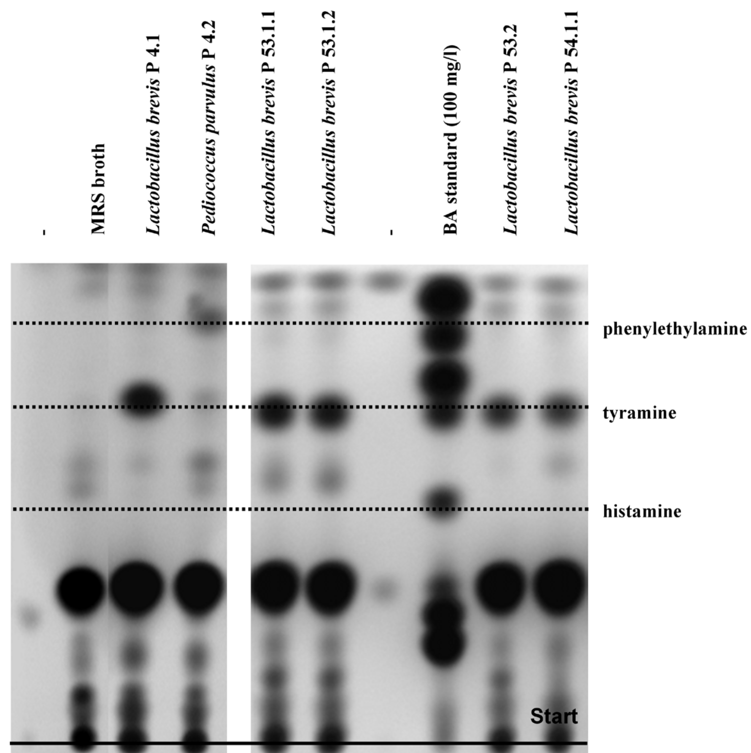

FIGURE 4
Separation of biogenic amines in the culture supernatant from different bacterial strains isolated from wine by hp-TLC (Silica 60 F254 plate) after derivatisation with dansyl chloride (negative image). The used solvent system was toluole, triethylamine, chloroform (10:7:6, v/v/v). - = negative control (water).

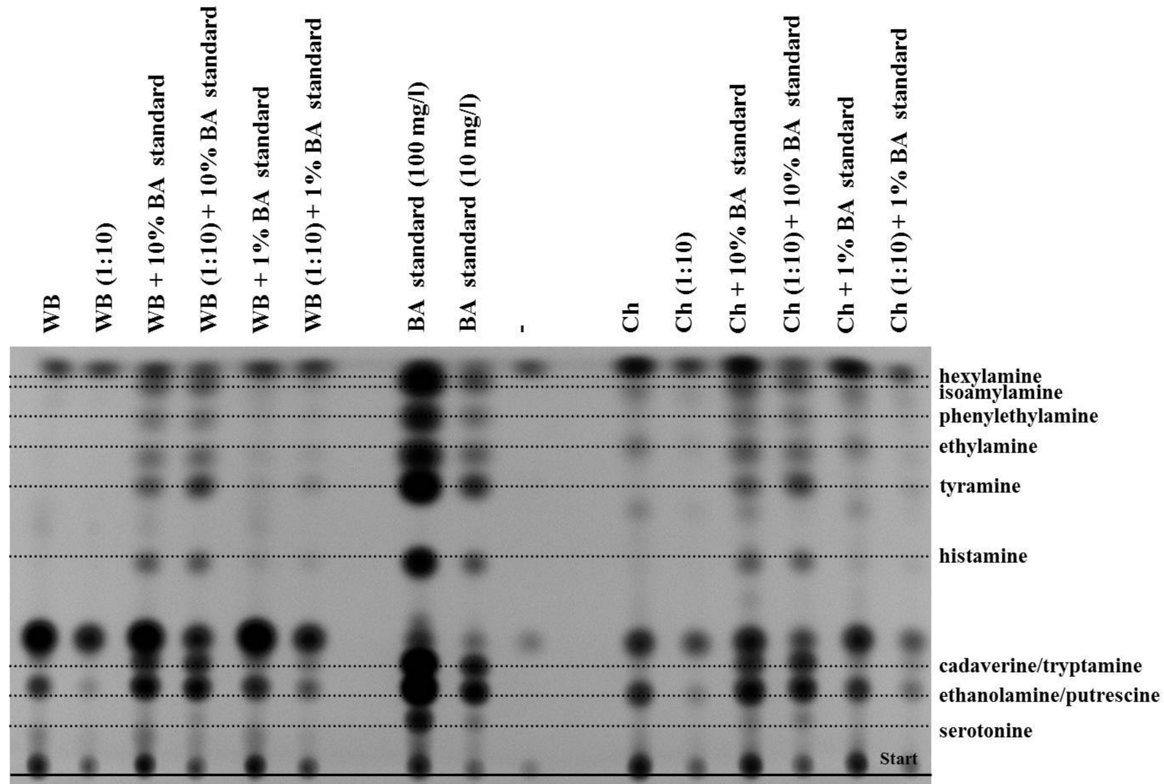

FIGURE 5
Separation of biogenic amines in two different white wines – with or without the addition of biogenic amines – by hp-TLC (Silica 60 F254 plate) after derivatisation with dansyl chloride (negative image). Some wine samples were diluted (1:10, with water) and added by different concentrations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

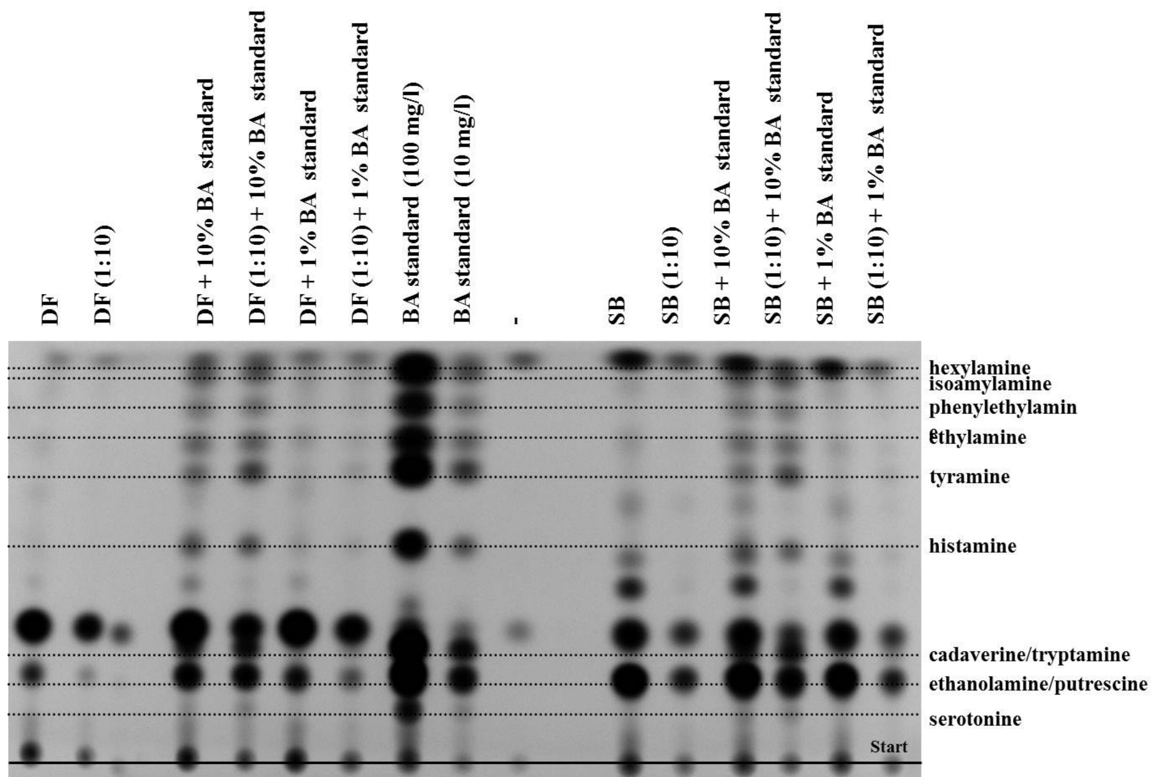

FIGURE 6
Separation of biogenic amines in two different red wines – with or without the addition of biogenic amines – by hp-TLC (Silica 60 F254 plate) after derivatisation with dansyl chloride (negative image). Some wine samples were diluted (1:10, with water) and added by different concentrations of biogenic amine standard (1% or 10%; the concentration of the standard was 100 mg/l). The used solvent system was toluole, 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 Weißburgunder) 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 Spätbugunder. 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 discounter 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.

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