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## ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA IN WINE PRODUCTION BY MALDI-TOF MS BIOTYPER

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The aim of this study was to identify lactic acid bacteria (LAB) in grapes, must and wines. A total amount of 90 samples including grape ( $n = 30$ ), must ( $n = 30$ ) and wine ( $n = 30$ ) were collected from vineyards in Slovakia. LAB were used cultured on MRS agar with subsequent confirmation with MALDI-TOF mass spectrometry (Bruker Daltonics). Altogether, 904 isolates were identified. Members of the family Lactobacillaceae were the most abundant in grape (60%), must (46%) and wine (51%). *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella* genera and 27 species of LAB were isolated from the examined samples. *Leuconostoc mesenteroides* spp. *mesenteroides* was the most abundant species in grape, must and wine.

**Keywords:** grape, must, wine, lactic acid bacteria, MALDI-TOF MS Biotyper

The wine production is a complex process in which the microorganisms, including lactic acid bacteria (LAB), contribute to the unique sensory characteristics of wine. In general, wine is expected to be an unavailable environment for microbiological growth due to the intrinsic and extrinsic factors of the products: low pH, high concentrations of ethanol and presence of sulfur dioxide (SO<sub>2</sub>) (Spano and Massa, 2006). The LAB associated with wine are represented by the phylum Firmicutes, class Bacilli, order Lactobacillales, families Lactobacillaceae and Leuconostocaceae (Garrity, Bell and Lilburn, 2004). The LAB of *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* genera may establish bacterial growth in wine (Miranda-Castilleja et al., 2016). The recognition of the groups of wine associated microbiota was established by a combination of methods for phylogenetic analysis and allowing defining the particular groups (Makarova et al., 2006). *Oenococcus oeni* is considered to be adapted for growth in the wine environment, hence, it is widely applied for use in commercial MLF starter cultures. Other *Lactobacillus* species have exhibited a capacity to survive in wine as well (Pozo-Bayon et al., 2005). *Lactobacillus plantarum* is another suitable candidate for application in starter cultures. The produced enzymatic complex of *L. plantarum* is specifically attributed to production of  $\beta$ -glucosidase, which could significantly influence the sensory characteristics of wine; thus, it is important in wine production (Mtshali et al., 2009). Description of protein profile with MALDI-TOF (Matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry is a prospective

method for identification of LAB. The results are compatible with those made by molecular methods. Therefore, MALDI-TOF can be considered a fast, accurate and low-cost method for identification of Gram-positive bacteria such as LAB (Rodríguez-Sánchez et al., 2016).

The aim of this study was to identify LAB collected from grape, must and wine with MALDI-TOF MS Biotyper during the technological process.

### Material and method

#### Materials

Samples of grapes ( $n = 30$ ) were taken gradually during the period of partial ripening of the fruit directly from the vineyards. Samples were put aseptically into polyethylene bags in August 2018 and stored at 8–10 °C for shipping to the laboratory. Grapes from vineyards of Central Slovakia and the Nitra wine region, and private vineyards were used. Rheinriesling ( $n = 3$ ), Welschriesling ( $n = 3$ ), Palava ( $n = 3$ ), Pinot Blanc ( $n = 3$ ), and Grüner Veltliner ( $n = 3$ ) of white varieties and Cabernet Sauvignon ( $n = 3$ ), Blaufränkisch ( $n = 3$ ), Blue Portugal ( $n = 3$ ), Merlot ( $n = 3$ ) and Pinot Noir ( $n = 3$ ) of red varieties were sampled. Samples of new wine “must” ( $n = 30$ ) were collected at the end of August 2018 and in the middle of September 2018 from the same winery as the grapes. Samples (apx. 100 mL) were collected into 200 mL sterile plastic bottles with screw caps and stored

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at 8–10 °C. An amount of 200 mL of each unfiltered wine ( $n = 30$  from same winery as grape and must) were collected before microfiltration) and stored at 4 °C in the refrigerator.

### Microbiological investigations

For microbiological analysis, the grape samples in physiological saline were processed in 24 h after collection. The Man Rogosa Sharpe agar (MRS, Conda, Spain) agar medium for LAB was applied for microbiological testing. The samples were diluted with sterile physiological saline (0.85%) and decimal dilutions were plated out onto MRS agar for incubation at 30 °C for 72 h microaerophilically. After incubation, the isolates were subcultured on Tryptone soya agar 90% (TSA, Basingstoke, UK) with MRS 10% at 30 °C for 24 h. Typical LAB colonies were identified with MALDI-TOF MS Biotyper (Bruker Daltonics, Germany) (Doan et al., 2012).

### Mass spectrometry identification of isolates

Qualitative analysis of LAB isolates was performed with MALDI-TOF Mass Spectrometry (Bruker Daltonics, Germany). Criteria for reliable identification were a score of  $\geq 2.0$  at species level (Doan et al., 2012).

### Statistical analysis

The statistical processing of the data obtained from each evaluation was done with Statgraphics Plus version 5.1 (AV Trading, Umex, Dresden, Germany). For each replication, the mean was calculated and data were log transformed.

## Results and discussion

In our study, the LAB counts isolated from grape berries ranged from 2.24 in Welchriesling to 3.33 log cfu.mL<sup>-1</sup> in Merlot. The LAB counts in must varied from 3.1 in Blue Portugal to 3.24 log cfu.mL<sup>-1</sup> in Welschriesling. For wine, the LAB counts were reported from 1.17 in Blue Portugal to 2.09 log cfu.mL<sup>-1</sup> in Palava (Table 1).

Winery environment is expected to be the main source of microorganisms associated with winemaking. Despite the significance of LAB in wine production, the limited number of studies describes their isolation from grapes (Fleet, 2001). The LAB counts were from 0.48 log cfu.mL<sup>-1</sup> to 2.06 log cfu.mL<sup>-1</sup> in Cabernet Sauvignon and Blaufränkisch, but negative results were obtained from white grape varieties (Kántor et al., 2015). Previously reported LAB counts for LAB ranged from 3.12 to 3.24 and 1.17 to 2.09 log cfu.mL<sup>-1</sup> for must and wine, respectively (Kántor et al., 2015). In total, five genera of LAB were identified in the present study (Table 2). *Lactobacillus* spp. was the most abundant (39%) while *Pediococcus* and *Weissella* the least abundant with 7% of distribution for each genera (Table 2).

Obligately homofermentative *L. alimentarius* with 36 isolates was the most abundant *Lactobacillus* (Table 3). The least abundant *Lactobacillus* spp. were *L. coryniformis* and *L. paracasei* with 10 isolates for each species. The predominant LAB were *Leuconostoc mesenteroides* ssp. *mesenteroides* with 251 isolates and *Lactococcus lactis* with 121 isolates. LAB species normally form a part of microbiota of grapes, musts and wines. Different LAB

**Table 1** Number of lactic acid bacteria in log cfu.mL<sup>-1</sup> (mean±SD)

Sample	Grape	Must	Wine
Rheinriesling	2.49±0.16	3.21±0.04	1.25±0.01
Welschriesling	2.24±0.11	3.24±0.02	1.27±0.04
Palava	2.52±0.16	3.20±0.01	2.09±0.03
Pinot Blanc	2.34±0.10	3.12±0.02	1.41±0.20
Grüner Veltliner	2.47±0.05	3.19±0.07	1.28±0.15
Cabernet Sauvignon	2.78±0.03	3.16±0.05	1.29±0.05
Blaufränkisch	2.74±0.15	3.17±0.04	1.27±0.04
Blue Portugal	2.72±0.14	3.10±0.02	1.17±0.07
Merlot	3.33±0.09	3.22±0.06	1.50±0.04
Pinot Noir	2.45±0.20	3.16±0.06	1.18±0.06

**Table 2** Number of isolated species from all samples together

Genera	No. of isolates	No. of species	% of isolates	% of species
<i>Lactobacillus</i>	356	21	39.38	77.78
<i>Lactococcus</i>	121	1	13.38	3.70
<i>Leuconostoc</i>	251	1	27.77	3.70
<i>Pediococcus</i>	119	2	13.16	7.41
<i>Weissella</i>	57	2	6.31	7.41
<b>Total</b>	<b>904</b>	<b>27</b>	<b>100</b>	<b>100</b>

species may be present in must and wines, and usually they include heterofermentative cocci of *Leuconostoc* and *Oenococcus*, homofermentative cocci of *Pediococcus* of Lactobacillaceae, homofermentative, facultative, and strict heterofermentative LAB of Lactobacillaceae family (Fugelsang and Edwards, 2007). In wine grapes of Australian vineyards, (Bae et al., 2006) there were detected *Lactococcus* and *Weissella* which is in line with our results.

*Lactobacillus acidophilus* in different wine samples of Slovak origin ranged from 1 to 105 cfu.mL<sup>-1</sup>

(Kačániová et al., 2012). *Lactobacillus crispatus* was captured by the RTQ PCR with sensitivity ranging from 1 to 105 cfu.mL<sup>-1</sup> and identification of *Lactobacillus salivarius* by the RTQ PCR method was done. In our study, only *Lactobacillus acidophilus* with 27 isolates was identified. The presence of *Lactobacillus brevis*, *L. casei*, *L. plantarum*, *L. hilgardii* and *Lc. mesenteroides* have been reported in the other studies (Ruiz et al., 2010) which corresponds to our results. A total of 382 isolates from grapes, 372 isolates from must and 150 isolates

from wine samples with score higher than 2 were identified (Table 3).

Altogether, 21 species of *Lactobacillus* were found in our study. The positive and negative properties of *Lactobacillus* sp. in wine have been intensively investigated in the recent years (Manes-Lazaro et al., 2009). Studies of wine-associated *Lactobacillus* species are necessary to recognize those responsible for wine spoilage. This fundamental information could be addressed through the identification and enumeration of the LAB at different stages of vinification (Dols-Lafargue, 2018).

In conclusion, the wine-making microbiota is associated with the microorganisms of grapes, must and winery environment; hence, the changes in the equilibrium of microbiota could alter the acceptance of wine with both the main quality and safety characteristics which may be affected (Capozzi et al., 2017). The microbiological composition of grape impacts the wine quality highlighting the effects of raw material in wine-making (Berbegal et al., 2019).

### Conclusions

In our study, ten different varieties ( $n = 90$ ) of grapes, musts and wines samples were evaluated. The numbers of isolated LAB ranged between the grape, must and wine samples with 904 isolates which were selected for further identification with MALDI-TOF Biotyper. In terms of LAB diversity, three family of LAB were isolated including Lactobacillaceae, Leuconostocaceae and Streptococcaceae represented by five different genera and 27 species. The most abundant species in our study were *Lactococcus lactis* and *Leuconostoc mesenteroides* spp. *mesenteroides* in all types of matrixes. MALDI-TOF MS Biotyper was the appropriate method for quick identification of LAB from grape, must and wine.

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**Table 3** Number of LAB isolates from grape, must and wine

Species of microorganisms	Grape	Must	Wine	Total
	No. of isolates			
<i>Lactobacillus acidophilus</i>	5	15	7	27
<i>Lactobacillus alimentarius</i>	10	20	6	36
<i>Lactobacillus amylolyticus</i>	15	0	10	25
<i>Lactobacillus brevis</i>	5	0	10	15
<i>Lactobacillus casei</i>	0	8	4	12
<i>Lactobacillus coryniformis</i>	3	5	2	10
<i>Lactobacillus delbrueckii</i> ssp. <i>delbrueckii</i>	5	8	5	18
<i>Lactobacillus fermentum</i>	18	0	0	18
<i>Lactobacillus fructivorans</i>	5	5	2	12
<i>Lactobacillus hilgardii</i>	5	5	2	12
<i>Lactobacillus nageli</i>	15	0	0	15
<i>Lactobacillus oligofermentans</i>	10	8	0	18
<i>Lactobacillus oris</i>	5	8	2	15
<i>Lactobacillus parabuchneri</i>	10	12	3	25
<i>Lactobacillus paracasei</i>	10	0	0	10
<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i>	10	6	0	16
<i>Lactobacillus paracasei</i> ssp. <i>tolerans</i>	10	5	0	15
<i>Lactobacillus pentosus</i>	15	0	0	15
<i>Lactobacillus plantarum</i>	5	6	5	16
<i>Lactobacillus saerimneri</i>	14	0	0	14
<i>Lactobacillus sakei</i>	12	0	0	12
<i>Lactococcus lactis</i>	35	65	21	121
<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i>	80	126	45	251
<i>Pediococcus acidilactici</i>	20	25	7	52
<i>Pediococcus pentosaceus</i>	20	35	12	67
<i>Weissella</i> spp.	15	10	7	32
<i>Weissella uvarum</i>	25	0	0	25
<b>Total</b>	<b>382</b>	<b>372</b>	<b>150</b>	<b>904</b>

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