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A Metagenetic Insight into Microbial Diversity of Spontaneously Fermented Polish Red Wines and an Analysis of Selected Physicochemical Properties

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Abstract: Due to changes in climatic conditions in Poland interest in viticulture and wine production has considerably boosted. One of the worldwide trends in winemaking is use of indigenous strains of yeast and lactic acid bacteria (LAB). The aim of the study was to analyse the microbial diversity of Polish red wines and their key properties and combine them for better understanding of the processes responsible for creating the sensory attributes. Metagenetic analysis was used to characterise the bacterial and yeast diversity of wines produced by spontaneous fermentation of grapes of the Regent variety, which came from three vineyards: "Dom Bliskowice" (DB), "Małe Dobre" (MD), and "Winnica Janowiec" (WJ). Among bacteria, *Tatumella ptyseos* was the most abundant species in DB and WJ wines and *Leuconostoc pseudomesenteroides* was the most abundant in MD wine. Among yeasts, *Saccharomyces cerevisiae* was found in DB and WJ wines, *Saccharomyces cariocanus* in MD wine, and *Hanseniaspora uvarum* in all samples studied. Studied wines had statistically significantly different antioxidant capacities and distinct glucose, fructose, and lactic acid concentrations. The presence of acetic and lactic acid bacteria was positively related to the concentrations of acetic and lactic acid, respectively, while the lack of malic acid was indicative of malolactic fermentation. This knowledge may be useful in the development of unique local starter cultures for the production of wines with specific characteristics.

Keywords: metagenetic analysis; wine microbiome; spontaneously fermented wine; Polish wine; wine properties

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1. Introduction

Scientists predict that, by 2050, climate change will lead to a 25% to 73% reduction in viticultural area in the world's major wine regions. Mediterranean regions will become less suitable for viticulture, while the viticultural suitability of western parts of North America, New Zealand, and Northern Europe, e.g., Poland, will increase [1]. The improvement in climatic conditions in Poland has considerably boosted interest in viticulture and wine production in the last decade. The number of producers increased from 26 in 2011/2012 to 380 in 2021/2022, the area under vines increased from 51.28 ha to 619.37 ha, and wine production increased from 428.47 hl to 17770.63 hl [2]. Since 2014, Poland has been listed in the world wine consumption rankings of the International Organization of Vine and Wine (OIV) [3]. The literature, however, offers only two studies devoted to the isolation, selection, and analysis of native strains occurring in Poland. Both of these papers focus on

the Małopolska Wine Region [4,5]. So far, no such research has been carried out in other Polish wine regions, e.g., the Małopolska Vistula Gorge, including the Lublin Province.

Nowadays, indigenous strains of yeast and lactic acid bacteria (LAB) are the preferred choice for wine production worldwide. These strains adapt better to the original environment and guarantee that the wines have sensory attributes typical of the region of origin [6–8]. The microbiome of grapes and wine depends on the grape variety, climatic conditions, agronomic practices, and the geographical location of the vineyard [9]. Grapes are the main source of non-*Saccharomyces* yeasts, some of which are unique to wines coming from specific world regions. Some of them, such as *Torulaspora delbrueckii*, *Schizosaccharomyces pombe*, *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, and *Pichia kluyveri*, have just started to be commercialised. Others, such as *Starmerella bacillaris* (syn. *Candida zemplinina*), *Kloeckera apiculata*, *Hanseniaspora vineae*, *Hanseniaspora uvarum*, *Starmerella stellata* (syn. *C. stellata*), *Kazachstania aerobia*, or *Schizosaccharomyces japonicus*, may follow a similar progress [10]. The fermentation of grape juice or must is a complex microbial reaction involving the sequential development of various species. Nowadays, the interest in these yeasts is growing, as they participate in the creation of the sensory attributes of wines through the production of various metabolites (alcohols, glycerol, organic acids, phenolic compounds, aromatic substances, and other products) and the secretion of enzymes (esterases, β-glucosidases, lipases, proteases). Numerous non-*Saccharomyces* can also reduce the concentrations of unwanted compounds, such as ochratoxin A, ethyl carbamate, and biogenic amines [11,12].

During the fermentation of wines, especially sour red wines, the presence of various bacteria, including LAB, has been observed. They represent genera such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Enterococcus*, *Weisella*, and the species *Oenococcus oeni*. Some of these bacteria are involved in malolactic fermentation (MLF). This kind of fermentation is an important process in red wine production as it reduces the acidity of wines [13]. MLF results in a rise in wine pH and microbial stability due to the removal of malic acid. Besides LAB, some acetic bacteria (AAB), e.g., *Gluconobacter* and *Acetobacter*, have been identified in wines. They dominate in low sulphite, uninoculated wine fermentations [14]. Other genera associated with the wine habitat are O*nus*, *Wolbachia*, *Komagateaibacter*, and *Shewanella*, all of which are an important part of the wine microbiome [15,16].

In recent years, metagenomic (including metagenetic) analysis has been widely used to determine microbial diversity. This method, also referred to as the high-throughput sequencing technique, has the advantage of being accurate and less time-consuming [17]. Metagenomic approaches have recently played a great role in the dissection of the contribution of the vineyard environment to wine fermentation, e.g., the impact of agronomical techniques, vineyard topologies, and climate on microbial populations found in vineyards and in fermentations, which can be responsible for the sensory characteristics of the resulting wine [18,19].

The aim of the present article was to analyse the bacterial and yeast diversity of three Polish red wines produced by spontaneous fermentation and to investigate some of their key characteristics, such as total polyphenol content, antioxidant capacity, pH, total acidity, and the concentrations of ethanol, sugars, and organic acids, and, finally, combine microbiological characteristics with physicochemical properties for better understanding of the processes responsible for creating the sensory attributes of the investigated Polish wines.

2. Materials and Methods

2.1. Materials

Grapes of the Regent variety came from three vineyards located in the Lublin Province in Poland: "Dom Bliskowice" (DB), "Małe Dobre" (MD), and "Winnica Janowiec" (WJ). They were collected in 2019 and used by permissions obtained from the above vineyards. The study involving plant materials was conducted in accordance with institutional, national, and international guidelines and legislation.

Chemicals: calcium hydroxide, sodium hydroxide solution (0.1 mol/L), Folin–Ciocalteu reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Poznań, Poland). Buffer solutions of pH 2, 4, and 7, sodium carbonate, methanol, sulphuric acid, glucose, fructose, lactic acid, acetic acid, and malic acid were purchased from POCH (Gliwice, Poland). All chemicals were of analytical grade.

2.2. Production of Wines

The grapes were stalked, crushed, portioned, and transferred to 5 L fermentation vessels in the laboratory. The experiments were performed in duplicate. No yeast was added to the pulp. The fermentation vessels were closed with stoppers and fermentation tubes. Spontaneous alcoholic fermentations were performed at 22–24 °C and monitored by weight loss. The weight was measured every 24 h until it was constant (12–14 days), and then the results obtained were converted into a concentration (g/L) of volatile CO_2. The samples were taken aseptically after main fermentation for metagenomic studies to obtain the full spectrum of microorganisms. Subsequently, each wine was pressed with a basket press and left for spontaneous MLF for 4 weeks. Wines were decanted and left to stand at 15 °C for two months. Then, the wines were decanted again, and the temperature was lowered to 8 °C for 4 weeks. After this, the wines were decanted, supplemented with potassium metabisulphite (60 mg/L), bottled, and stored at 15 °C until chemical analysis.

2.3. Isolation of DNA

Wine samples (100 mL volume each) were centrifuged and DNA was isolated from the sediment. Isolation was performed according to the procedure of Genomic Mini AX Bacteria and Genomic Mini AX Yeast (with the lytic enzyme Lyticase) (A&A Biotechnology, Gdańsk, Poland).

2.4. Metagenetic Analysis

A metagenetic analysis of bacterial and archaeal populations was performed by sequencing the hypervariable region V3-V4 of the 16S rRNA gene. A metagenetic analysis of the fungal population was performed by sequencing the hypervariable region ITS1.

The specific 341F and 785R (16S), and ITS1FI2 and 5.8S (ITS1) primer sequences were used to amplify the selected region and prepare the library. A PCR reaction was performed with Q5 Hot Start High-Fidelity 2X Master Mix; the reaction conditions were in line with the manufacturer's recommendation. Sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA) using paired-end (PE) technology. An automatic data analysis was carried out on the MiSeq using MiSeq Reporter (MSR) v2.6 software (Illumina, San Diego, CA, USA); it included the following steps: (1) demultiplexing of samples and (2) generating fastq files containing raw reads.

A bioinformatic analysis allowing the reads to be assigned to the species level was performed using QIIME software (http://qiime.org, accessed on 20 October 2021) and SILVA 138 SSU (16S reference sequences database) (https://www.arb-silva.de, accessed on 20 October 2021) or UNITE ver. 8.2 (ITS reference sequences database) (http://unite.ut.ee, accessed on 20 October 2021). This analysis consisted of the following steps:

(1) adapter sequences removal—cutadapt, (2) quality reads analysis and low-quality sequence removal (quality < 20, minimum length 30)—cutadapt, (3) merging paired reads—fastq-join algorithm, (4) clustering based on the reference sequences database—uclust algorithm, (5) filtering out chimeric sequences—usearch61 algorithm, and (6) taxonomy assignment with the selected reference database—uclust algorithm.

The diagrams were plotted using the ggplot2 package (https://ggplot2.tidyverse.org, accessed on 31 January 2022) [20].

2.5. Chemical Analysis of Wine Parameters

2.5.1. Determination of Alcohol Content (ETH)

The alcohol content in the wines was determined according to the OIV-MA-AS312-01A method by measuring the density of the distillate with a pycnometer [21].

2.5.2. Determination of pH and Total Acidity (TA)

The pH was determined according to the OIV-MA-AS313-15 method [22]. The analysis of total acidity was performed according to the OIV-MA-AS313-01 method using potentiometric titration. TA was expressed as g of tartaric acid in L [23].

2.5.3. Determination of Total Polyphenol Content (TPC)

The total polyphenol content was determined according to Folin–Ciocalteu method with modifications [24]. Wines were diluted 1:10 (*v*/*v*) with distilled H_2O. A total of 2340 µL of distilled H_2O was mixed with 150 µL of Folin–Ciocalteu reagent and 60 µL of diluted wine. After 1 min, 450 µL of 20% (*w*/*v*) Na_2CO_3 was added. The mixture was stored for 1 h in the dark at room temperature. The absorbance was read on a UV-1280 spectrophotometer (Shimadzu, Kyoto, Japan) at 765 nm. Standard solutions of gallic acid at a concentration of 100–500 mg/L were used to prepare a calibration curve. The results were expressed as mg/L gallic acid equivalent (mg GAE/L).

2.5.4. Determination of Antioxidant Activity (DPPHA)

Antioxidant activity was determined using the DPPH assay with modifications [24]. Wines were diluted 1:15 (*v*/*v*) with distilled H_2O. A solution of DPPH in methanol at a concentration of 0.006 mmol/L was prepared. A total of 75 µL of the diluted wine was mixed with 2925 µL of DPPH solution and left for 30 min in the dark. The absorbance was read on a UV-1280 spectrophotometer (Shimadzu, Kyoto, Japan) at 515 nm. Standard solutions of Trolox at a concentration of 0.2–1 mmol/L were used to prepare a calibration curve. The results were expressed as mmol/L Trolox equivalent (mmol TE/L).

2.5.5. Analysis of Sugars and Organic Acids

Wine samples were filtered and then analysed using a high-performance liquid chromatography system (Gilson Inc., Middleton, WI, USA) equipped with an ion exchange column (Aminex HPX-87H, 300 mm × 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA) with a Micro-Guard precolumn Cation H cartridge 30 × 4.6 mm (Biorad), a diode array detector (220 nm) for organic acids analysis, and a refractive index detector (Knauer GmbH, Berlin, Germany) for sugars analysis. A 0.03 M sulphuric acid was used as the mobile phase at 42 °C and the flow rate was set to 0.5 mL/min [25]. Chromatograms were integrated and analysed using Chromax 2007 software, ver. 1.0a (Pol-lab, Warsaw, Poland), and Gilson UniPoint ver.2.0 (Gilson, Inc. Middleton, WI, USA). Solutions of glucose, fructose, and malic, lactic, and acetic acids of HPLC purity were used as standards.

2.5.6. Statistical Analysis

The effects of bacterial and yeast diversity on the investigated Polish red wines were analysed using a two-step ordination method called transformation-based PCA (tb-PCA). Relative bacterial and yeast abundance data were divided by margin total before the classical PCA. This procedure made the results similar to those from principal co-ordinate analysis (PCoA) [26]. PCA was also applied to identify the features characteristic of the wines studied. The significance of differences in feature means among the Polish red wines was tested by one-way ANOVA, followed by Tukey's HSD test, except acetic acid concentration which was analysed by T test because it was not detected in DB wine. Analyses were performed and plots were generated using Statistica ver. 13.1 statistical software package (StatSoft, Cracow, Poland) and R ver. 4.0.5, (The R Foundation for Statistical Computing, Vienna, Austria) [27] (stats, vegan, and factoextra packages) applications.

3. Results

3.1. Abundance and Diversity of Microorganisms in Red Wines

Samples of the red wines were clustered on the basis of sequences of a fragment of 16S rDNA (bacteria) and region ITS1 rDNA (fungi). The red wines differed in the number of raw reads, the number of Operational Taxonomic Units (OTUs), and microbial diversity (Table 1). The largest number of bacterial OTUs was found in DB wine, followed by WJ wine; MD wine contained a small quantity of bacterial OTUs, but was the most abundant in fungal OTUs, followed by DB and then WJ. However, the number of OTUs was not an indicator of the microbial diversity of the investigated wines, as the largest numbers of bacterial species were found in MD wine, while fungal diversity was the highest in DB wine and the lowest in WJ wine. Chao 1 indexes and the numbers of species for each wine were also much higher for bacteria than for fungi, and they were similar for MD and DB, 44/29 and 43/30, respectively. Simpson's and Shannon's diversity indexes reflect the uniformity of distribution of microorganisms. The former is a measure of population evenness indicating the probability of two randomly sampled individuals belonging to two different taxa. The latter combines both evenness and richness, but it quantifies the uncertainty in the taxon identity of a randomly chosen individual [19]. High values of these indexes are suggestive of a better uniformity of distribution, which means that MD was the best one in this respect. On the other hand, WJ and DB wines showed the lowest values of the mentioned indexes for fungi and bacteria, respectively.

Table 1. Sequence abundance and microbial diversity of wine samples.

Wine Sample ID	Raw Read		Number of OTU		Species Number		Chao1 Index		Shannon Index		Simpson Index		Inv Simpson	
	Bact.	Fungi	Bact.	Fungi	Bact.	Fungi	Bact.	Fungi	Bact.	Fungi	Bact.	Fungi	Bact.	Fungi
DB	174,068	168,709	145,041	154,682	43	30	162	68.75	1.133	1.061	0.450	0.487	1.818	1.95
MD	126,428	194,953	94,992	180,301	44	29	169	81	2.184	1.435	0.825	0.659	5.746	2.938
WJ	150,406	165,063	125,424	115,409	39	14	120	44	1.359	0.588	0.577	0.24	2.369	1.316

Table 2 shows relative abundance of the genera of bacteria and yeast identified by metagenetic analysis of the Polish red wines. A total of 19 bacterial genera and 15 yeast genera were detected. The numbers of bacterial genera for each wine were as follows: 13 for DB, 14 for MD, and 12 for WJ, but the numbers of genera with RA above 10% were 2, 3, and 2, respectively. *Lactobacillus* (before changes in taxonomy of LAB) was present in all wines at 14.98% to 28.53%. The bacterium *Tatumella* was also found in all wines, but it was observed in large amounts in DB and WJ wines only. *Lactococcus* and *Gluconobacter* were present in each wine, but their RA was below 10%. As shown in Figure 1 and Table S1, the three wines differed significantly in bacterial species composition. *Tatumella ptyseos* was the main species in DB and WJ wines (74 and 63%, respectively), *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) was the most highly represented in DB and MD wines (14.6 and 21.8%, respectively), and *Apilactobacillus kunkeei* (formerly *L. kunkeei*) was the most abundant in WJ wine (17.7%). On the other hand, *Leuconostoc pseudomoesenteroides* (29.6%) and *Komagataeibacter saccharivorans* (18.3%) were found only in MD wine.

Among yeasts (Table 2 and Table S2), the genus *Saccharomyces* (RA between 47 and 90%) was the most abundant taxon, which is normal for wines after fermentation, but there were large differences in RA values between the wine samples. This genus was represented by the species *S. cerevisiae* (in DB and WJ, 76.6 and 89.7%, respectively) and *S. cariocanus* (in MD, 40%). On the other hand, wines DB and MD, contained fairly abundant groups of *Saccharomycetales* which could not be identified, probably due to missing reference sequences in databases. The genus *Hanseniaspora*, represented by the species *H. uvarum*, was identified in all the wines at 4.4–8.5%. Another yeast characteristic of the fermentation environment, *Torulaspora delbrueckii*, was present only in WJ wine (above 1%). Each wine also contained other fungi: *Aureobasidium pullulans*, *Alternaria infectoria*, *A. rosae*, *A. tenuissima*, and *Mycosphaerella tasiana*. Figure 1B shows bar plots of RA of the main yeast species for the three wines studied.

Table 2. Relative abundance (RA) of bacterial and yeast genera identified in the Polish red wines.

RA of Bacterial Genera (Code)	DB Wine (%)	MD Wine (%)	WJ Wine (%)
Curtobacterium (C)	0.02	0	0.04
Bacillus (B)	0.03	0.01	0.03
Lactobacillus (L)	14.98	28.53	17.74
Fructobacillus (F)	0	8.87	0
Leuconostoc (Le)	0.2	31.27	0
Lactococcus (La)	2.57	7.84	0.04
Cohnella (Co)	0.02	0	0
Acetobacter (A)	0	0.17	0
Gluconobacter (G)	3.53	2.26	8.25
Komagataeibacter (K)	0	18.37	0
Methylobacterium (Me)	0.05	0.01	0.01
Sphingomonas (S)	0.46	0.02	0.1
Ralstonia (R)	0.03	0.01	0.01
Polaromonas (Po)	0.04	0	0
Massilia (Ma)	0	0	0
Escherichia-Shigella (ES)	0	0.01	0.04
Erwiniaceae (E)	0.01	0	0
Enterobacter (En)	0	0	0.01
Pantoea (Pa)	0.01	0	0.06
Tatumella (T)	77.54	1.22	68.6
Pseudomonas (P)	0.04	0.01	0.02
other (O)	0.46	1.39	5.1
RA of yeasts genera			
Meyerozyma (Mey)	0.008	0.001	0.001
Metschnikowia (Mt)	0.068	0.087	0
Pichia (Pi)	0.03	0.001	0
Kazachstania (Ka)	0	0	0.027
Lachancea (Lac)	0	0.097	0.039
Saccharomyces (Sc)	77.037	47.299	90.007
Torulaspora (To)	0.023	0	1.093
Candida (Ca)	0.235	0.003	0.065
Starmerella (Ss)	0	0.015	0
Hanseniaspora (Ha)	5.997	4.446	8.507
Saccharomycetales (Sun)	16.271	46.735	0
Symmetrospora (Sy)	0.005	0.003	0
Curvibasidium (Cu)	0.01	0.001	0
Filobasidium (Fi)	0.023	0.008	0
Vishniacozyma (Vi)	0.018	0.004	0
Bulleromyces (Bu)	0.007	0	0
Other fungi (OF)	0.26	1.2	0.25

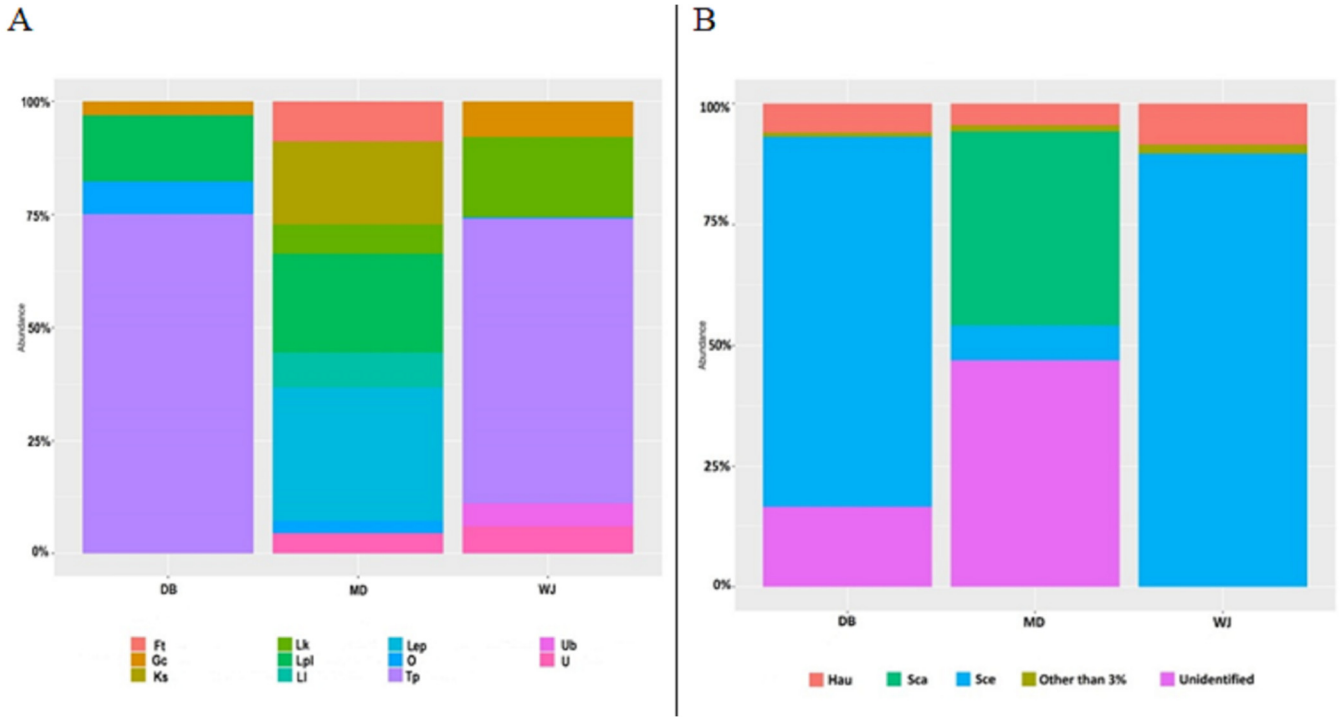

Figure 1. Bar plots showing relative abundance of bacterial (**A**) and yeast (**B**) species in wine samples. Abbreviations (**A**): Ft—*Fructobacillus tropaeoli*, Gc—*Gluconobacter cerinus*, Ks—*Komagataeibacter saccharivorans*, Lk—*Lactobacillus kunkeei* (*Apilactobacillus kunkeei*), Lpl—*Lactobacillus plantarum* (*Lactiplantibacillus plantarum*), Ll—*Lactococcus lactis*, Lep—*Leuconostoc pseudomesenteroides*, Tp—*Tatumella ptyseos*, Ub—uncultured bacteria, U—unidentified, O—other bacteria, (**B**) Hau—*Hanseniaspora uvarum*, Sca—*Saccharomyces cariocanus*, Sce—*Saccharomyces cerevisiae*, O—other than 3%, U—unidentified. Bar plots were performed using ggplot2 software (https://ggplot2.tidyverse.org, accessed on 20 October 2021).

The tb-PCA biplots (Figure 2) allow the communities of bacteria and yeasts typical of the particular wines to be identified. The term "typical" does not refer here to the number of microorganisms, but to their uniqueness, i.e., the fact that they occur in only one wine, which distinguishes them from one another. The factors shown in biplot 2A explain over 98.2% and those shown in biplot 2B explain exactly 100% of the variability of the system under consideration. Among bacteria, the following genera and species were typical of MD wine: *Lactococcus* sp., *Fructobacillus* sp., *Komagateibacter* sp., *F. tropaeoli*, *L. citreum*, *L. pseudomesenteroides*, *L. mesenteroides*, *A. cerevisiae*, *G. oxydans*, *K. hansenii*, and *K. saccharivorans*. Bacteria that were typical of WJ wine included *Enterobacter* sp., *Tatumella* sp., *Pantoea* sp., *E. coli*, *C. herbarum*, and *L. kunkeei* (*A. kunkeei*). In turn, DB wine was unique in containing the following bacteria: *Erwiniaceae*, *Polaromonas* sp., *Conella* sp., *Sphingomonas* sp., *T. punctata*, *P. rhizosphaere*, *G. frateurii*, and *L. pentosus*. Among the yeasts, the following species were peculiar to the particular wines: DB—*B. albus*, *M. pulcherrima*, *P. kluyveri*, and *T. pretoriensis*; MD—*S. paradoxus*, *S. cariocanus*, and *Starmerella* sp.; and WJ—*T. delbrueckii* and *K. servazzi*. The pattern of points lying between the wine vectors in the biplots also provided information on the species that were characteristic of two or three of the wines studied, e.g., *Curtobacterium* sp., *Pseudomonas* sp., *B. subtilis*, *P. gingerii*, and *T. ptyseos* occurred mostly in DB and WJ wines, while *L. plantarum* and the yeasts *S. coprosmae*, *F. wieringae*, *F. stepposum*, *M. chrysoperlae*, and *M. sinensis* were found mainly in MD and DB.

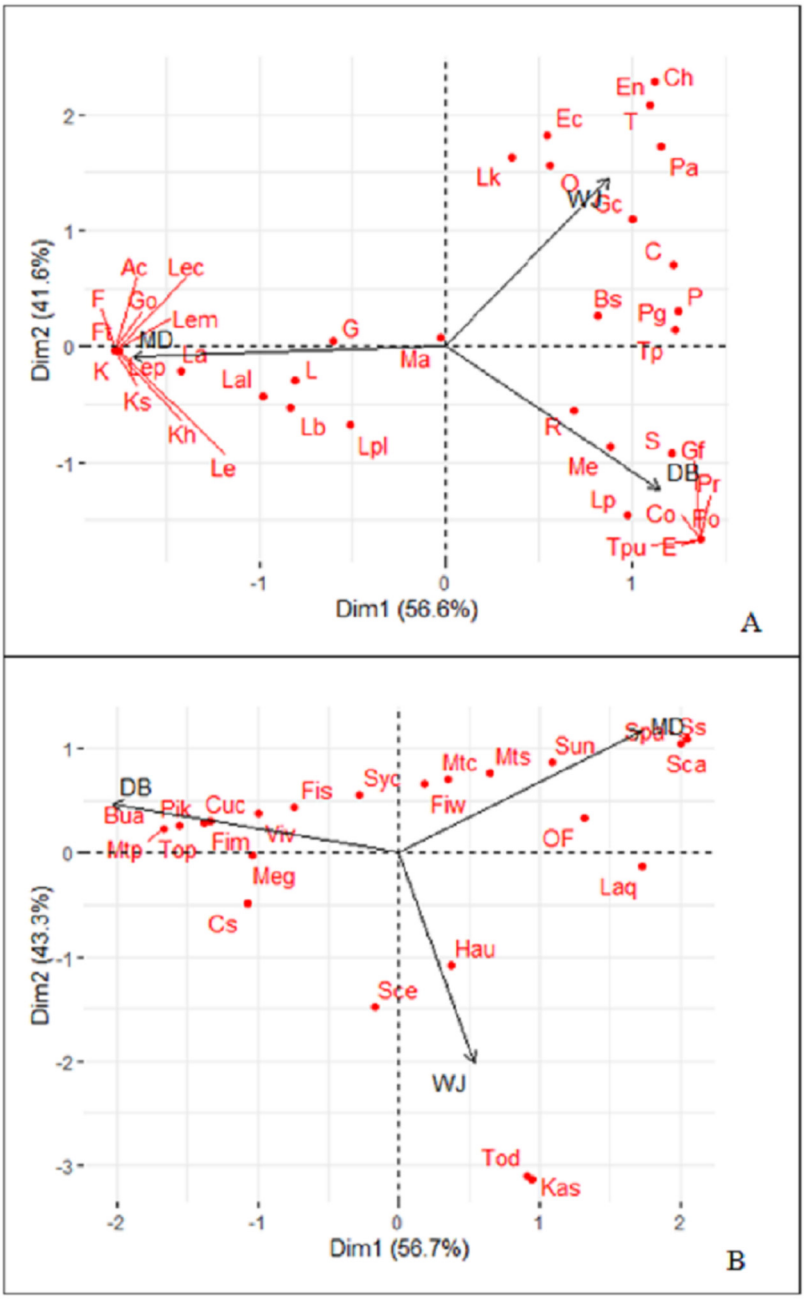

Figure 2. tb-PCA biplots of bacterial (**A**) and yeast (**B**) communities typical for DB, MD, and WJ wines. Abbreviations (**A**) F-*Fructobacillus* sp., Ft-*Fructobacilus tropaeoli*, Ac-*Acetobacter cerevisiae*, Go-*Gluconobacter oxydans*, Lec-*Leuconostoc citreum*, Lem-*Leuconostoc mesenteroides*, Lep-*Leuconostoc pseudomesenteroides*, La-*Lactococcus* sp., Lal-*Lactococcus lactis*, *L*-*Lactobacillus* sp., Lb-*Lactobacillus brevis (Levilactobacillus brevis)*, Lpl-*Lactobacillus plantarum (Lactiplantibacillus plantarum)*, K-*Komagataeibacter* sp. Ks-*Komagataeibacter saccharivorans*, Kh-*Komagataeibacter hansenii*, Le-*Leuconostoc* sp., G-*Gluconobacter* sp., Ma-*Massilia* sp., Lk-*Lactobacillus kunkeei (Apilactobacillus kunkeei)*, Ec-*Escherichia coli*, En-*Enterobacter* sp., Ch-*Curtobacterium herbarum*, O-other bacteria, T-*Tatumella* sp., Gc-*Gluconobacter cerinus*, Pa-*Pantoea* sp., C-*Curtobacterium* sp., P-*Pseudomonas* sp., Bs-*Bacillus subtilis*, *Pg-Pseudomonas gingeri*, Tp-*Tatumella ptyseos*, R-*Ralstonia* sp., Me-*Methylobacterium* sp., S-*Sphingomonas* sp., Gf-*Gluconobacter frateurii*, Pr-*Pseudomonas rhizosphaerae*, Po-*Polaromonas* sp., Lp-*Lactobacillus pentosus (Lactiplantibacillus pentosus)*, Co-*Cohnella* sp., Tpu-*Tatumella punctata*, E-*Erwiniaceae*,

(**B**) Bua-*Bulleromyces albus*, Mtp-*Metschnikowia pulcherrima*, Pik-*Pichia kluyveri*, Top-*Torulaspora pretoriensis*, Cuc-*Curvibasidium cygneicollum*, Viv-*Vishniacozyma victoriae*, Fim-*Filobasidium magnum*, Meg-*Meyerozyma guilliermondii*, Cs-*Candida* sp., Fis-*Filobasidium stepposum*, Syc-*Symmetrospora coprosmae*, Fiw-*Filobasidium wieringae*, Mtc-*Metschnikowia chrysoperlae*, Mts-*Metschnikowia sinensis*, Sun-*Saccharomycetales* unident., Spa-*Saccharomyces paradoxus*, Ss-*Starmerella* sp., Sca-*Saccharomyces cariocanus*, OF-other fungi, Laq-*Lachancea quebecensis*, Hau-*Hanseniaspora uvarum*, Sce-*Saccharomyces cerevisiae*, Tod-*Torulaspora delbrueckii*, Kas-*Kazachstania servazzii*. Biplots were performed using R software (The R Foundation for Statistical Computing, Vienna, Austria).

3.2. Comparative Analysis of the Characteristics of the Investigated Red Wines

The parameters of the individual Regent variety grape musts used for fermentation were as follows: MD must—extract value 22 °Blg, pH 3.63, total acidity as tartaric acid 5.01 g/L; WJ must—extract value 21 °Blg, pH 3.56, total acidity as tartaric acid 5.64 g/L; and DB must—extract value 20 °Blg, pH 3.62, total acidity as tartaric acid 5.7 g/L. Fermentation was controlled by weight loss related to CO_2 release until a constant weight was achieved (Table 3). Table 4 shows the key parameters of the DB, MD, and WJ wines obtained. The differences in the ethanol content between the wines were not statistically significant, with a mean of 11.09% vol. The pH values were significantly lower for DB and MD wines (3.7–3.72) compared to WJ wine (3.88). The total acidity (TA) of DB and WJ wines (4.72–4.92 g/L) was significantly lower than that of MD wine (7.24 g/L). DB and WJ wines were characterised by significantly higher TPC values (about 25–30%) than MD wine. DB, MD, and WJ wines statistically differed in DPPH values, glucose, fructose, and lactic acid contents. The DPPH value was the lowest for MD wine and the highest for WJ wine, which was correlated with TPC values. Malic acid was not detected in any of the wines, whereas lactic acid was present in all the wines, which means that MLF took place. Charts showing differences in the above parameters among the wines studied are included in Supplementary Materials (Figures S1–S5).

Table 3. Kinetics of CO_2 releasing in DB, MD, and WJ musts.

Fermentation Time (Days)	DB	MD	WJ
		CO_2 g/L	
2	12.77 ± 4.27 *	8.31 ± 3.13	7.65 ± 2.20
4	41.17 ± 1.32	8.31 ± 3.13	8.31 ± 3.13
6	17.65 ± 2.63	14.23 ± 11.50	5.73 ± 1.20
8	6.90 ± 2.32	24.79 ± 4.02	20.91 ± 24.39
10	4.97 ± 3.13	15.63 ± 7.22	8.41 ± 6.72
12	0.61 ± 0.86	7.41 ± 6.76	24.07 ± 22.87
14	0.00 ± 0.00	0.00 ± 0.00	1.02 ± 0.41
The sum of released CO_2	84.08	78.69	76.10

* Mean values ± standard deviation. The sums of CO_2 are given as sums of mean values.

Table 4. Analysis of key parameters of red wines.

Wine	Eth% vol. *	pH *	TA g/L *	TPC mg GAE /L *	DPPHA mmol TL/L *	Glu g/L *	Fru g/L *	LA g/L *	AA g/L *
DB	11.02 ± 0.51 ^{ab}	3.72 ± 0.08 ^{a}	4.92 ± 0.08 ^{a}	3369 ± 122.82 ^{a}	10 ± 0.34 ^{b}	2.01 ± 0.06 ^{c}	0.81 ± 0.02 ^{b}	3.21 ± 0.09 ^{a}	nd
MD	11.48 ± 0.27 ^{b}	3.70 ± 0.06 ^{a}	7.24 ± 0.42 ^{b}	2677 ± 259.87 ^{b}	7.43 ± 0.39 ^{a}	1.89 ± 0.06 ^{b}	3.89 ± 0.07 ^{c}	4.41 ± 0.10 ^{c}	1.40 ± 0.05 ^{b}
WJ	10.78 ± 0.25 ^{a}	3.88 ± 0.05 ^{b}	4.72 ± 0.19 ^{a}	3495 ± 234.65 ^{a}	11.63 ± 0.43 ^{c}	1.23 ± 0.05 ^{a}	0.69 ± 0.03 ^{a}	4.02 ± 0.05 ^{b}	0.66 ± 0.03 ^{a}

* Mean values ± standard deviations; values with the same superscript letters in the same column and the same variable are not significantly different ($p < 0.05$). Abbreviations: Eth—concentration of ethanol, TA—total acidity, TPC—total polyphenol concentration, DPPHA—analysis of antioxidant activity, Glu—glucose concentration, Fru—fructose concentration, LA—lactic acid concentration, AA—acetic acid concentration; nd—not detected.

The data presented in Table 4 were analysed using tb-PCA, and the results are shown in Figure 3. MD wine was characterised by the highest values of total acidity, and fructose and lactic acid concentrations, WJ had the highest pH and DPPH values, and DB had the

highest glucose concentration. Figure 3 also shows that the lowest glucose and ethanol contents were found for WJ, the lowest lactic acid content was detected in DB, and the lowest TPC value was recorded for MD wine.

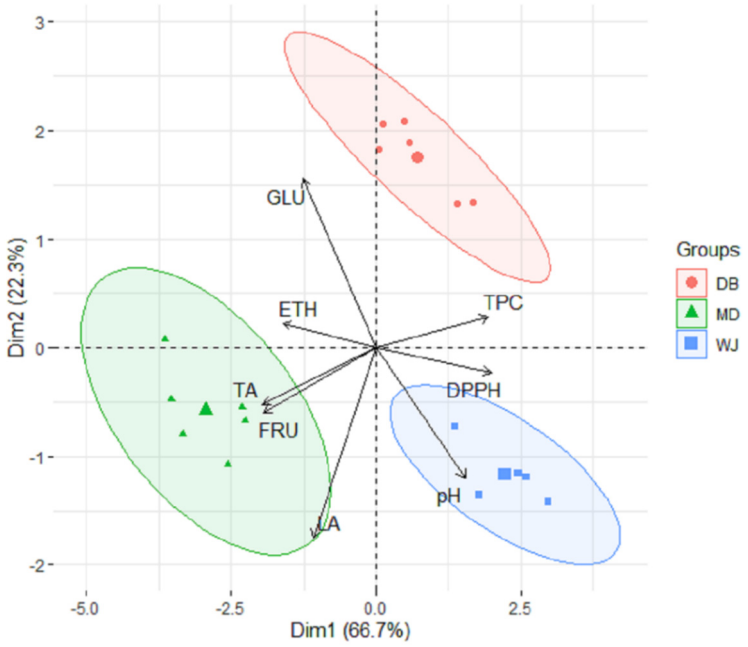

Figure 3. A tb-PCA biplot of key parameters of the particular wines. Abbreviations: ETH-concentration of ethanol, TA-total acidity, TPC-total polyphenol concentration, DPPH-analysis of antioxidant activity, GLU-glucose concentration, FRU-fructose concentration, LA-lactic acid concentration. Biplot was performed using R software.

3.3. Unique Features of the Investigated Wines

Table 5 summarises the physicochemical characteristics of the wines identified on the basis of statistically significant differences and lists microorganisms unique to the particular wines. It turns out that each wine has a unique set of characteristics and microorganisms, which may be a consequence of the specific metabolic reactions taking place in the cells of the individual microorganisms and the interactions between these microorganisms. The relationships between the wines' physicochemical parameters and their microbial communities are not obvious, but the highest content of acetic and lactic acids in MD wine clearly corresponds to the unique presence of acetic acid bacteria and several species of lactic acid bacteria in this wine. This observation is additionally corroborated by the highest value of TA in MD wine. It is interesting that ethanol concentrations in the wines were not statistically significant, so this feature was not connected with a specific species or genus peculiar to only one kind of wine.

Table 5. Unique features of the studied wines.

Feature	DB Wine	MD Wine	WJ Wine
Characteristic with the highest statistical significance	Glu	TA, Fru, LA, AA	pH, DPPH
Characteristic with the lowest statistical significance	LA	TPC, DPPH	Glu, Fru
Unique bacteria	Pr, Po, Gf, S, Co, Tpu, E, Lp	La, F, Ft, Ac, Go, Lec, Lem, Lep, K, Ks, Kh	En, C, Ch, Lk, T, Pa, Ec
Unique yeasts	Bua, Mtp, Pik, Top	Spa, Ss, Sca	Tod, Kas

Abbreviations are the same as under Figures 2 and 3.

4. Discussion

The high-throughput sequencing technique provide a very useful tool for the rapid and exhaustive characterisation of microbial populations connected with wineries and wine production [19]. Recently, there have been numerous reports from many countries with wine-making traditions on the influence of various factors on the microbiota of grapes, wines, and the environments connected with them [28,29]. This is the first report from Poland in which the reader can find information about Polish red wines as analysed using a metagenetic approach. The literature provides evidence that the abundance and diversity of microorganisms in spontaneously fermented wines depend on grape microbiota, the course of fermentation, the metabolites produced, and environmental conditions [18,19]. Generally, among fungal populations found in vineyard grapes, the most frequently occurring yeast genera are *Hanseniaspora*, *Saccharomyces*, *Candida*, *Debaryomyces*, *Metschnikowia*, *Pichia*, *Torulaspora*, *Zygosaccharomyces*, *Saccharomycodes*, *Brettanomyces*, *Cryptococcus*, *Isaatchenkia*, and *Starmerella* [30–32]. Many of these yeasts have also been reported to be present in musts during alcoholic fermentation [33–35]. The Polish red wines had similar populations of yeasts, among which *Saccharomyces*, represented by *S. cerevisiae* and *S. cariocanus*, was the most abundant genus. Moreover, *Hanseniaspora* and *Candida* were present in all the wines, while *Pichia*, *Torulaspora*, *Metchnikowia*, and *Lachancea* were detected in two of the three wines. Bacterial communities are usually more plentiful and varied than yeast communities on grapes, in must, and in wine. According to the literature, the most abundant bacteria found on vineyard grapes belong to the taxa *Firmicutes*, *Lactobacillales*, *Bacillales*, *Enterobacteriales*, *Pseudomononadales*, *Rhodospirilliales*, *Pasteurellales*, *Bacteroides*, and *Actinobacteria* [33,34,36]. On the other hand, bacterial populations in musts are limited to *Lactobacillales*, *Bacillales*, *Enterobacteriales*, *Pseudomononadales*, *Rhodospirilliales*, and *Oenococcus oeni* [33]. In the present study, we detected many of these groups of bacteria, namely *Lactiplantibacillus*, *Bacillus*, *Enterobacter*, and *Gluconobacter*. *Oenococcus* was not found in any of the wines, while *Tatumella* was present in all samples. It has been stated that many bacterial genera and species have a wide range of occurrence. Nevertheless, fermentation usually reduces microbial diversity in favour of *Saccharomyces* yeasts.

The Polish red wines obtained from grapes of the Regent variety had different TPC and DPPH radical scavenging activities and distinct glucose, fructose, and lactic acid concentrations, but they did not differ significantly in ethanol concentration. Generally speaking, the investigated wines contained similar concentrations of ethanol and organic acids, and had pH, TA, TPC, and DPPH values similar to those determined by other authors in wines of the same grape variety, independent of the country of origin [37–40]. It is interesting that ethanol concentration values in our wine samples, which were obtained using spontaneous fermentation, were similar to those reported by Dobrowolska-Iwanek et al. [39] and Kapusta et al. [38], in whose studies the active yeasts *S. cerevisiae* were used to run the fermentation, and the values reported in Liu et al.'s work [40], in which sequential fermentation with *Metchnikowia* or *Hanseniaspora* cultures together with *S. cerevisiae* was carried out.

The associations between the characteristic microbiota and the physicochemical parameters of wines are interesting but rarely touched upon in the scientific literature. In the present study, we suggest that there exists a relationship between the bacteria and yeasts uniquely inhabiting the individual red wines and the specific characteristics of those wines, as shown in Table 5. The most prominent of those is the relationship between organic acids (acetic and lactic) and specific AAB and LAB bacteria. Probably, the lack of malic acid is indicative of MLF. Our wines did not contain *Oenococcus oeni*, which is generally responsible for this type of fermentation, but it has been demonstrated that *L. plantarum* is also capable of performing MLF [41], and this species was present in the wines tested, especially in MD and DB; in WJ wine, we detected large counts of *L. kunkei (A. kunkeei)*, which could also be connected with MLF. Two interesting observations made with regard to yeasts were the presence of *S. cariocanus* and *S. paradoxus* in MD wine and the absence of *S. cerevisiae*. Siren et. al. [42] reported the presence of several species besides *S. cerevisiae* in

wines, *S. paradoxus*, among others, and they explained that there was a close evolutionary relationship between these two yeast species, so it is possible that *S. paradoxus* is an artefact driven by close sequence homology.

5. Conclusions

Metagenetic analysis shows the multitude of microorganisms inhabiting the environment of vineyards and fermentation niches. The chemical and physical properties and the composition of the microbiota of the Polish red wines are generally in line with European and world trends. In our wine samples, the most abundant bacterial species were *T. ptyseos*, *L. pseudomesenteroides*, and *L. plantarum* and the most highly represented yeast species were *S. cerevisiae*, *S. cariocanus*, and *H. uvarum*. The red wines studied showed significant differences in antioxidant capacity, sugars, and lactic acid concentrations, ethanol contents were similar, and malic acid was absent. The growing interest in the production of spontaneous fermentation wines gives a chance to obtain wines with exceptional organoleptic characteristics. Another possibility of using the knowledge acquired in this study may be the development of unique local starter cultures to control fermentation so that the wines produced can have unique individual characteristics similar to spontaneously fermented wines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12094373/s1, Figure S1: Ethanol concentrations in wines (MD, WJ, DB) compared using Tukey's HSD test, Figure S2: pH (a) and total acidity (b) in wines (MD, WJ, DB) compared using Tukey's HSD test, Figure S3: Total polyphenol contents (a) and antioxidant activity measured by DPPH assay (b) in wines (MD, WJ, DB) compared using Tukey's HSD test, Figure S4: Residual glucose (a) and fructose (b) concentrations in wines (MD, WJ, DB) compared using Tukey's HSD test, Figure S5: Lactic acid (a) and acetic acid (b) concentrations in wines (MD, WJ, DB) compared using Tukey's HSD test and T test, respectively, Table S1: Relative abundance (RA) of bacterial species identified in the Polish red wines, Table S2: Relative abundance (RA) of yeast species identified in the Polish red wines.

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