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Research Article

Analysis of the microbiological composition of the domestic winery in Rzeszów

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Aim of this study is to analyse the microbiological composition of grapes and soil from a home vineyard located in Rzeszów. During the research, the following tasks were undertaken: determination of the total number of microorganisms, isolation of the microbial cultures from grapes and soil, and identification of the bacterial strains and yeast using the MALDI-TOF MS Biotyper mass spectrometry. Natural microbiota of grapes and soil are very diverse. Microbiological analysis showed that the total number of microorganisms is higher in the soil, compared to grapes. As the result of the analysis of the microbiota of the home vineyard using the MALDI-TOF MS Biotyper mass spectrometry, five species of yeasts and eight species of bacteria were identified. Microbiological evaluation of the tested vineyard showed the presence of the yeast strains as *Saccharomyces* spp., *Dekkera anomala*, and *Candida* spp., and the strains of bacteria as *Lactobacillus* spp., *Pantoea agglomerans*, *Lactococcus* spp., *Staphylococcus warneri*, and *Acetobacter* spp.

Keywords: microbiological composition, grapes, soil, vineyard, mass spectrometry

1 Introduction

The process of alcoholic fermentation is already very well understood, and winemaking is based on carefully planned stages. In most parts of the world, strains of specific yeasts and lactic acid bacteria are used in the production of a wine beverage. The qualitative characteristics of wine depend not only on climatic conditions such as temperature, insolation, soil type, or rainfall but also on the used microorganisms. Currently, there is a lot of competition on the wine market, and winemakers are developing more and more original recipes to obtain unique wines, organoleptically more favourable than those already existing on the market. Therefore, more and more attention is paid to selecting yeast strains that allow better control of the fermentation process (Cordero-Bueso et al., 2018). The must fermentation process can occur both through the deliberate introduction of the selected strains of microorganisms and spontaneously, as the result of the microbiota present in the grapes. Its composition is naturally varied and depends on many factors such as the location of the vineyard, its climate, groundwater level, used chemical protection measures, agrotechnical treatments, method of grape harvesting, and storage. The most common yeasts include *Hanseniaspora*, *Pichia*, *Schizosacharomyces*, *Kluyveromyces*, or *Rhodotorula*. It was also found that most of these yeast species intensively participate in the first phase of the fermentation process, and then, due to the increase in the concentration of ethyl alcohol and their physiological characteristics, they disappear in favour of the yeast with more favourable fermentation characteristics (Marsit & Dequin, 2015).

Non-*Saccharomyces* yeasts involved in fermentation were initially considered undesirable and responsible for the failure of winemaking. They usually got into the grape must as a result of the lack of sterility of the process or as a result of the unplanned treatments during cultivation. The roles of microorganisms responsible for wine spoilage, mainly related to the formation of unfavourable sensory compounds, were attributed to them. In addition to the dominant species of *S. cerevisiae*, *Hanseniaspora*, and *Candida* species can also be found, and they have a positive effect on the winemaking process. In recent years, many studies have been conducted that have

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proved that some species of yeast can have a positive effect on the organoleptic characteristics of the wine, especially those related to shaping a favourable profile of aroma, taste, colour, and clarity (Padilla et al., 2016). Although many microorganisms can participate in alcoholic fermentation, wine production is invariably dominated by *S. cerevisiae* strains belonging to the fungi. Yeasts are single-celled fungi belonging to many taxonomic groups. Even though people of yesteryear did not understand this phenomenon, the fermentation process has been used for thousands of years, both to produce alcoholic beverages and as an effective way to ensure food safety and quality (Sicard & Legras, 2011). Yeasts have been used also for their ability to cope with difficult environmental conditions such as high concentrations of organic acids, low pH, and limited amount of oxygen and nutrients. In addition to that, *S. cerevisiae* strains are able to grow in difficult anaerobic conditions with low nitrogen concentrations. In connection with the above-mentioned aspects, this species belongs to the microorganisms capable of effective alcoholic fermentation, and showing the ability to quickly process sugars contained in the must, yielding relatively large amounts of ethyl alcohol. The fermentation process itself is fast and uncomplicated, and the obtained wines are of high quality and have the desired bouquet of flavours, and do not have undesirable impurities or other disadvantages (Albergaria & Arneborg, 2016). Moreover, lactic acid fermentation can last throughout the entire process or start at the very end of alcoholic fermentation. One or several species of lactic acid bacteria participate in it. There are mainly the species as *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Oenococcuc*, and *Weisella*. However, not all of them are able to participate in the fermentation process under difficult environmental conditions. Despite this, many studies have shown that *Lactobacillus* spp. bacteria are able to survive in difficult conditions prevailing during the process. The bacteria also exhibit certain biological properties that positively affect the quality and shelf life of the wine. It has been proved that the products of their metabolism have a stabilizing effect on wine by deacidifying it, and thus improving the organoleptic characteristics of the drink, especially the taste and aroma (Berbegal et al., 2016).

The aim of this study is to analyse the microbiological composition of grapes and soil from a home vineyard located in Rzeszów, Poland. During the research, the following tasks were undertaken: determination of the total number of microorganisms, isolation of the microbial cultures from grapes and soil, and identification of the bacterial strains and yeast using the MALDI-TOF MS Biotyper mass spectrometry.

2 Material and methods

2.1 Research material

The material used for the research consisted of grapes and soil samples, which were collected on October 4, 2018 from a local, backyard vineyard located on Jazowa Street in Rzeszów. This vineyard is located in a fruit garden, covering an area of about 10 ares . In this area, there were 14 grape varieties cultivated, from which 6 grape varieties were collected and used for the research, together with 6 soil samples from the depth of about 0.01 m.

2.2 Determination of the total number of microorganisms

To obtain microbiota from the ground, 1 g of soil was weighed and placed in a porcelain mortar, gently ground with fine quartz sand, and then 5 mL of 0.89% saline solution was added with a pipette and carefully rubbed. Appropriate serial dilutions were made from the resulting suspension (10^{-4} and 10^{-5}), sequentially taking 100 µL from each sample and spreading it with a sterile glass spreader onto Petri dishes with already prepared Tryptone soya agar (TSA) for bacteria, and Potato dextrose agar (PDA) for yeast. The prepared samples were incubated in an incubator at 30 °C for 72 hours, and 25 °C for 5 days, respectively. From the harvested grapes, 5 g of fruit was weighed and placed in a sterile test tube, and 45 mL of 0.89% saline solution was added, followed by thorough shaking for about 20 minutes at 200 rpm. Tenfold dilution was obtained, followed by appropriate serial dilutions (10^{-2} and 10^{-3}), and then 100 µL of each sample was sequentially taken using a pipette, and placed and spread with a sterile glass spreader onto Petri dishes with already prepared TSA for bacteria and PDA for yeast. The prepared samples were incubated in an incubator at 30 °C for 72 hours, and 25 °C for 5 days, respectively.

2.3 Isolation of the microbial material

After the specified incubation time, cultures of microorganisms were successively separated from the substrates and single colonies were inoculated with a sterile inoculation loop using reduction inoculation onto sterile Petri dishes with an appropriate medium. The prepared samples were incubated at 30 °C for 72 hours, and 25 °C for 5 days, respectively.

2.4 Identification of microbiota using the MALDI – TOF MS Biotyper mass spectrometry

To identify the vineyard microbiota, the obtained single colonies were transferred to Eppendorf filled with 300 µL of distilled water, and shaken. Then 900 µL

of absolute ethanol were added, and it was centrifuged for 2 min at 10,000 rpm, and the remaining ethanol was removed with a pipette. The pellet was left to dry. Successively, we added 30 µL of 70% formic acid and mixed it thoroughly, and then 30 µL of acetonitrile was added and gently shaken. Subsequently, the samples were centrifuged at the maximum speed for 2 min. Then, 1 µL of the supernatant from each sample was placed in the appropriate measuring places on the MALDI plate (MALDI target), and after being dried, it was covered with 1 µL of the matrix, and again allowed to dry. For identification, the plate was placed in the MALDI-TOF MS Biotyper. The spectra were obtained by 40 laser shots with the power needed to ionize the sample. Each spectrum was automatically read and analysed, and the results were compared in real time with the database using the appropriate software program.

3 Results and discussion

3.1 Determination of the total number of microorganisms

During the research, a microbiological analysis of grapes and soil was performed. After 72 hours of incubation at 30 °C on the TSA medium, it was discovered that the highest number of bacteria – 2.71 ±1.06 log CFU.g^{-1} – was present in the soil for the Marechal Foch cultivar. Contrarily, the lowest number of bacteria was found in grapes of the Chasselas variety, and it was 2.00 ±1.28 log CFU.g^{-1} (Table 1). As a result of the tests carried out, it was also found out that a greater number of microorganisms was present in the soil than in the grapes themselves (Table 1).

In the case of the PDA, after 5 days of incubation at 25 °C, the highest number of microorganisms was found in the soil of the Concord variety, and it was 2.75 ±3.11 log CFU.g^{-1}. Contrarily, the smallest number of bacteria was found again in the case of grapes of the Chasselas variety on the PDA medium, and it was 1.97 ±1.21 log CFU.g^{-1} (Table 2). As a result of the research carried out, the same was found as before – more microorganisms are present in the soil than in the grapes themselves (Table 2).

From the previously obtained results, using the Statistica. v10.0 program, the correlation coefficient was calculated using the Pearson Method. Based on the result of this parameter, it can be concluded that there is a fairly strong correlation between the total number of microorganisms in all grape samples and their presence in the soil, taking into account the type of the substrate (TSA and PDA). The calculations show that a stronger relationship between the total number of soil microorganisms and grapes occurred on the TSA medium, where it was 0.89, while on the PDA, it was 0.87 (Table 3).

Table 3 Correlation coefficient between the type of the substrate and the total number of microorganisms

Agar	Correlation coefficient
PDA	0.87
TSA	0.89

Table 1 Total number of bacterial colonies in soil and grapes on the substrate

Name of the sample	Number of colonies in 1g of grapes (log CFU.g^{-1})	Number of colonies in 1 g of soil (log CFU.g^{-1})
1. Aurore	2.15 ±1.12	2.50 ±1.70
2. Chasselas	2.00 ±1.28	2.31 ±2.95
3. Marechal Foch	2.23 ±3.50	2.71 ±1.06
4. Concord	2.17 ±1.50	2.68 ±1.52
5. Opal	2.12 ±1.62	2.58 ±1.50
6. Iza Zaliwska	2.13 ±2.00	2.63 ±1.26

Table 2 Total number of yeast colonies in soil and grapes on PDA

Name of the sample	Number of colonies in 1g of grapes (log CFU.g^{-1})	Number of colonies in 1 g of soil (log CFU.g^{-1})
1. Aurore	2.08 ±2.24	2.62 ±1.52
2. Chasselas	1.97 ±1.21	2.38 ±1.12
3. Marechal Foch	2.17 ±3.03	2.72 ±0.50
4. Concord	2.22 ±2.07	2.75 ±3.11
5. Opal	2.04 ±2.24	2.65 ±2.00
6. Iza Zaliwska	2.01 ±2.99	2.54 ±2.23

3.2 *Identification of vineyard microbiota using the MALDI-TOF MS Biotyper*

Isolated single yeast colonies were identified with the MALDI-TOF MS Biotyper. As a result of the analysis, five yeast strains were found, including *Saccharomyces* spp., *Dekkera anomala*, *Candida* spp., and other strains, with 100% certainty. Presence of *Kluyveromyces* spp., and *Hanseniaspora* spp. was confirmed by additional tests (Table 4).

Isolated single bacterial colonies were identified with the MALDI-TOF MS Biotyper. The analysis revealed the presence of eight bacterial strains, including *Pantoea agglomerans*, *Lactococcus* spp., *Lactobacillus* spp., *Staphylococcus warneri*, and *Acetobacter* spp. and others, with 100% certainty. The presence of *Bacillus megaterium*, *Cellulosimicrobium celluans*, and *Bacillus cereus* was confirmed by additional tests (Table 5).

The chemical composition of grapes and the natural microbiota of the vineyard make the fruit good raw material for carrying out alcoholic fermentation and obtaining wine. Research by Kačániová et al. (2018) showed that red grape varieties were characterized by a higher number of microorganisms than white grape varieties, which is comparable to the research carried out in this work. In addition, it was also shown that the total microbial count on both TSA for bacteria and PDA for yeast was higher in the soil than in the fruit itself. The tests carried out using the MALDI-TOF MS Biotyper technique allowed the identification of the microbiota from the tested vineyard. Due to this method, the presence of *Acetobacter* gram-negative bacterial strains was proved, among others. Such bacterial strains were detected also by Bokulich et al. (2012). They also confirmed that acetic bacteria were common on grapes, and they were considered undesirable. They cause wine spoilage and can also be the cause of wine defects and diseases. On the other hand, they can also have a beneficial effect on the sensory characteristics of the wine. They originate mainly from soil and water, but they can also be carried by insects or introduced with natural fertilizers (Bokulich et al., 2012).

In their research, Loureiro et al. (2012) dealt with the subject of the diversity of microorganisms found both on the vine and in the wine. Using various analytical methods, they confirmed that the Lactobacillales, which constitute 1–10% of the grape microbiota on average, were the most frequently represented group of bacteria in that environment. Moreover, there were also other types among them that were observed during the studies, including gram-positive bacteria *Lactobacillus* spp., *Lactococcus* spp., *Staphylococcus warneri* or *Bacillus megaterium*, and *B. cereus*, which was found mainly in the soil. The presence of *Pantoea agglomerans*, and *Cellulosimicrobium cellulans*, whose characteristic place of occurrence are the woody parts of the vine, was also detected (Loureiro et al., 2012). In the soil, and both on the surface of leaves and in berries, there are many species of yeast in addition to bacteria. The research carried out by Malfeito-Ferreira (2011) mentioned mainly the strains that had a significant influence on the production of wine. They included *Hanseniaspora*, located primarily in grapes, but also *Candida*, and *Kluyveromyces*, inhabiting mainly the soil. However, they are considered to cause impurities in the technological process that may cause adverse organoleptic changes in the wine. These strains were identified also by this study in both fruit and field samples. Research by Barata et al. (2012) showed that the yeast genus *Saccharomyces* was one of the most important strains necessary for grape must fermentation. This strain is quite common and it occurs on the surface of the berries of almost all grape varieties. The tests carried out

Table 4 Results of the comparison of the tested samples with the database, indicating the probable presence of the yeast strains

Number of the sample	Species of yeast	Result
1	*Saccharomyces* spp.	2.110
2	*Dekkera anomala*	2.121
3	*Candida* spp.	2.021
4	*Kluyveromyces* spp.	1.952
5	*Hanseniaspora* spp.	1.951

Table 5 Results of the comparison of the tested samples with the database, indicating the probable presence of the bacterial strains

Number of the sample	Species of bacteria	Result
1	*Lactobacillus* spp.	2.032
2	*Bacillus megaterium*	1.932
3	*Cellulosimicrobium cellulans*	1.911
4	*Pantonea agglomerans*	2.074
5	*Bacillus cereus*	1.904
6	*Lactococcus* spp.	2.096
7	*Staphylococcus warneri*	2.006
8	*Acetobacter* spp.	2.077

using the MALDI TOF MS Biotyper proved the presence of *S. cerevisiae* in the tested samples, which was also confirmed by the subsequent microscopic examinations (Barata et al., 2012). Due to the microscope, photos of the individual strains of yeast that have been flourishing in the tested vineyard were obtained. On this basis, the presence of *S. uvarum*, and *S. bayanus*, as well as *Dekkera anomala*, which are considered to be the ones of the most numerous in both fruit and soil, was detected. These strains were also identified and defined as natural grapevine microbiota by Kantor (2016), and their beneficial effect on the alcoholic fermentation process and wine production was confirmed. Microorganisms are a diverse, common and natural part of our environment. In the case of winemaking technology, they are necessary to obtain excellent liquors appreciated by the greatest gourmets, which is confirmed by the research carried out in the work as well as by the references to many literary sources. Bacteria and yeast shape the physical, chemical, and sensory characteristics of the wine. However, it must be kept in mind that they can have both positive and negative effects.

4 Conclusions

Natural microbiota of grapes and soil are very diverse. Microbiological analysis has shown that the total number of microorganisms is higher in soil than in grapes. The MALDI-TOF MS Biotyper mass spectrometry is an effective and fast device for identifying microorganisms. As the result of the analysis of the microbiota of the home vineyard using the MALDI-TOF MS Biotyper mass spectrometry, five species of yeast and eight species of bacteria were identified. Microbiological evaluation of the tested vineyard showed the presence of the yeast strains as *Saccharomyces* spp., *Dekkera anomala*, *Candida* spp. and the strains of bacteria as *Lactobacillus* spp., *Pantoea agglomerans*, *Lactococcus* spp., *Staphylococcus warneri*, and *Acetobacter* spp.

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