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# BACTERIA AND YEASTS ISOLATED FROM DIFFERENT GRAPE VARIETIES

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## ABSTRACT

The aim of this study was to isolate and identify bacteria and yeasts in different grape samples. The samples were collected in September 2017. Used 13 grape samples in this study (9 white and 4 red) were from the local Slovak winemakers. Alibernet, Irsai Oliver, Dornfelder, Blue Frankish, Feteasca regala, Green Veltliner, Pálava, Mūller Thurgau, Rhinriesling, Cabernet Savignon, Pinot Blanc, Savignon Blanc and Welschriesling. Two cultivation media were used for detection of bacteri and yeasts in grape samples. Malt extract agar base (MEA) and Tryptone Soay agar (TSA) were used for the cultivation of bacteria and yeasts. Cultivation was performed by spread plate method. Ethanol/formic acid extraction procedure was used for preparation of samples. MALDI-TOF Mass Spectrometer (Microflex LT/SH) (Bruker Daltonics, Germany) was used for identification of bacteria and yeasts. In total, 8 genera of yeasts, 8 genera of Gram-negative bacteria and 10 of Gram-positive bacteria were identified. Together 333 isolates, yeasts, Gram-negative and Gram-positive bacteria were identified.

**Keywords:** bacteria; yeasts; grape; mass spectrometry

## INTRODUCTION

Different physical and chemical parameters of environment determine the growth of plants in geographical region (e.g., temperature, humidity, precipitation, soil nutrient concentrations and solar radiation) **(Droždž et al., 2015)**. These factors also have a significant impact on the biogeography of the bacteria and fungi in the ecosystems. Studies focused on the bacteria associated with grapes by directly sampling during the initial stage of fermentation of the wine must were underatken **(Bokulich et al., 2014; Nedemová et al., 2016)**. Although not previously determined, it stands to reason that the same

The most common bacteria of grapes were *Oenococcus oeni, Leuconostoc mesenteroides, Pediococcus parvulus, P. pentosaceus, P. damnosus,* and different species of *Lactobacillus* (*L. brevis, L. plantarum, L. fermentum, L. buchneri, L. hilgardii, and L. trichodes*) **(Fleet, 2007; Du Toit et al., 2010)**. Malolactic fermentation, in addition to deacidification, contributes the favor characteristics of wine and has a impact on microbial stabilization **(Pozo-Bayon et al., 2005)**.

Previous studies of grapes and grape musts microflora revealed valuable indigenous yeast strains, which could serve as the contributors to the regional character of wines specific to different winemaking regions **(Varela and Borneman, 2016; Raymond Eder et al., 2017)**.

Non-*Saccharomyces* were the predominant yeast species isolated at the early stages of the spontaneous fermentation of *Vitis vinifera* L. grape musts **(Padilla et al., 2016)**. Among these, *Hanseniaspora, Candida, Pichia,* and *Metschnikowia* were the most important genera **(Jolly et al., 2013; Varela and Borneman, 2016).** The population of non-*Saccharomyces* species decreases in fermentation processes and the wine yeast *Saccharomyces cerevisiae* completes the fermentation **(Albergaria and Arneborg, 2016)**. The ability of *S. cerevisiae* to replace non-*Saccharomyces* species is associated with its higher fermentative power, alcohol tolerance and secretion of killer-like compounds **(Albergaria and Arneborg, 2016)**. Previous studies provide an overview on yeast microbiota of *Vitis vinifera* L. grape musts **(Padilla et al., 2016)**, but still is less known about the microorganisms present on grapes from other species of *Vitis*. The potential existence of various grapevine and microbial species communities is an aim of research interest **(Wolfe and Dutton, 2015)**.

A rapid and high-throughput identification method based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced in bacterial taxonomy and in yeast and mold identification **(Pan et al. 2011; Hendrickx et al. 2011)**. MALDI-TOF MS idenfification is bases on measuring complex mixtures of proteins showing a unique fingerprint

for each species. Basically the ribosomal proteins, which are expressed at high level, the phenotypic technique is less influenced by expression variability **(Wieser et al. 2012)**. ,Nowaday, MALDI-TOF became an important method for microorganism identification.

The aim of our study was to find bacteria and yeasts from the surface of grape berries and identify them by MALDI-TOF Mass Spectrometry.

### Scientific hypothesis

The scientic hypothesis of this study was that the surfaces of different grape beries were contaminated with different bacterial and yeasts species, which could be found and identified with mass spectrometry.

## MATERIAL AND METHODOLOGY

### Grape collection samples

In total, 13 grape samples were used in experiment. Ripe grape bunches were collected into sterile polyethylene bags and transported to laboratory for microbiological analysis. The grape samples were collected from the Lesser Carpathian wine region. The grape samples of following varieties were investigated: Alibernet, Irsai Oliver, Dornfelder, Blue Frankish, Fateasca reagla, Grüner Veltliner, Pálava, Mūller Thurgau, Rheinriesling, Cabernet Savignon, Pinot Blanc, Savignon Blanc and Welschriesling. One sample consisted from one grape.

### Microbiological analyses of grape berries samples

Five grams of berries from each grape variety were diluted with 45 ml of sterile physiological saline (0.85%). Berries were stirred on a horizontal shaker for 30 minutes. After that, the dilutions of $10^{-2}$ and $10^{-3}$ were prepared for cultivation with spread plate method. A 0.1 ml of each dilution ($10^{-2}$, $10^{-3}$) was placed on the surface of a solid cultivation medium. Bacteria were cultivated on Plate count agar (PCA) (Oxoid, UK), yeasts on Malt extract agar base (MEA) (Oxoid, UK) supplemented with bromocresol green (0.020 $g.L^{-1}$) (Centralchem®, Slovakia). Bacteria were cultivated at 37 °C for 24 – 48 h in aerobic condition, but yeasts at 25 °C for five days in aerobic conditions. Growing colonies with macroscopic morphological differences were recultivated on TSA (Tryptic Soya agar, Oxoid®). Inoculated plates were cultivated at 30 °C for 48 h (TSA). After cultivation, the proteins were extracted from fresh bacterial colonies.

### Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial and yeast isolate was transferred into an Eppendorf vial and mixed with 300 µL of sterile water. After addition of ethanol (900 µL), the suspension was mixed and centrifuged (13 000 g, 2 min). After removal of supernatant, the pellets were dried at room temperature at least for 5 min. The bacterial and yeast pellets were resuspended in 20 – 50 µL of formic acid (70 %) and the same amount of acetonitrile. After centrifugation (2 min at 13 000 g), a 1 µL of supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. A 1 µL of MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50 % acetonitrile/2.5 % trifluoro-acetic acid) was added to the spot and dried.

The MALDI target plate was introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, log(score) ≥2.0 was set as the threshold for a match at the species level. Isolates with ≥2.0 were accepted as a correct identification.

## RESULTS AND DISCUSSION

From the surface of grape berries a total of 33 species of 18 bacterial genera (8 Gram negative $G^-$ and 10 Gram positive $G^+$) and 10 species of yeasts belonging to 8 genera were identified with MALDI-TOF Mass Spectrometry. From a total of 333 isolates, the percentage representation of each microbial group ($G^-$, $G^+$ and yeasts) reached the following values: 69 isolates of G- (20.72%), 105 isolates of $G^+$ (31.53%), and 159 isolates of yeasts (47.74%). Table 1 shows that the most common microorganisms isolated from grapes were yeasts. The highest number of yeast species were identified from grape varieties Irsai Oliver (10.06%), Pálava, Pinot Blanc and Rheinriesling (9.43%). Yeast and bacteria were isolated from each grape variety. Bacterial species were identified in highest counts. The number of species of the three main groups of microorganisms in different grape varieties are given in Table 1.

Yeasts and bacterial genera were identified by MALDI-TOF. Percentages of the number of isolates of each genus are shown in Figure 1 for yeasts, in Figure 2 for $G^-$ and in Figure 3 for $G^+$. The most abundant $G^-$ bacterium was *Stenotrophomonas maltophilia* and *Ignatzschineria indica.* Within 22 different species of $G^+$ bacteria, the highest percentage representation (of isolates) was found for *Bacillus endophyticus, Paenibacillus glucanolyticus, Paenibacillus lautus* and *Staphylococcus succinus. Rhodotorula mucilaginosa* was the most abundant among of yeasts.

**Kántor et al. (2017)** found in 19 Slovak grape samples 11 genera of $G^-$, 11 of $G^+$ bacteria and nine of yeasts. Among 200 isolates, $G^-$, $G^+$ bacteria and yeasts represented 11%, 27% and 62% of the total number of isolates studied. The most common genera of isolated yeasts were *Hanseniaspora* (37%), *Metschnikowia* (31%), and *Rhodotorula* (10%). The most frequently isolated among $G^-$ bacteria were *Acinetobacter* (22%), *Pseudomonas* (22%) and *Sphingomonas* (13%). The most common genera of $G^+$ bacteria were *Bacillus* (20%), *Lactobacillus* (19%), *Leuconostoc* and *Staphylococcus* (11%), respectively. In our study, from 333 isolates both different and similar species of microorganisms to **Kántor et al. (2017)** results were identified.

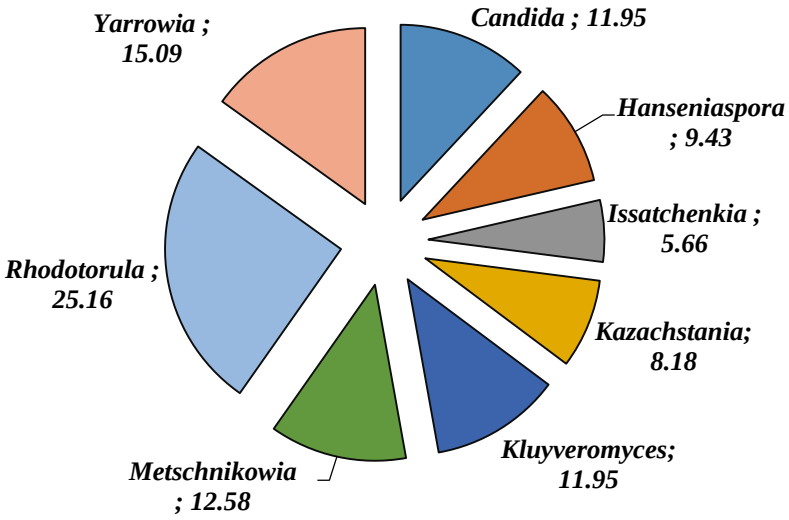


**Figure 1** Yeast were isolated from grapes (%).

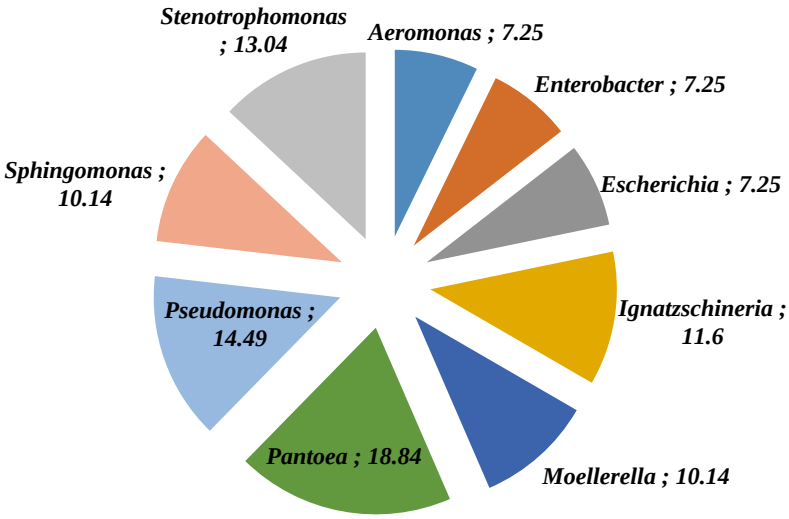


**Figure 2** $G^-$ bacteria were isolated from grapes (%).

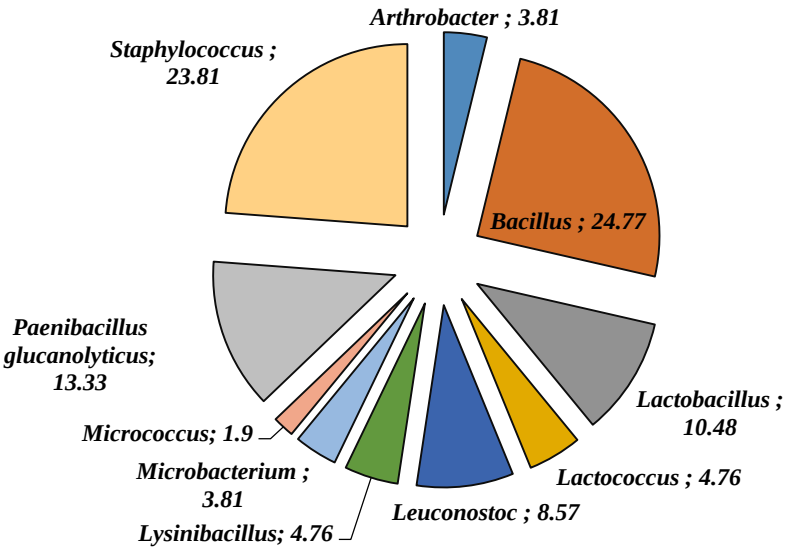


**Figure 3** $G^+$ bacteria were isolated from grapes (%).

Similar results were decribed in research of **Kántor et al. (2016)**, where the microorganisms in similar grape wine varieties were studied. The most dominant species was *Saccharomyces cerevisiae* isolated from all 15 new wine samples, that was a very good wine quality indicator. With mass spectrometry, were identified seven different *S. cerevisiae* strains. The second most common species was *Kloeckera apiculata (Hanseniaspora uvarum)* found in seven new wine samples (2 strains). They also identified other non – *Saccharomyces* yeasts such as *Metschnikowia pulcherrima* (1 strain), *Pichia occidentalis* (1 strain) and *Pichia kluyveri* (1 strain).

Lactic acid bacteria are a minor part of grape microbiota. They are the typical microorganisms of malolactic fermentation and they representatives, including *Oenococcus oeni*, has been seldom isolated from grapes in the vineyard. *Enterobacter* spp., *Enterococcus* spp., *Bacillus* spp., *Burkholderia* spp., *Serratia* spp., *Staphylococcus* spp. are widely distributed in the environment and are among others have been isolated from grapes, while a wine s a not suitable substrat for their growth **(Barata et al., 2012)**.

The community of microorganisms found by **Renouf et al. (2007)** was complex and diverse. It could be divided into 3 groups: 1) species without fermentation ability, e.g. *Auresbasidium* and *Burkholderia*, previously had not been isolated from wine; 2) species with some fermentation ability, e.g. *Lactobacillus, Pichia, Candida, Metschnikowia,* which could act during the first stages of winemaking; and 3) species that are the main fermentation microorganisms - *Saccharomyces cerevisiae* and *Oenococcus oeni*.

**Kántor and Kačániová (2015)** identified 12 yeasts and 30 species of bacteria species by MALDI TOF MS Biotyper. The dominant genera of microorganisms were *Bacillus, Candida, Lactobacillus, Staphylococcus* and *Aureobasidium.* They also identified 4 different strains of *Saccharomyces cerevisiae* (**Kántor and Kačániová, 2015)**.

**Table 1** Microorganismus in different grape berries.

| Grape variety | Gram positive bacteria | Gram negative bacteria | Yeasts | Total |
|---|---|---|---|---|
| Alibernet | 8 | 3 | 5 | 16 |
| Blue Frankish | 12 | 2 | 12 | 26 |
| Cabernet Savignon | 3 | 5 | 10 | 18 |
| Dornfelder | 5 | 6 | 12 | 23 |
| Feteasca regala | 7 | 5 | 12 | 12 |
| Green Veltliner | 8 | 4 | 12 | 12 |
| Irsai Oliver | 11 | 8 | 16 | 35 |
| Mūller Thurgau | 6 | 5 | 13 | 24 |
| Pálava | 8 | 7 | 15 | 15 |
| Pinot Blanc | 9 | 9 | 15 | 33 |
| Savignon Blanc | 12 | 5 | 12 | 29 |
| Rheinriesling | 10 | 7 | 15 | 32 |
| Welschriesling | 6 | 3 | 10 | 19 |
| **Total** | 105 | 69 | 158 | 333 |

**Table 2** Number of isolates identified with maldi tof ms biotyper in grape.

| Microorganisms | White grape | Red grape | Total |
|---|---|---|---|
| *Candida magnoliae* | 5 | 4 | 9 |
| *Candida parapsilosis* | 5 | 5 | 10 |
| *Hanseniaspora uvarum* | 8 | 7 | 15 |
| *Issatchenkia orientalis* | 4 | 5 | 9 |
| *Kazachstania exigua* | 7 | 6 | 13 |
| *Kluyveromyces marxianus* | 12 | 7 | 19 |
| *Metschnikowia pulcherrima* | 15 | 5 | 20 |
| *Rhodotorula glutinis* | 10 | 8 | 18 |
| *Rhodotorula mucilaginosa* | 15 | 7 | 22 |
| *Yarrowia lipolytica* | 18 | 6 | 24 |

**Table 2** (continue)

| **Yeast** | **99** | **60** | **159** |
|---|---|---|---|
| *Aeromonas hydrophila* | 3 | 2 | 5 |
| *Enterobacter cloacae* | 2 | 3 | 5 |
| *Escherichia coli* | 3 | 2 | 5 |
| *Ignatzschineria indica* | 4 | 4 | 8 |
| *Moellerella wisconsensis* | 5 | 2 | 7 |
| *Pantoea agglomerans* | 2 | 4 | 6 |
| *Pantoea dispersa* | 2 | 5 | 7 |
| *Pseudomonas frederiksbergensis* | 3 | 2 | 5 |
| *Pseudomonas* sp. | 2 | 3 | 5 |
| *Sphingomonas* sp. | 5 | 2 | 7 |
| *Stenotrophomonas maltophilia* | 7 | 2 | 9 |
| **Gram negative bacteria** | **38** | **31** | **69** |
| *Arthrobacter koreensis* | 2 | 2 | 4 |
| *Bacillus cereus* | 3 | 2 | 5 |
| *Bacillus endophyticus* | 5 | 2 | 7 |
| *Bacillus licheniformis* | 2 | 0 | 2 |
| *Bacillus safensis* | 4 | 2 | 6 |
| *Bacillus simplex* | 1 | 2 | 3 |
| *Bacillus thuringiensis* | 3 | 0 | 3 |
| *Lactobacillus acidophilus* | 2 | 0 | 2 |
| *Lactobacillus fermentum* | 1 | 2 | 3 |
| *Lactobacillus paracasei* | 2 | 4 | 6 |
| *Lactococcus lactis* | 4 | 1 | 5 |
| *Leuconostoc mesenteroides ssp. mesenteroides* | 7 | 2 | 5 |
| *Lysinibacillus fusiformis* | 2 | 3 | 5 |
| *Microbacterium oxydans* | 2 | 2 | 4 |
| *Micrococcus luteus* | 2 | 0 | 2 |
| *Paenibacillus glucanolyticus* | 5 | 2 | 7 |
| *Paenibacillus lautus* | 6 | 1 | 7 |
| *Staphylococcus epidermidis* | 3 | 2 | 5 |
| *Staphylococcus hominis* | 2 | 4 | 6 |
| *Staphylococcus saprophyticus* | 2 | 2 | 4 |
| *Staphylococcus succinus* | 5 | 2 | 7 |
| *Staphylococcus warneri* | 2 | 1 | 3 |
| **Gram positive bacteria** | **67** | **38** | **105** |
| **Total** | **204** | **129** | **333** |

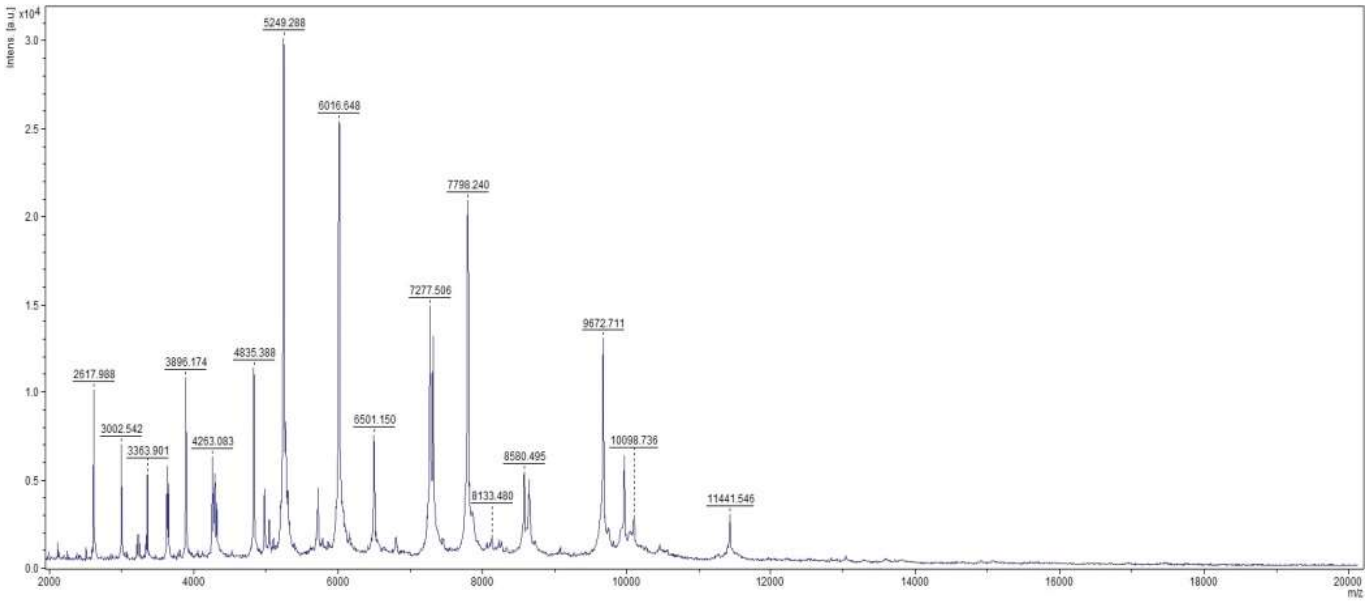


**Figure 4** Spectrum of G⁻ bacteria *Ignatzschineria indica* identified with MALDI TOF mass spectrometry.

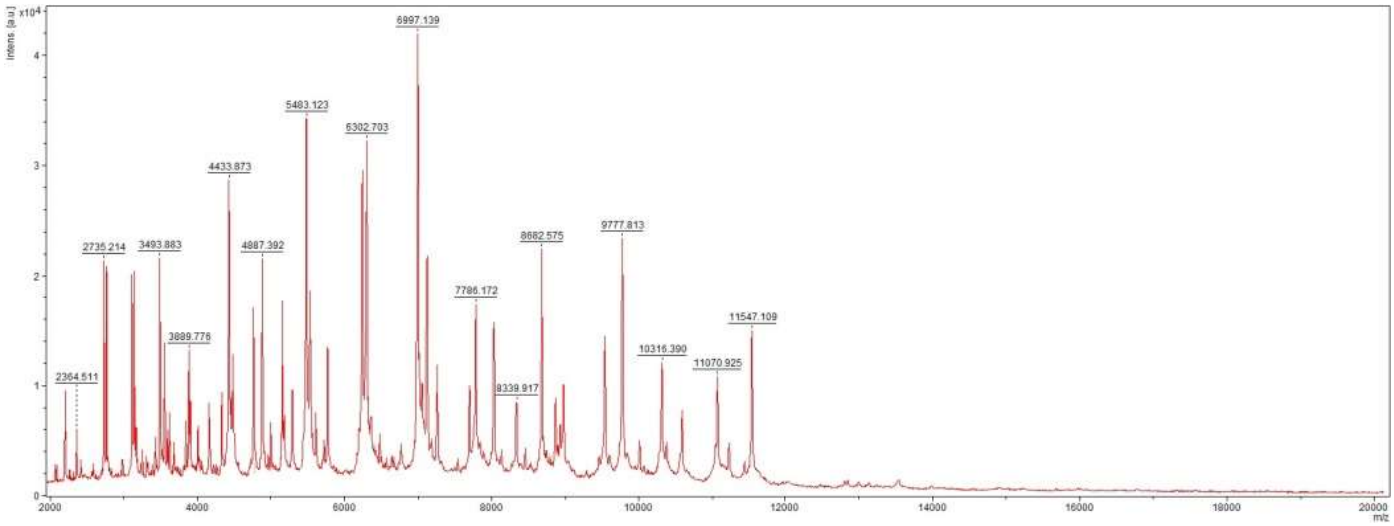


**Figure 5** Spectrum of G⁻ bacteria *Moellerella wisconsensis* identified with MALDI TOF mass spectrometry.

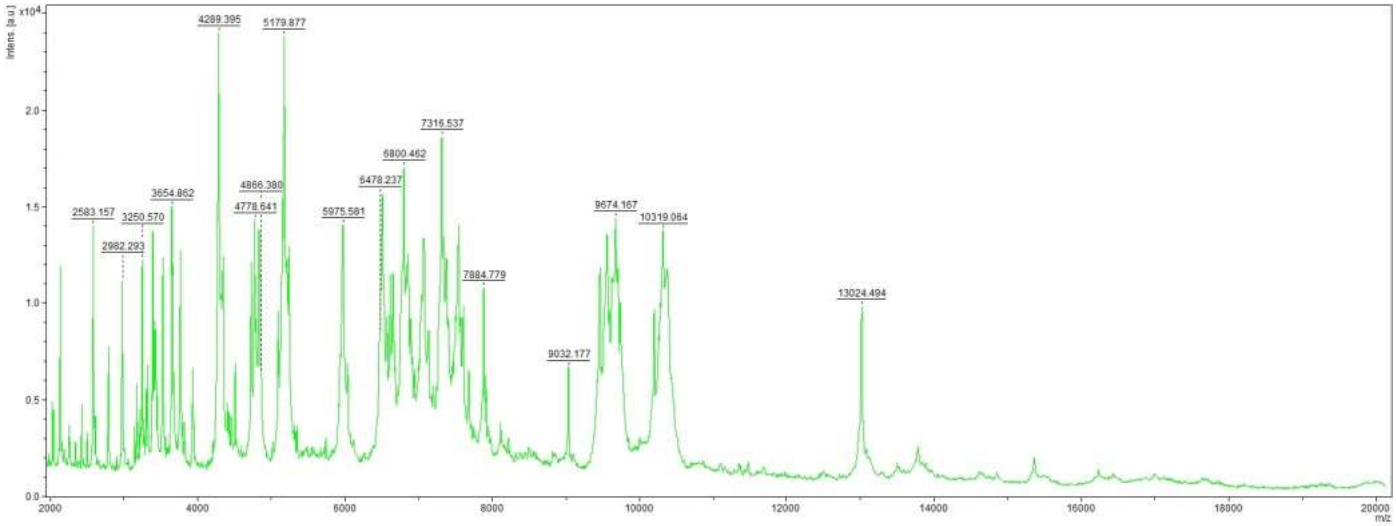


**Figure 6** Spectrum of G⁺ bacteria *Paenibacillus glucanolyticus* identified with MALDI TOF mass spectrometry.

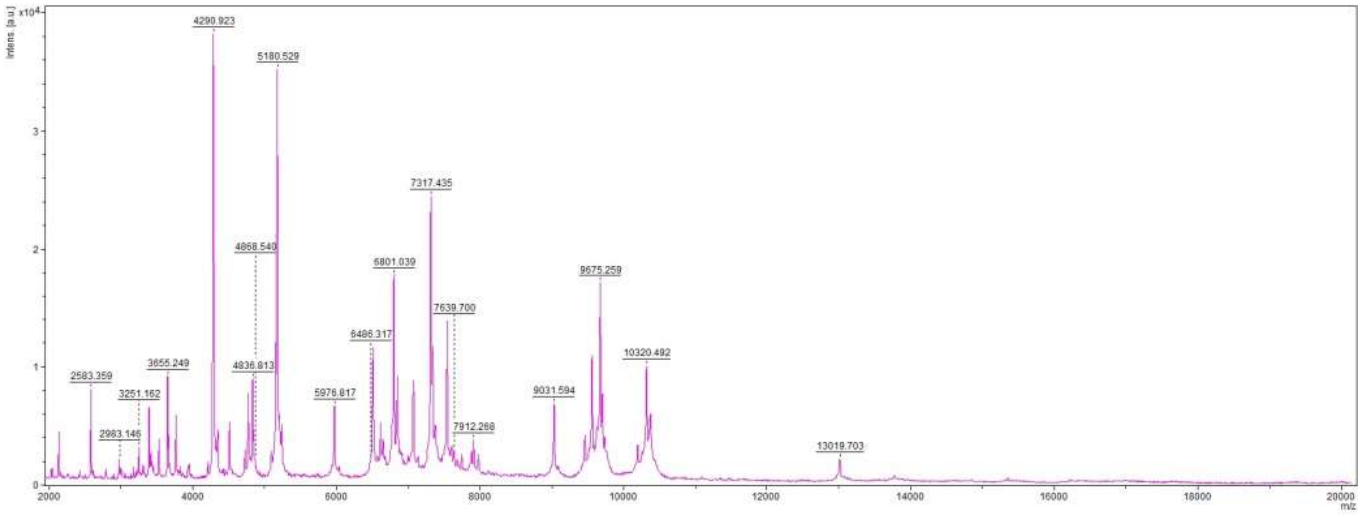


**Figure 7** Spectrum of $G^+$ bacteria *Paenibacillus lautus* identified with MALDI TOF mass spectrometry.

## CONCLUSION

Microbiological analysis of 13 grape samples revealed the three main groups of microorganisms:11 species of $G^-$ and 22 species of $G^+$ bacteria and 10 species of yeasts. In total, 333 isolates were analysed by MALDI-TOF. From white grapes 204 microbial species and 129 from blue grape varieties were isolates, among which the yeasts, representing 47.74% of the all isolates, were the most abundant group.

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