

SHORT COMMUNICATION

Culturable microorganisms during fermentation of Veltlínske zelené (Grüner Veltliner) ice wine**KATARÍNA ŽENIŠOVÁ – MÁRIA BUČKOVÁ – ANDREA PUŠKÁROVÁ –
LUCIA KRAKOVÁ – LUBICA PIKNOVÁ – DOMENICO PANGALLO****Summary**

Limited information related to the microbial communities responsible for fermentation of central European ice wine made by Veltlínské zelené grape is available in the scientific literature. In this study, various culture media for fungi and bacteria were used to isolate a large and diverse panel of microorganisms occurring in frozen berries and in two steps of wine fermentation. The isolated microorganisms were clustered through internal transcribed spacer polymerase chain reaction (ITS-PCR) and consequent electrophoretic amplicons separation by QIAxcel system (Qiagen, Hilden, Germany). One or more fungal and bacterial representatives of each cluster were identified by sequencing of ITS fragment and 16S rRNA gene, respectively. The microbiological analysis displayed a complex bacterial community composed by *Staphylococcus* spp., *Gluconobacter* spp., *Lactobacillus* spp., *Ewingella americana*, *Leuconostoc* spp., *Okibacterium* sp., *Lactococcus lactis*, *Carnobacterium maltaromaticum*, *Sanguibacter* sp., *Terrabacter terrae*, *Enterococcus faecium*, *Micrococcus* spp., *Bacillus* spp., *Acetobacter* sp., *Propionibacterium* spp., *Gordonia soli* and *Paenibacillus* sp. The fungal microbiota were very similar to usual microflora previously detected in wine-related samples, where *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Pichia* spp. were the dominant yeasts.

Keywords

ice wine; frozen berries; cultivation; fungal community; bacterial microbiome

Ice wine is a sweet fermented product manufactured from frozen grape berries left on the vine until winter hard freeze [1]. Such kind of wine is produced in Europe, mainly in Central European countries, in North America (where Canada is the biggest world producer) and also in Japan [2]. Many factors are important for the production of a good ice wine, but the characteristics of the berries and, of course, the microorganisms participating in wine fermentation play major roles.

Although ice wine is a popular product in Central Europe, until now only limited amount of information is available about the microflora present during its fermentation stages. Therefore, investigation of microbiome is very important in order to understand which kinds of microorganisms are involved in the ice wine production. In order to give a first response to such

ecological question, the culturable microflora of frozen berries and of different fermentation phases of Veltlínské zelené ice wine from Modra (Slovakia) were studied.

Fungal and bacterial communities were isolated from three different samples from January–February 2015: winter grapes (B), middle fermenting must (M1) and must in the end-fermentation phase, almost wine (M2).

Various microbiological agar media were used for the isolation of fungal members, namely, yeast extract peptone dextrose (YPD; medium more specific for the isolation of yeasts), potato dextrose agar (PDA; culture medium appropriate for the recovery of moulds) [3] and, for bacterial strains, R2A (medium suitable for isolation of a broad spectrum of bacteria), GYC (for cultivation of acetic acid bacteria; glucose 50 g·l⁻¹, yeast extract

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10 g·l⁻¹, calcium carbonate 20 g·l⁻¹, agar 20 g·l⁻¹, 50 mg·l⁻¹ nisin), de Man–Rogosa–Sharpe (MRS; generic medium for isolation of lactic acid bacteria in anaerobic conditions) and MRS-Tomato (MRS-T; this medium was used in order to widen the isolation spectrum of lactic acid bacteria, in particular regarding *Oenococcus* strains; anaerobic conditions) [4]. The dehydrated media were purchased from HiMedia (Mumbai, India), the chemicals used for media preparation were provided by Sigma-Aldrich (St. Louis, Missouri, USA).

The isolates were clustered by the internal transcribed spacer polymerase chain reaction (ITS-PCR) using the fungal primers: ITS3 (GCA TCG ATG AAG AAC GCA GC) and ITS4 (TCC TCC GCT TAT TGA TAT GC) [3], and bacterial primers: G17 (GTG AAG TCG TAA CAA GG) and the 1:1 mixture of GplusR (CGT CCT TCA TCG GCT) and GminusR (CGT CCT TCA TCG CCT) (all synthesized by Microsynth, Balgach, Switzerland) [4]. The ITS-PCR amplicons were separated by QIAxcel electrophoresis equipment (Qiagen, Hilden, Germany). Representatives of each cluster were identified by sequencing the yeast ITS fragment using the primers: ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 [3], or by sequencing the bacterial 16S rRNA gene amplified by the primers 27f (AGA GTT TGA TCM TGG CTC AG) and 685r (TCT ACG CAT TTC ACC GCT AC) [4]. The bacterial and fungal ITS-PCR coupled to the QIAxcel amplicon separation permitted clustering of isolated microorganisms. It

was possible to observe that each species produced its characteristic QIAxcel profile (Tab. 1, Tab. 2).

A larger fungal diversity was displayed in the berries sample (B) than in the fermentation specimens (M1 and M2). The fungal microbiome from berries was composed by *Hanseniaspora uvarum*, three *Pichia* species, *Metschnikowia* spp., *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. In sample M1, only fungal representatives of *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* were recovered, while in sample M2, the only species isolated was *Saccharomyces cerevisiae* (Tab. 1).

Bacterial community was found to be more differentiated than the fungal counterpart, as different *Staphylococcus* and *Gluconobacter* species, as well as *Lactococcus lactis* strains were identified at all the three sampling stages. The stage M1 showed the largest bacterial diversity with 10 genera and 17 species identified, followed by the microbiota isolated from berries with 10 genera and 11 species identified, and from M2 (end-fermentation phase, almost wine) with 8 species belonging to 6 genera (Tab. 2).

The media used in this study for isolation of fungi had a similar efficiency regarding isolation of different fungal members, while the bacterial media were not so specific. In fact, MRS and MRS-T media, which should have been selective for lactic acid bacteria, were also useful for the isolation of *Gluconobacter* and different other genera, such as *Propionibacterium*, *Terrabacter* and *Gordonia*, belonging to the phylum *Actinobacteria*. Moreover, the medium GYC, used for the isolation of acetic

Tab. 1. Yeast microbiota isolated from winter grapes and from production steps of ice wine.

| QIAxcel profile No. | Species identification on the basis of the highest internal transcribed spacer similarity score | Percentage of isolates | | | | | |
|---------------------|--|------------------------|-----|---------|-----|---------|------|
| | | Berries B | | Must M1 | | Must M2 | |
| | | YPD | PDA | YPD | PDA | YPD | PDA |
| 387 | KJ706285 <i>Hanseniaspora uvarum</i> 100%/100% FR751341 <i>Hanseniaspora uvarum</i> 100%/100% | 11% | 20% | ni | 33% | ni | |
| 291 | KJ706777 <i>Pichia kluyveri</i> 100%/100% | 11% | ni | ni | | ni | |
| 275 | KT029806 <i>Pichia fermentans</i> 100%/100% KM402064 <i>Pichia fermentans</i> 100%/100% | 11% | 12% | ni | | ni | |
| 240 | KM603624 <i>Pichia anomala</i> 100%/99% | ni | 6% | ni | | ni | |
| 248 | JX234570 <i>Metschnikowia pulcherrima</i> 100%/100% KT029787 <i>Metschnikowia pulcherrima</i> 100%/100% | 39% | 32% | ni | | ni | |
| 258 | KM243745 <i>Metschnikowia</i> sp. 100%/99% | 11% | 6% | ni | | ni | |
| 423 | CP006454 <i>Saccharomyces cerevisiae</i> 100%/100% KT732653 <i>Saccharomyces cerevisiae</i> 100%/100% KU131579 <i>Saccharomyces cerevisiae</i> 100%/100% KP204935 <i>Saccharomyces cerevisiae</i> 100%/100% | 17% | 12% | 100% | 67% | 100% | 100% |
| 461 | JX458097 <i>Zygosaccharomyces bailii</i> 100%/100% | ni | 12% | ni | | ni | |

B – winter grapes; M1 – middle fermenting must; M2 – must in the end-fermentation phase, almost wine; YPD, PDA – microbiological media for the isolation of yeasts; ni – not isolated.

Tab. 2. Bacterial microbiota isolated from winter grapes and from production steps of ice wine.

| QIAxcel profile No. | Species identification on the basis of the highest 16S rRNA similarity score | Percentage of isolates | | | | | | | | | | | |
|---------------------------|--|------------------------|-----|-----|---------|-----|-----|-----|---------|-----|-----|-----|-------|
| | | Berries B | | | Must M1 | | | | Must M2 | | | | |
| | | R2A | GYC | MRS | MRS-T | R2A | GYC | MRS | MRS-T | R2A | GYC | MRS | MRS-T |
| 415 | HQ436427 <i>Staphylococcus</i> sp. 99%/99% | | | ni | | ni | ni | ni | 9% | 16% | ni | ni | ni |
| 393 | KJ642472 <i>Staphylococcus</i> sp. 100%/100% | | | | | | | | | | | | |
| 631 | LT571449 <i>Staphylococcus epidermidis</i> 100%/100% | 6% | ni | ni | ni | | | | | | | | |
| 631 | EU379290 <i>Staphylococcus pasteurii</i> 100%/100% | | | ni | | 33% | ni | ni | ni | 12% | ni | ni | ni |
| 694 | KU821699 <i>Staphylococcus warneri</i> 100%/100% | | | ni | | | | ni | | 36% | ni | ni | ni |
| 653 | LC103259 <i>Gluconobacter</i> sp. 100%/99% | | | ni | | ni | 40% | ni | ni | | | | |
| 681 | X80775 <i>Gluconobacter cerinus</i> 100%/100% | | | | | | | | | | | | |
| 681 | KP234004 <i>Gluconobacter cerinus</i> 100%/100% | ni | ni | 11% | ni | | | ni | | 20% | ni | ni | ni |
| 548 | KM485570 <i>Lactobacillus plantarum</i> 100%/100% | ni | ni | 11% | ni | ni | ni | 25% | ni | | | | |
| 548 | LC144969 <i>Lactobacillus plantarum</i> 100%/100% | | | | | | | | | | | | |
| 521 | KU945826 <i>Lactobacillus pentosus</i> 100%/100% | ni | ni | 15% | ni | | | ni | | | | | |
| 555 | KT983990 <i>Ewingella americana</i> 100%/100% | | | | | | | | | | | | |
| 555 | HE585220 <i>Ewingella americana</i> 99%/100% | 23% | 15% | ni | ni | | | ni | | | | | |
| 433 | KT952390 <i>Leuconostoc</i> sp. 100%/100% | | | ni | | ni | ni | ni | 25% | | | | |
| 462 | KT952384 <i>Leuconostoc pseudomesenteroides</i> 100%/100% | | | ni | | ni | ni | 19% | ni | ni | ni | 30% | ni |
| 550 | KR067643 <i>Okibacterium</i> sp. 100%/99% | 6% | ni | ni | ni | | | ni | | | | | |
| 405 | LC096207 <i>Lactococcus lactis</i> 100%/100% | | | | | | | | | | | | |
| 405 | KU324909 <i>Lactococcus lactis</i> 100%/99% | | | | | | | | | | | | |
| 405 | DQ171718 <i>Lactococcus lactis</i> 100%/100% | | | | | | | | | | | | |
| 405 | AB593359 <i>Lactococcus lactis</i> 100%/99% | | | | | | | | | | | | |
| 405 | KR858832 <i>Lactococcus lactis</i> 100%/99% | | | | | | | | | | | | |
| 405 | KC845210 <i>Lactococcus lactis</i> 100%/100% | | | | | | | | | | | | |
| 513 | KT767913 <i>Carnobacterium maltaromaticum</i> 100%/100% | 12% | 7% | ni | ni | | | ni | | | | | |
| 601 | HG942154 <i>Sanguibacter</i> sp. 100%/100% | ni | 15% | ni | ni | | | ni | | | | | |
| 423 | JX517277 <i>Terrabacter terrae</i> 100%/100% | ni | 23% | 4% | ni | | | ni | | | | | |
| 446 | KM495938 <i>Enterococcus faecium</i> 100%/100% | | | | | | | | | | | | |
| 446 | CP011281 <i>Enterococcus faecium</i> 100%/100% | ni | ni | 7% | 38% | | | ni | | | | | |
| 546 | GQ246667 <i>Micrococcus</i> sp. 100%/99% | | | ni | | ni | 6% | ni | ni | | | | |
| 540 | LN774579 <i>Micrococcus yunnanensis</i> 100%/99% | | | ni | | ni | 6% | ni | ni | | | | |
| 494 | KJ184943 <i>Bacillus</i> sp. 100%/100% | | | ni | | ni | 18% | ni | ni | | | | |
| 494 | KP992151 <i>Bacillus</i> sp. 100%/100% | | | | | ni | | | | | | | |
| 307 | KT200500 <i>Bacillus sonorensis</i> 99%/99% | | | ni | | ni | 6% | ni | ni | | | | |

Tab. 2. continued

| QIAxcel profile No. | Species identification on the basis of the highest 16S rRNA similarity score | Percentage of isolates | | | | | | | | | | | |
|---------------------------|--|------------------------|-----|-----|-------|---------|-----|-----|-------|---------|-----|-----|-------|
| | | Berries B | | | | Must M1 | | | | Must M2 | | | |
| | | R2A | GYC | MRS | MRS-T | R2A | GYC | MRS | MRS-T | R2A | GYC | MRS | MRS-T |
| 371 | KP761420 <i>Bacillus altitudinis</i> 100%/100% | | ni | | | ni | 18% | ni | ni | 8% | ni | ni | ni |
| 470 | KT985478 <i>Bacillus subtilis</i> 100%/100% | | ni | | | 23% | ni | ni | ni | | | | |
| 532 | KM085445 <i>Acetobacter</i> sp. 100%/100% | | ni | | | 33% | ni | ni | ni | | | | |
| 390 | KP944184 <i>Propionibacterium acnes</i> 100%/99% | | ni | | | ni | ni | ni | 14% | | | | |
| 410 | KM507346 <i>Propionibacterium</i> sp. 100%/100% | | ni | | | ni | ni | ni | 9% | | | | |
| 581 | NR_043331 <i>Gordonia soli</i> 100%/100% | | ni | | | ni | ni | ni | 9% | | | | |
| 238 | HQ703905 <i>Paenibacillus</i> sp. 100%/99% | | ni | | | | | ni | | 8% | ni | ni | ni |

B – winter grapes; M1 – middle fermenting must; M2 – must in the end-fermentation phase, almost wine; R2A, GYC, MRS, MRS-T – microbiological media for the isolation of bacteria; ni – not isolated.

acid bacteria and mainly for the *Gluconobacter* group, facilitated also the recovery of lactic acid bacteria (*Carnobacterium* and *Lactococcus lactis*) and members of the genera *Sanguibacter*, *Ewingella*, *Terrabacter*, *Micrococcus* and *Bacillus*. Members of the genus *Acetobacter* were exclusively isolated on R2A medium. This discrepancy between the bacterial media and the expected isolated species was already evidenced by GODALOVA et al. [4] and pointed again to the need of proper identification of the isolated bacteria, in order to better understand the microbial ecology of wine-related samples.

Comparing the fungal diversity of ice wine with our previous results [3] on Veltlínské zelené wine from the same region, it can be noted that *Saccharomyces cerevisiae* is present already in the iced berries. Moreover, it seems that the genera *Pichia* and *Metschnikowia* in iced berries are represented by different species. Another difference between the two types of wine is the presence of *Hanseniaspora uvarum* also during the fermentation of ice wine. In fact, this species was isolated in the middle fermenting stage (M1), while the fungal microbiome of normal wine was composed exclusively by *S. cerevisiae* strains.

Different lactic acid bacteria were isolated from iced berries, the diversity being similar to that of Veltlínské zelené grapes [4]. The difference regarded mainly the large presence of *Lactococcus lactis* members on iced berries instead of *Leuconostoc* strains. In this survey, *Leuconostoc* species were isolated only during the fermentation steps. It is well known that lactic acid bacteria from the genus *Oenococcus* play an important role during malolactic fermentation of red wine [5]. Unfortunately, only limited information is available in the literature regarding the specific functions of *Lc. lactis* strains in wine fermentation. Generally, various kinds of lactic acid bacteria can produce several types of enzymes, which contribute to the organoleptic characteristics of wine [6].

Largest community of *Actinobacteria* and *Gammaproteobacteria* was identified on iced berries and on previously analysed grapes [4], respectively. This was another characteristic of the bacterial community of iced berries, which could be influenced by the different climatic conditions. We had more chances to isolate *Actinobacteria* than *Gammaproteobacteria* members from this kind of cold environment. In fact, *Actinobacteria* are frequently isolated from extreme and cold habitats [7].

To our knowledge, different *Actinobacteria* such as *Okibacterium* sp., *Sanguibacter* sp. and

Terrabacter terrae were recovered in this study from grape berries for the first time, although these bacteria were shown, by previous studies, to be associated with soil and plants [8–10]. The bacterium *Carnobacterium maltaromaticum* can be considered as an unusual isolate for this kind of environment, as it is frequently detected on meat, seafood and fermented products [11–13].

This paper is a rare survey of the culturable microbial community of frozen Veltlínské zelené grapes and its fermentation stages for the production of ice wine. The fungal microbiome appeared similar to that detected in our previous investigation oriented to yeast dynamics during the production of normal Veltlínské zelené wine. On the other hand, the bacterial culturable community exhibited predominance of different lactic acid bacteria and also a diffused presence of *Actinobacteria* instead of *Gammaproteobacteria* members. We think that other studies should be carried out using a culture-independent approach to obtain a detailed picture of the microbiome of ice wine samples.

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