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Influence of microbial communities on the chemical and sensory features of Falanghina sweet *passito* wines

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ABSTRACT

Natural (N) as well as starter inoculated (S, inoculated with *Saccharomyces cerevisiae* M3–5; CZS, *Candida zemplinina* T13, *Zygosaccharomyces bailii* NS113 and *Saccharomyces cerevisiae* M3–5) fermentations of Falanghina must from dehydrated grape were monitored. Culture dependent analyses and amplicon-based high-throughput sequencing targeting 18S rRNA and 16S rRNA genes were used to monitor the fungal and bacterial communities (8 sampling points during 65 days). The resulting wines were subject to both sensory evaluation and volatile organic compounds analysis.

Fungal community of un-inoculated musts (N) at beginning of the fermentation was mainly represented by *Aureobasidium*, *Cladosporium*, *Sclerotinia*, while *Candida*, *Debaryomyces*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Saccharomyces* and *Zygosaccharomyces* showed a very low occurrence. The dominance of *Hanseniaspora vineae* and/or *Hanseniaspora uvarum* was clear up to 29th days of fermentation. *S. cerevisiae* occurred in all the phases but become dominant only at the end of the process.

The odour profiles as evaluate by Quantitative Descriptive Analysis (QDA) highlighted a significant impact of the fungal populations on the olfactory profiles of the wines. Raisins, dried fruits, Sherry and liqueur were stronger in both S and CZS, while N was mostly discriminated by solvent/chemical and floral features. Outcomes underpin the impact of microbiota on the chemical and odour traits of Falanghina *passito* wines.

1. Introduction

From the *Vins de paille* of France to Austrian *Strohwein*, wines made from dried grapes are a centuries-old tradition. From this perspective, Italy can boast excellence of many notorious and time-honored sweet or dry *passito* wines, both from white or red grapes dehydrated on-vine or post-harvest under the sun, in shaded aerated rooms or in fully controlled environmental conditions. Withering (*Appassimento*) is a lost-in-time process whereby grapes are partially dried mostly to concentrate sugars and flavors prior to vinification. Moreover, the production technologies may be deeply variable and may include important parameters, such as slow/long multiple fermentations, the addition of fresh must during the fermentation, the wine ageing in oak barrels and many others. All these gives rise to different wine styles, corresponding to typical products characterized by distinctive sensory characteristics and flavor complexity (Moio & Piombino, 2013).

The basic *Appassimento* styles differ markedly depending on the area. Different grape varieties are dried in distinctive ways and for a variable time, depending on the quality of the harvest, as well as on the local tradition or DOC/DOCG (Appellation of Controlled Origin/Appellation of Controlled and Guaranteed Origin) regulations. In other words, the high number of variables involved makes such phase hard to standardize. As grapes are slowly dehydrated in *fruttaio* without environmental conditioning in the traditional *passito* wine production, the incidence of noble rot on the grapes can be extremely variable, according to the weather conditions.

Indeed, a crucial aspect in *passito* wines production is the fermentation step. Such phase is often delegated to the fresh must microbiota although cellar residential yeasts can play an important role as shown by different researchers (Aponte & Blaiotta, 2016a; De Filippis, La Storia, & Blaiotta, 2017; Tofalo et al., 2009). However, in *Vin Santo* production, a natural starter culture (known as the mother sediment) is used to enrich the must (Domizio et al., 2007). Musts for *passito* wine production are characterized by a high sugar content (over 300 g/L),

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coupled to a high concentration of acids, polyphenols, metal ions and Maillard reaction's by-products, that may contribute to stuck or sluggish fermentation (Tofalo et al., 2009). To address this problem, many winemakers inoculate pure yeast cultures into the must after pressing instead.

Only a few works have focused on the selection of autochthonous yeast strains to efficiently lead the main fermentation. Aponte and Blaiotta (2016a) adopted indigenous yeast strains for the fermentation on a pre-competitive scale of *Moscato di Saracena*, a *passito* wine produced in Southern Italy, while Azzolini and co-workers (2013) coupled the use of selected yeast strains in fermentation, with the use of *Botrytis cinerea* cultures during drying in the vinification of *Amarone* and *Recioto* wines. Moreover, Rantsiou et al. (2012) demonstrated that mixed starters including *S. cerevisiae* and *C. zemplinina* could be profitably used to reduce the production of acetic acid by *S. cerevisiae* in sweet wine fermentations.

In the present study, natural and starter-inoculated fermentations of musts of sun dried Falanghina grapes were monitored. This typical grape cultivar of the Campania Region (Southern Italy) is characterized by a middle trunk-conical bunch, winged with medium-sized grapes provided with thick and waxy peel, crispy pulp from slightly floral flavor, which gives rise to musts with a strong acidity. Physico-chemical and microbiological evaluations were performed from the beginning until the end of the fermentation to describe the kinetics and to depict fungal and bacterial population changes. Finally, we evaluated the sensorial profile and the volatile organic compounds (VOCs) of the resulting wines to investigate the impact of the microbial populations on the chemical and odour profiles of this wine.

2. Material and methods

2.1. Passito must production

Grapes used for this study were cultivated in Castelvenere (Benevento province) at an altitude of about 150 m above the sea level by Geneva Double Courtain (GDC) farmed system, planted 2.40 × 1.40 m. Guyot pruning was carried out by leaving three bosses in fruit per plant. Soil was characterized by steep slope and medium-low fertility. The grapes were harvested in the second half of October, already over-ripened and, precisely, characterized by an average sugar content of 25.5°Brix. Bunches quality was excellent nevertheless a further selection was made by removing berries exhibiting small lesions or evidence of alteration. Grapes were placed in perforated plastic trays sized 50 × 30 × 20 cm (about 2.5 kg each), covered with a thin non-woven sheet and brought into the so-called *fruttaio*: a well-ventilated drying room. After about one month of withering, a further selection was carried out by eliminating berries evidently affected by *Botrytis* not in the noble form. Before pressing (beginning of March), withered grapes were subjected to a final selection. Bunches were de-stemmed and lightly crushed before being pressed with a steel mini wine press. We extracted about 50 L of *Passito* must starting from 140 Kg withered grapes (about 35%). The must obtained had the following main features: total sugars, 37°Brix; pH, 3.81; total acidity, 8.9 g/L (expressed as tartaric acid).

2.2. Fermentations trials

Must was sulphitated (100 mg/L of potassium metabisulphite) and divided into six batches. Four batches were inoculated with selected yeast starters. Specifically, two batches “S” were added with *S. cerevisiae* M3–5 (about 10^6 CFU/mL), previously isolated from Passito of Moscato di Saracena (Aponte & Blaiotta, 2016a); two batches “CZS” were inoculated with *Candida zemplinina* T13 (about 10^6 CFU/mL) isolated from Aglianico of Taurasi (Aponte & Blaiotta, 2016b) and *Zygosaccharomyces bailii* NS113 from Falanghina wine (Aliberti, Aponte, & Blaiotta, 2012), both at a populations level of about 10^6 CFU/mL, and *S. cerevisiae* M3–5 at about 10^5 CFU/mL. The remaining two batches “N” were left to spontaneous fermentation, thus serving as controls. Strains were previously grown in a medium with high sugar content (yeast extract 10 g/L, peptone 20 g/L, sucrose 350 g/L, pH 3.5) at 25 °C for 3 days. At the onset of fermentation, all batches were supplemented with 100 mg/L of Nutistart (Laffort, Bordeaux Cedex), containing assimilable nitrogen and thiamine, was used as fermentation activator (100 mg/L provides 14 mg/l of total available nitrogen and 0.13 mg/L of thiamine). Such addition was repeated after 7 days (at 50 mg/L). Fermentations were carried out at 18–20 °C. After 49 days, a first racking was performed and lees were discarded. At 66 days, after a second racking, wines were further sulphitated (50 mg/L), bottled and stored at 10 °C. Fermentations were physico-chemically and microbiologically monitored by collecting samples at 0, 4, 7, 13, 20, 29, 34, 42, 63, and 66 days of fermentation. After one month of storage at 10 °C, wines were subjected to sensory evaluation and volatile organic compounds (VOCs) analysis.

2.3. Physico-chemical analyses

Brix degrees and pH were assessed by using a portable refractometer (0–80% Sper Scientific, PBI International) and a lab pH-meter (XS, model pH 50), respectively. Total acidity of samples was estimated by titration with NaOH 0.25 N on 25 mL of must/wine samples, and expressed as tartaric acid (g/L). Glucose, fructose, glycerol, and ethanol contents were evaluated by HPLC analyses as previously described (Aponte & Blaiotta, 2016a). A Gilson 307 Series HPLC system equipped with a refractive index detector (RID 133, Gilson) coupled to a Met-Carb68H column (6.5 × 300 mm, Varian) was used. Tartaric, malic, acetic and lactic acids were estimated HPLC by using an Agilent 1100 Series HPLC system equipped with a DAD detector (Agilent 1100 series G13114B) and a Spherisorb ODS2 column (5 µm 4,6 × 250 mm, Sigma-Aldrich).

Color intensity and tonality were evaluated by spectrophotometer reading the absorbance at 420, 520 and 620 nm (Eppendorf Basic BioSpectrometer), after dilution (1:5) in tartaric acid solution (9 g/L of tartaric acid, pH 3.2). Color intensity was calculated as the sum of A420, A520, A620 values, while the color tonality was assessed by ratio A420/A520. All analyses were performed in triplicate. Significant differences ($p < .05$) among the three fermentations were tested by ANOVA and *post-hoc* Tukey's test, carried out in R environment.

2.4. Microbiological analyses

Must and wine samples were serially diluted in quarter strength Ringer's solution (Oxoid, Basingstoke, UK) and spread-plated (in duplicate) on WL-nutrient agar (Oxoid) supplemented with 100 mg/L of chloramphenicol and on MRSm1 medium, for yeast and lactic acid bacteria (LAB) counts, respectively. MRSm1 was developed ad hoc, adding to 26 g/L of MRS broth (Oxoid) the following ingredients: yeast extract (5 g/L), fructose (5 g/L), L-cysteine hydrochloride monohydrate (0.5 g/L), calcium chloride (0.25 g/L), bromocresol green (22 mg/L), agar (20 g/L), 170 mg/L of cycloheximide and 10 mg/L of pantothenic acid (all from Sigma-Aldrich). pH was adjusted to 5.2 with 20% malic acid solution. Plates were incubated at 30 °C for 5 days.

2.5. DNA isolation from must/wines, high-throughput sequencing analysis (HTS) and data analysis

Total DNA extraction from samples collected at the different sampling points was carried out by using the Biostic Bacteremia DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA), starting from the pellet obtained from 2 mL of must/wine (10,000 × *g*, 2 min). Before DNA isolation, pellets from the two replicates for each sample, were joined, washed twice with 1.5 mL of STE (Sodium Chloride-Tris-EDTA, pH 8.0) buffer and centrifuged at 12,000 × *g* for 2 min.

The bacterial and eukaryotic diversity were studied through HTS. Fungal communities were studied by sequencing of a portion (V4 subregion) of the small subunit of ribosomal RNA gene (18S rRNA), using a recently described primer set (18S-580f/18S-997r) (De Filippis, Laiola, Blaiotta, & Ercolini, 2017). Library preparation and sequencing on a 454 GS Junior platform were carried out as previously reported (De Filippis, Laiola, et al., 2017). Bacterial community was studied through sequencing of the V3-V4 region of the 16S rRNA gene amplicons obtained by using primer S-D-Bact-0341F/S-D-Bact-0785R (Klindworth et al., 2013). Libraries were prepared and sequenced on an Illumina MiSeq platform as recently described (Berni Canani et al., 2017). Paired-end reads were joined by using FLASH (Magoc & Salzberg, 2011).

Quality filtered reads (average quality score below 25, length < 250 or 300 bp, for Bacteria and Fungi, respectively) were then analyzed and further filtered by using QIIME 1.9.1 software, with a pipeline recently described (Berni Canani et al., 2017). OTUs defined by 97% similarity threshold were identified by using the Silva SSU/LSU rRNA gene release 119 for Fungi (Quast et al., 2013) and the Greengenes 13.5 database for Bacteria (McDonald et al., 2012), respectively.

Statistical analyses and plotting were carried out in R environment (http://www.r-project.org). Principal Component Analysis (PCA) was carried out on the Log transformed abundance table by using *dudi.pca* function in *made4* package. Spearman's pairwise correlations were computed between fungal and bacterial OTU abundance or microbiota and VOCs (*corr.test* function in *psych* package) and plotted by using the *corrplot* function. Correction of *p*-values for multiple testing was performed (Benjamini & Hochberg, 1995).

The 18S and 16S rRNA gene sequences are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under accession number SRP149165.

2.6. Sensory analysis

A quantitative descriptive sensory analysis (QDA) of the experimental wines was performed. The odour profiles (orthonasal evaluation) were obtained employing apanel composed of 8 judges (4 males and 4 females, 21 to 48 years old). They were recruited from the staff and the students of the Department of Agricultural Sciences of the University of Naples Federico II, selected on the basis of their sensory abilities, trained in recognize and describe odours (chemical standards), in performing sensory descriptive analysis of wine and with extensive experience in sensory descriptive analyses of various wine typologies including sweet wines. Thirty mL of each wine were served at 10 °C in black tulip-shaped glasses, coded with random three-digit codes. Samples were evaluated in duplicate (two duplicate sessions). Each judge evaluated all the wines in each session and the wines were served according to a randomized service design. The judges were asked to focus on the perceived odour descriptors and rate the corresponding intensities on a 9- point scale (1 = very weak, 2 = weak, 3 = medium, 4 = strong, 5 = very strong, half values being allowed). They were provided with a list of 25 odour descriptors (the order was randomized among the judges) associated with sweet wines (Reboredo-Rodríguez, González-Barreiro, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2015). Furthermore, under the item “More” the judges had the opportunity to add and rate descriptors eventually perceived and not included in the list.

Only the descriptors cited at least 4 times (citation frequency ≥ 50%) in one sample and one repetition were retained for data elaboration. Sensory data were computed as the geometric mean of frequency and mean intensity (Mean Sensory Modified Frequency - MF) as described by Dravnieks (1982): $MF = (F * I)^{1/2}$; where *F* is the frequency of citation expressed as a percentage of the maximum frequency of citation (i.e. total number of judges) and *I* is the mean intensity expressed as a percentage of the maximum rate (i.e. the value of 5). This method takes into account both types of values produced by assessors for each descriptor: frequency of citation of a sensory term and intensity assigned to it. In this way are properly considered cases in which a term has been used frequently but with low scores, and cases in which the same descriptor has been poorly cited but with high scores. Data were analyzed by one-way ANOVA, and the mean scores for each descriptor of the three experimental wines were compared by a Tukey *t*-test ($p < .05$) (XLStat 2012.6.02 statistical pocket, Addinsoft Corp., Paris, France).

2.7. VOCs analysis

For VOCs extraction, 100 mL of wine were extracted by liquid-liquid extraction with 5 mL of CH_2Cl_2 as a solvent spiked with 250 µL of an alcoholic solution of 2-octanol (258 ppm/EtOH) as internal standard. The mix was kept under magnetic stirring for 1 h at the constant temperature of 21 °C. After 12 h at 2 °C, the organic phase was recovered through a separating funnel, dried over Na_2SO_4, and stored at −20 °C until the GC/MS analysis. Each extraction was carried out in triplicate.

For High Resolution Gas-Chromatography/Mass Spectrometry (HRGC/MS) analysis, 2 µL of organic extract were injected in splitless mode, while the injection port of a GC/MS-QP2010 quadrupole mass spectrometer (Shimadzu, Shimadzu corp., Kyoto, Japan) was maintained at 250 °C. The GC/MS was equipped with a DB-WAX column (60 m, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific Inc., Folsom, CA 95360, USA). The carrier gas was helium (1.3 mL/min) and the temperature program used was the following: 40 °C for 5 min, raised up to 220 °C at a rate of 2 °C/min, and held for 20 min at the maximum temperature. Electron impact mass spectra were recorded with ion source energy of 70 eV, while the temperature was kept at 230 °C. The peak areas were measured using a GC/MS solution program Shimadzu version 2.30 (Shimadzu corp., Kyoto, Japan). Compounds concentrations and identification were computed and performed as previously reported (Piombino et al., 2010). In a few cases, the pure chemical standard was not available, and the identified compounds were labelled as tentative (t). Significant differences among the wines were tested by ANOVA ($p < .05$) performed with the XLStat 2012.6.02 statistical pocket (Addinsoft Corp., Paris, France).

3. Results and discussion

Viable yeasts in uninoculated, sulphitated must (sample N, t0) were about 4.4 Log CFU/mL at the beginning of the fermentation, thus lower than those usually recorded for other *passito* musts (5–6 Log CFU/mL), such as “Vino cotto”, “Vinsanto”, “Mondeuse Black” and “Erbaluce” (Alessandria et al., 2013; Domizio et al., 2007; Rantsiou et al., 2013; Tofalo et al., 2009). This may be due to the high quality of the sun dried berries used, that were manually selected.

As expected, in inoculated batches (S and CZs), fermentation started with a higher level of viable yeasts: 6.0 ± 0.01 and 6.8 ± 0.06 Log CFU/mL in S and CZS, respectively (Supplementary Table 1). After 4 days of fermentation, yeasts reached about 8 Log CFU/mL in both S and CZS trials, and then remained quite stable up to 20 days of fermentation (Supplementary Table 1). From the 20th day on, the yeast microflora in inoculated fermentations showed a different behavior: both experienced a drop, but the decline was faster in CZS if compared to S (Supplementary Table 1). In sample N, yeast microflora reached about 7 Log CFU/mL after one week and did not change significantly up to 44 days (Supplementary Table 1; 7.2–6.5 Log CFU/mL). At the end of monitoring (66 days), yeasts were below one Log CFU/mL in starter-led fermentations, while they reached about 4 Log CFU/mL in the natural fermentation (Supplementary Table 1). In all batches, LAB were undetectable (< 10 CFU/mL) up to the 66th day (Data not shown). Just few cycloheximide-resistant yeast colonies were retrieved on MRSm1 agar plates used for LAB counting (Data not shown).

A culture-independent approach, based on HTS analyses, was applied to monitor both fungal and bacterial communities during

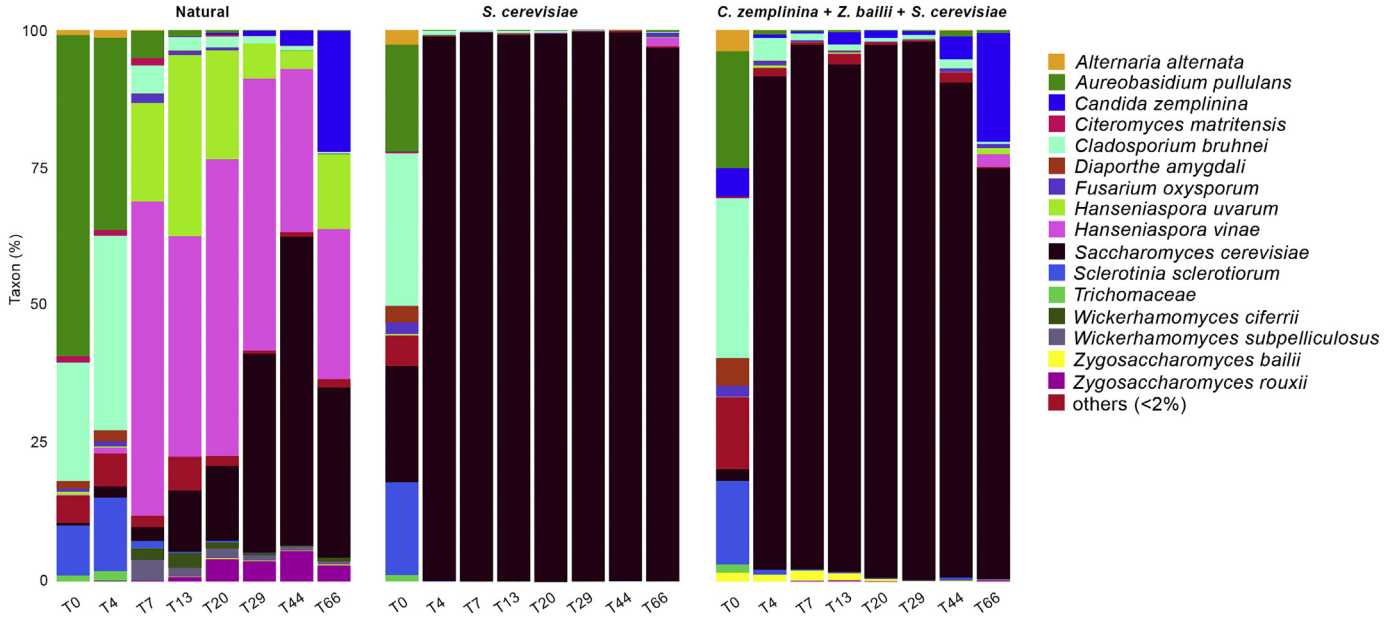

Fig. 1. Stacked bar showing the eukaryotic populations in Passito musts and wines analyzed in this study: N, natural fermentation; S, samples inoculated with *S. cerevisiae*; CZS, samples inoculated with *C. zemplinina, Z. bailii, S. cerevisiae.*

fermentations. HTS technologies have become the tool of choice in deciphering both vineyard and wine microbiome (De Filippis, La Storia, & Blaiotta, 2017; De Filippis, Parente, & Ercolini, 2018; Morgan, du Toit, & Setati, 2017). These technologies have revealed more filamentous fungal species than yeast species especially those associated with the grape berry surface, and a higher diversity compared to other culture-independent methods (Morgan et al., 2017).

In this study, filamentous fungi, such as *Aureobasidium*, *Cladosporium* and *Sclerotinia* spp., prevailed in all the samples at the beginning of the fermentation (Fig. 1). These genera are part of the fungal microbiota of *Vitis vinifera* showing different types of life modes (pathogen, endophyte, saprotroph) and are responsible for diseases like “Fruit rot” and “Shoot blight” (Jayawardena et al., 2018). A high occurrence of *Aureobasidium* (*A.*) *pullulans* was also shown by Alessandria et al. (2013) during on-vine withering of Mondeuse black cultivar winegrapes by culture-dependent methods. However, *A. pullulans* is a technologically irrelevant species that as soon as the fermentation started has disappeared (Alessandria et al., 2013; this study Fig. 1).

Yeasts retrieved in sample N at time 0 included *Hanseniaspora* (*H.*) *uvarum*, *Saccharomyces cerevisiae* (*S.*), *Metschnikowia fructicola*, *Candida* (*C.*) *etchellsii*, *Hanseniaspora vineae*, *Debaryomyces* (*D.*) *hansenii*. Also other yeast genera were previously retrieved in natural musts from dehydrated grapes, such as *Pichia*, *Zygosaccharomyces*, and *Kluyveromyces* (Alessandria et al., 2013; Aponte & Blaiotta, 2016a; Domizio et al., 2007; Tofalo et al., 2009). Besides other factors, the higher sugar content (about 400 g/L) may explain the different microbial composition of our *passito* must compared to that previously reported.

About 20% of *S. cerevisiae* was found in sample S at time 0, while its abundance was about 10 times lower in CZS, consistent with the starter inoculum (Fig. 1). Both *C. zemplinina* and *Zygosaccharomyces* (*Z.*) *bailii* were found at t0 only in CZS. Naturally fermented samples showed a different fungal community and clearly clustered apart from the inoculated samples (Fig. 2). *S. cerevisiae* prevailed in both the inoculated fermentations after 4 days, but *Z. bailii* was still detectable in CZS until 20 days, as well as *C. zemplinina*, which increased in abundance at the end of the fermentation. Higher diversity was found in natural fermentation. It was mainly carried out by *H. uvarum* and *H. vinae* until 29 days, when *S. cereviasiae* abundance started increasing. Moreover, *Z. rouxii* persisted from day 13th until the end of the process and *C. zemplinina* increased from 44 to 66 days (Fig. 1). The persistence, until the end of the fermentation, of *Candida*, *Zygosaccharomyces* and *Pichia* was also shown in Vinsanto and Vincotto *passito* wines by Domizio et al. (2007) and Tofalo et al. (2009), respectively.

Bacterial community was dominated by Proteobacteria and Actinobacteria, while Firmicutes showed lower levels (Supplementary Fig. 1). Most abundant genera in all the fermentations included *Methylobacterium*, *Sphingomonas*, *Propionibacterium* and *Acinetobacter*. Previous HTS analyses of the grapevine phyllosphere, flowers and grape berry surface, demonstrated that the bacterial communities were predominated by Proteobacteria followed by Firmicutes, Actinobacteria, Acidobacteria, and Bacteroidetes (Morgan et al., 2017). In our fermentations *Propionibacterium* and *Staphylococcus* showed an increasing trend up to 66 days. *Acinetobacter* reached abundance > 10% in N fermentation after 29 days, while it developed faster in S (11.3% at 13 days) and CZS (16.9% at 20 days). *Acinetobacter* as well as other bacterial genera including *Methylobacterium*, *Sphingomonas*, *Pseudomonas*, *Wolbachia*, and *Paracoccus* were detected until the end of alcoholic fermentation in previous studies (Bokulich, Joseph, Allen, Benson, & Mills, 2012; Portillo & Mas, 2016). A low abundance of both LAB and acetic acid bacteria was shown during fermentations (Supplementary Fig. 1). Similar results were previously reported (Bokulich et al., 2012). Among LAB, *Lactobacillus* and *Streptococcus* occurred in all fermentation phases, while the other genera were found sporadically only. Both *Gluconobacter* and *Gluconacetobacter* were often found, while *Acetobacter* was never detected.

Finally, we evaluated the co-occurrence pattern between bacterial and fungal communities (Supplementary Fig. 2). *Acinetobacter* significantly (FDR < 0.05) co-excluded with filamentous fungi (*Sclerotinia*, *Cladosporium*), while *Methylobacterium* and *Sphingomonas* showed an opposite correlative pattern. *S. cerevisiae* co-occurred with *Streptococcus* spp. and *C. zemplinina* showed only one significant negative correlation with *Pseudomonas*.

Consequently, different kinetics in sugars consumption, were detected along the three fermentations (Supplementary Table 1). Must at t0 showed a level of total sugars of about 400 g/L (388–397 g/L). Fermentation N showed a one week long lag phase, since during this period only 21 g/L of sugars were consumed. By contrast, S and CZs fermentations promptly began, and > 100 g/L of sugars were consumed in the same time frame: about 105 and 137 g/L, in S and CZS,

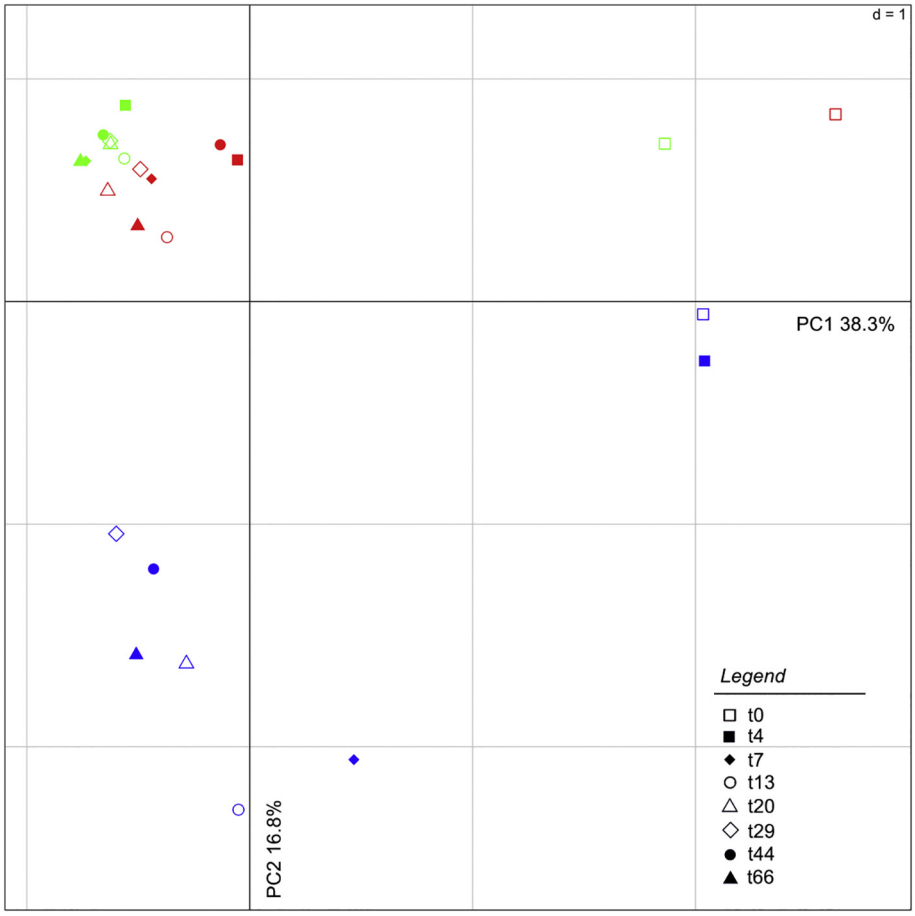

Fig. 2. Principal Component Analysis (PCA) based on eukaryotic community composition. The two principal components were plotted using the *vegan* package in R. Samples were coloured according to the type of inoculum: blue, natural fermentation (N); green, *S. cerevisiae* (S); red, *C. zemplinina, Z. bailii, S. cerevisiae* (CZS). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively. Such difference in sugar consumption (about 30–40 g/L) between fermentations S and CZS continued until 66th days and could be ascribed to the fructophilic character of some yeasts inoculated in CZS. Finally, all the samples at 66 days were characterized by different residual sugar content: 230, 144 and 105 g/L for N, S and CZS, respectively (Supplementary Table 1). High residual sugar is a typical feature of most wines obtained from over-ripened/dehydrated grapes (Alessandria et al., 2013; Aponte & Blaiotta, 2016a; Domizio et al., 2007; Moio & Piombino, 2013; Rantsiou et al., 2012; Tofalo et al., 2009). However, also dry wines with low residual sugars can be produced from *passito* musts by using technological and/or microbiological tools such as starter cultures (Alessandria et al., 2013; Azzolini et al., 2013). Differences in the residual sugars of this type of wine can be mainly related to the initial sugar content of musts that generally ranges from around 280 g/L (eg. Vinsanto must; Domizio et al., 2007) to about 400 g/L (Erbaluce must; Rantsiou et al., 2012), similarly to that used in this study.

The fructose/glucose ratio (F/G) at the onset of the fermentation was that typical of over ripened grapes: 1.16 ± 0.02 (Supplementary Table 1). In spontaneous fermentation N, such value remained unchanged until the day 13th, then slightly increased up to 2 at the day 66th. On the other hand, F/G ratio showed a similar trend in both yeast-inoculated trials, reaching values around 2 after 20 days. Values significantly diverged later, reaching 4.2 and 5.2 at the end of monitoring, in CZS and S respectively (Supplementary Table 1). The lower F/G ratio in CZS compared to S may be related to the occurrence of *C. zemplinina* in the starter culture. In fact, as reported by Rantsiou et al. (2012) and Aponte and Blaiotta (2016b), *C. zemplinina* strains are characterized by a higher consumption of fructose than glucose, and some strains show a total fructophilic character. Data obtained in this study further confirm this behavior. Indeed, S and CZS wines showed similar glucose content (20–23 g/L) but very different fructose concentration (121 g/L in S and 85 g/L in CZS) at the end of fermentation (Supplementary Table 1).

Kinetics of ethanol production substantially reflected those of sugar depletion (Supplementary Table 1). Wines showed variable ethanol content, with the lowest value in N (8.9 ± 0.4%, *v*/v) and the highest in CZS (16.5 ± 0.2%, v/v). Ethanol yield, namely the ratio ethanol/sugar consumed (g/g) was variable as well: 0.42, 0.48 and 0.46 in N, S and CZS, respectively. As expected, glycerol was already detectable in musts at the beginning of fermentation, with values ranging from 3.0 ± 0.2 in CZS to 3.6 ± 0.2 g/L in S (Supplementary Table 1). Indeed, due to water loss stress, the cell metabolism of dehydrated grape shift from aerobic to anaerobic and the osmotic potential due to higher sugar levels improve the synthesis of glycerol together with acetic acid (Cirilli et al., 2012). Although the glycerol content of wines at 66 days did not differ significantly (14.2–14.5 g/L), different production kinetics were highlighted depending on the fermentation: glycerol was produced mainly during the first week in S and CZS, while it reached the same value in the natural fermentation N after 20 days (Supplementary Table 1). This suggest that in the inoculated musts, yeast cells were able to quickly counteract osmotic stress, by producing glycerol at higher rates in order to limit water loss from the cytoplasm and prevent dehydration of the yeast (Erasmus, van der Merwe, & van Vuuren, 2003). Acetic acid was low (0.18–0.24 g/L) in musts, but reached levels ranging from 1.15 ± 0.07 in N to 1.39 ± 0.07 g/L in S after 66 days (Supplementary Table 1). Moreover, regarding acid acetic content, no significant differences were found between S and CZS wines. The concentration of acetic acid may seems too high compared to others *passito* wine previously studied such as Vinsanto, Amarone, Mondeuse Blanck and Moscato of Saracena (Alessandria et al., 2013; Aponte & Blaiotta, 2016a; Azzolini et al., 2013; Domizio et al., 2007). However, the acetic acid produced during alcoholic fermentation

should be correlated with both initial sugar content of must and the sugar consumed during fermentation (ethanol content of wine) also in relation to the winemaking steps, that can importantly differ in the production of the mentioned wines. Sugar content of musts used to produce Vinsanto, Amarone Mondeuse Blanck and Moscato of Saracena wines was about 300–310 g/L (Alessandria et al., 2013; Aponte & Blaiotta, 2016a; Azzolini et al., 2013; Domizio et al., 2007), lower than the must fermented here (400 g/L). Moreover, the fermentation purity index [(acetic acid g/L)/(ethanol % vol)], as defined by Comitini et al. (2011), of the wines produced during this study (0.130, 0.092, and 0,075 for N, S and CZS, respectively) supports the idea that mixed starter cultures including *C. zemplinina* and *Z. bailii* could be a means to manage the alcoholic fermentation in musts from withered grapes. *C. zemplinina* strains are osmotolerant and fructophilic and generally produce low amounts of acetic acid, together with relevant quantities of glycerol from sugar fermentation (Magyar & Toth, 2011), its presence in co-culture with *S. cerevisiae,* consuming sugars at the beginning of fermentation, contributes to reduce the osmotic stress, therefore reducing production of acetic acid by *S. cerevisiae* (Rantsiou et al., 2012). *Z. bailii* is also considered an osmotolerant species (Martorell, Fernàndez-Espinar, & Querol, 2005) and often occurs and persists during *passito* wine fermentations (Domizio et al., 2007; Tofalo et al., 2009). Rantsiou et al. (2012) performed laboratory scale fermentations of Erbaluce dried grape musts (about 400 g/L of total sugars) with 15 different combination of *C. zemplinina* (5 strains) and *S. cerevisiae* (3 strains) by co-inoculum and sequential inoculum. Co-inoculation technique led to higher production of both ethanol (10.6 to 14.9% *v*/v) and acetic acid (0.96–1.62 g/L); by contrast, wine produced through sequential inoculum, were characterized by lower ethanol (7.7 to 11.9% v/v) and acetic acid (0.37–1.03 g/L). In this study the use of *S. cerevisiae* M3–5 alone (fermentation S) or in co-inoculum with *C. zemplinina* T13 and *Z. bailii* NS113 (fermentation CZS) allowed production of wines with higher ethanol content and acceptable acetic acid concentration. *S. cerevisiae* M3–5 was isolated from *passito* wine and previously showed good performances in high sugar environments in both laboratory scale fermentation (38°Brix) and during of the fermentation of *passito* must (30°Brix) (Aponte & Blaiotta, 2016a).

Lactic acid, detected at 0.31 ± 0.09 g/L in must samples, remained almost constant until the day 66th reaching 0.27 ± 0.04 (data not shown). This is in accordance with the low occurrence of LAB in all samples analyzed by HTS.

Finally, wines presented different color features (data not shown). N wines showed the highest intensity (0.544 ± 0.001) coupled to the lowest hue (2.278 ± 0.016); by contrast, CZS wines exhibited the lowest intensity (0.283 ± 0.001) and the highest hue (3.146 ± 0.031).

Mean concentrations of volatiles quantified by GC/MS in the wines are reported in Supplementary Table 2. The three wines differed in their volatile profiles: differences in total alcohols, acids and esters suggest a diverse course of the fermentation process. N was the richest in alcohols and the poorest in acids and esters, while S and CZS were quite similar in terms of total amount of VOCs belonging to these chemical classes.

The concentration of 3-methyl-1-butanol and 2-phenylethanol, which are the main alcohols produced during the alcoholic fermentation, differed in the three wines, reaching the highest levels in N and the lowest in CZS. Although significantly different in their concentrations, alcohols linked to the enzymatic oxidation of fatty acids, 1-hexanol and 3-hepten-1-ol, showed comparable levels in the three wines. Differently, 2,3-butanediol was significantly higher in CZS. Finally, 4-penten-1-ol was not detected in N, while 4-methyl-1-pentanol was detected only in S.

The lowest levels of each of the identified volatile acids were detected in N. Isovaleric and 9-decenoic acids showed the highest concentrations in CZS, while isobutyric, hexanoic and octanoic acids in S; the amounts of propanoic, decanoic and dodecanoic acids were similar in S and CZS.

S resulted as the richest in esters, followed by CZS (−5.8%) and then by N (−32%).

S and CZS were both richer in ethyl esters compared to N, but ethyl hexanoate, ethyl octanoate and ethyl decanoate were higher in S. Acetates were higher in N, where isoamyl acetate and β-phenylethyl acetate, two important wine odour-active molecules, were around two or twenty times more concentrated compared to S and CZS. Ethyl vanillate was detected only in S and CZS.

Three aldehydes linked to the lipoxygenase activity were identified. The two saturated, hexanal and heptanal, showed the same concentration in N, S and CZS, while the unsaturated cis-3-hexenal was detected only in S. The concentration of benzaldehyde was significantly affected by yeasts conducting the alcoholic fermentation, with the highest amount detected in S, followed by CZS (−16%) and finally N (−46%).

Among ketones, 2-pentanone was detected only in N. Other two ketones directly related to the alcoholic fermentation were detected in the three wines: diacetyl was not significantly different but, comparing the spontaneous fermentation to the inoculated wines, it showed an increasing trend; acetoin was significantly different among the three samples; however, it was much higher in N compared to S and CZS. Accordingly, 2,3-butanediol, a compound directly metabolized from acetoin, showed higher levels in CZS compared to N, in line with the higher levels of glycerol. Indeed, 2,3-butanediol is linked to yeasts metabolism and glycerol production, as well as to ethanol oxidation and acetaldehyde. *C. zemplinina* could be responsible for the production of this compound. Indeed, its ability to produce high amount of glycerol was recently confirmed and associated to the low production of ethanol and acetic acid (Englezos et al., 2015). However, from a sensory point of view, we have to consider that both acetoin and 2,3-butanediol have detection threshold values of about 150 mg/L in wine (Romano & Suzzi, 1996) therefore at the detected levels they cannot influence the aroma of any of the studied wines.

γ-Butyrolactone, probably formed by yeast catabolism of glutamic acid (Wurz, Kepner, & Webb, 1988) was the most abundant lactone in all samples, with a significantly higher concentration in CZS; δ-hexalactone was detected in N and S at similar concentrations, while γ-nonalactone was present only in N.

The level of total furans importantly differs among the wines, being CZS the richest both at quantitative and qualitative level, followed by S (−42%) and finally N (−89%). Furfural and 5-hydroxymethylfurfural were the most abundant furans in both the inoculated wines. Sotolon was detected at concentrations higher than its odour detection threshold (10 μg/L in wine) in all the wines, at similar levels and with the highest value in CZS.

Acetals were detected in all the wines and S showed the highest total amount. 2,4,5-Trimethyl-1,3-dioxolane was the most abundant in the three samples, while 2-methyl-1,3-dioxane was detected only in N.

The wine CZS was the richest in sulphur compounds. Methionol was identified in the three wines at very different levels: the highest in CZS, followed by S (−56%) and then N (−90%). Furthermore, an unknown thiol was detected in N and CZS but not in S; its concentration was > 3.5 times higher in CZS compared to N.

Linalool, 4-terpineol, α-terpineol and citronellol were identified in N and S, while only α-terpineol and citronellol were detected in CZS in similar amounts.

The odour sensory profiles (Fig. 3) of the three wines showed some differences that seem in accord with results on VOCs. The three wines showed common odour traits, namely the dominance of the descriptors “raisins”, “dry fruits”, “Sherry/Marsala” and “liquor” (Fig. 3). All these descriptors are commonly elicited in the odour profile of sweet wines (Piombino et al., 2010; Reboredo-Rodríguez et al., 2015). Despite these common characteristics, the fermentative microbiota had a significant impact in differentiating the odour profiles of the wines. Indeed, the CZS wine got significantly higher values for the descriptors “plastic/gum” and “woody”, being woody notes related to the oxidative

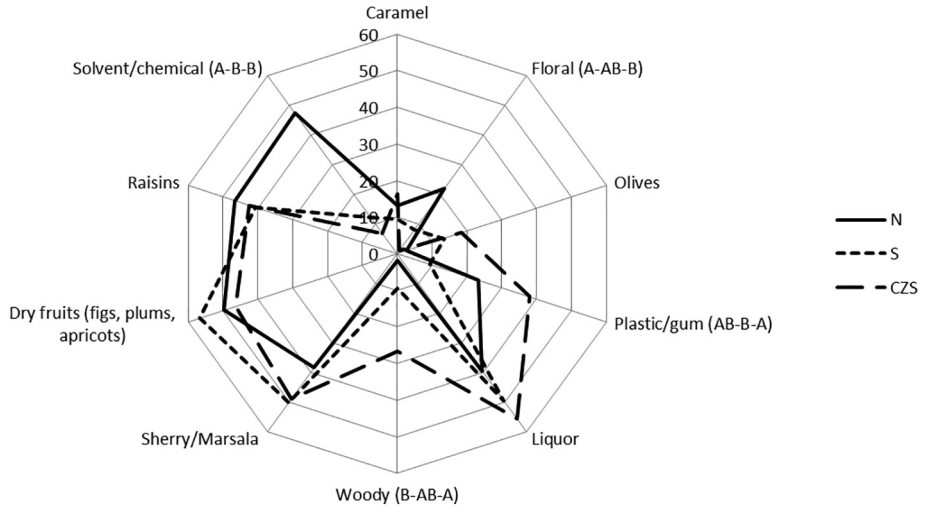

Fig. 3. Odour profiles of the experimental Falanghina sweet passito wines (Mean Sensory Modified Frequency %).

evolution of wine (Escudero, Asensio, Cacho, & Ferreira, 2002), while N wine received significantly higher scores for "solvent/chemical" and "floral" descriptors. The chemical odour of sweet wines has been previously associated to higher alcohols (Ruiz, Zea, Moyano, & Medina, 2010), while terpenes are the major responsible for floral notes (Del Caro et al., 2012; Genovese, Gambuti, Piombino, & Moio, 2007).

The major persistence of *Aureobasidium pullulans* and the clear dominance of *Hanseniaspora* spp. in wine N may be related to both higher alcohols and terpenes production. Increased concentration of higher alcohols have been previously observed by several authors in different wines fermented by non-*Saccharomyces* yeast (Herjavec, Podgorski, Redzepovic, & Mirosevic, 2003; Varela et al., 2009; Vilanova & Sieiro, 2006) having a very active synthetic or Ehrlich pathway for aminoacids metabolism. Moreover, a recent study (Davis, Boundy-Mills, & Landolt, 2012) showed that the major headspace volatiles emitted by *Aureobasidium pullulans* isolated from apples, were 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol.

The higher concentration of 2-phenylethanol and terpenes (linalool, 4-terpineol, α-terpineol and citronellol) in N, could also be ascribed to the hydrolysis of odourless aroma precursors, as a consequence of the high β-glucosidases activity of non-*Saccharomyces* yeasts driving the natural fermentation (Varela et al., 2009).

The "plastic/rubber" descriptors were significantly more perceivable in CZS which was the wine richest in sulphur compounds. Sulphur compounds may be synthesised by yeasts and are associated with unpleasant notes like rubber, garlic and cabbage (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). High sulphur compounds production has been previously associated to *Star. bacillaris* strains (synonym *Candida zemplinina*) (Aponte & Blaiotta, 2016b). Moreover, the woody notes were perceived as stronger in CZS and this could be related to its higher furans concentration. Indeed, furfural was correlated with the "cooked vegetables" descriptor and could also contribute to the "woody note" of aged wines (Escudero et al., 2002). Wine sugars may be converted to furfural, 5-hydroxymethylfurfural, and other correlated volatiles through acid-catalyzed degradation, a pathway of particular importance to sweet wines (Waterhouse, Sacks, & Jeffrey, 2016). Furfural has also been identified as being one of the compounds linked with the tendency of wine to brown (Ferreira, Escudero, Fernańdez, & Cacho, 1997). The woody notes and higher furans concentration in CZS lead to suppose that in this wine a stronger oxidation process occurred. This hypothesis, is also supported by color parameters above reported: CZS wines exhibited the highest hue.

4. Conclusions

This study is the first describing the impact of microbial community on both physico-chemical parameters and odour active compounds in *passito* wines produced from Falanghina wine-grapes cultivar. Indigenous yeasts of the fresh must are often inadequate or too stressed by the high osmotic conditions of the must (hyperosmotic shock) to efficiently carry out the fermentative process, leading to a low-quality wine (high residual sugar content, low alcohol content and with high "solvent/chemical" notes). The use of selected starter cultures, especially if acclimated to the high sugar environment, is confirmed as an optimal solution for *passito* wines production. Moreover, the co-inoculum of osmotolerant *C. zemplinina* and *Z. bailii* with *S. cerevisiae* is confirmed a good solution for production *passito* wines with higher ethanol and relative lower acetic acid. However, the wine produced by mixed starter cultures, during this study, was rich in sulphur compounds and some negative descriptors as "plastic/gum" and "woody" were highly perceived.

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