Highlights

-Culture dependent and independent analysis was performed during fermentations

-Healthy, rotten and botrytized grapes were used as starting material

-Massive sequencing revealed higher diversity at any fermentation stage

-*Saccharomyces* and *Oenococcus* were found in all the musts by massive sequencing

-Healthy grapes showed the lowest biodiversity, at mid and end of fermentation

Microbiome dynamics during spontaneous fermentations of sound grapes in comparison with sour rot and *Botrytis* infected grapes

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ABSTRACT

The main losses in viticulture around the world are normally associated with rotten grapes affecting both the chemical composition and the grape microbiota that later might affect the alcoholic fermentation. We analyzed the population in musts obtained from sour rotten, botrytized and healthy Macabeo grapes and the population dynamics during the spontaneous alcoholic fermentation by culture dependent and various culture independent methods including, for the first time, qPCR and massive sequencing. Grape health state affected the fermentation kinetics and also the microbial diversity and composition. Unexpectedly, the fermentation proceeded the fastest in the rotten must followed by the healthy and the botrytized grapes. As in previous studies, plate cell counts and qPCR results confirmed the increase in the number of both bacteria and fungi in the musts from damaged grapes. Massive sequencing detected higher biodiversity than the other techniques at each stage, with *Saccharomyces* and *Oenococcus* found already in the grape must. *Hanseniaspora osmophila* replaced to *Hanseniaspora uvarum* as the predominant yeast during the mid-fermentation stage for both damaged grapes. Furthermore, musts and beginning of fermentation from rotten and botrytized grapes consistently had a higher presence of the fungi *Zygosaccharomyces, Penicillium* and *Aspergillus* while high abundance of *Botrytis* were observed just for botrytized grapes. As expected, the acetic acid bacteria number increased in musts from rotten and botrytized grapes, mostly due to changes in proportion of the genus *Gluconoacetobacter* which remained more abundant during damaged grapes fermentation than during healthy ones. Interestingly, the presence of *Oenococcus oeni* at the end of the alcoholic fermentation was strongly affected by the health status of the grapes.

Keywords: wine; sour rot; *Botrytis*; massive sequencing; lactic acid bacteria; acetic acid bacteria.

1. INTRODUCTION

The grape berry surface hosts a microbiota of filamentous fungi, yeast, and bacteria that can have an impact on grape and wine quality (Fleet, 2003; Riberéau-Gayon et al., 2006). When the grape surface is altered (e.g. by damaged skin of the berry, highly compact bunches, excess of humidity, phytopathogen infections) the diversity and the population sizes of the microbiota are affected and can lead to the spoilage of the berry. Grape damage of the harvested bunches and the alteration of the grape ecological balance may compromise the vinification process and the final wine quality typically adding off-flavors (Steel et al., 2013). Thus, it is important to further investigate the microbiota diversity changes in damaged grapes and its influence on the alcoholic fermentation.

Sour rot and *Botrytis* infections are the most common causes of heavy grape berry crop losses. The sour rot affects mostly dense bunches close to harvesting and is typically characterized by vinegar odour and brown berries (Barata et al., 2012b). Disease aetiology is related with the skin rupture of the berry caused by physical factors (e.g. rain, hail, berry abrasion) or biological factors (e.g. insects, birds, moulds). The injuries on grape skin contribute to the development of yeasts and bacteria considered as the main responsible agents of this rot (Huber et al., 2011). Moreover, insects are an important source of microorganisms that can colonize grapes and proliferate once the injury in the skin is done (Barata et al., 2012c). *Botrytis* infection (also known as grey mold) is frequent in vineyards exposed to cold and wet conditions during the ripening period (Nigro et al., 2006). In the case of sweet wines, where the presence of *Botrytis cinerea* is desired, the grapes are subjected to an extended ripening before harvesting and to a prolonged period of drying before crushing to enhance the abundance of *B. cinerea* (Stefanini et al., 2016).

Previous studies have documented the microbiota in sound and damaged grapes, including sour rotten and Botrytis-affected grapes (Barata et al., 2008, 2012c; Mateo et al., 2014; Nisiotou et al., 2007, 2011). The results described how grape spoilage affects the grape microbiota, with damaged grapes harboring the highest yeast and acetic acid bacteria (AAB)

population (Barata et al., 2008, 2012a; Mateo et al., 2014). However, most of these studies use culture based techniques probably leading to underestimation of the microbial species involved. Currently, it is accepted that culture-isolated microorganisms are not necessarily representative of the microbial diversity (Amann et al., 1995; Rantsiou et al., 2005). Thus, the reported species selected during grape damaged by sour rot or *Botrytis* might be biased by the composition of culture media and the capacity of the microbes to grow on them (Cocolin et al., 2000; Millet and Lonvaud-Funel, 2000).

Recently, several culture-independent methods based on the genetic background have been used to analyze the microbial diversity from grapes to wine (reviewed in Cocolin et al., 2011). Generally, the use of molecular biology methods has not only endorsed the traditional results but has also been able to identify higher microbial diversity than previously expected (Nocker et al., 2007). Despite the potential of molecular techniques, we have just found one work where these were applied to study the microbial diversity of *Botrytis*-affected grapes (Nisiotou et al., 2011). Specifically, these authors used PCR-DGGE to monitor the yeast population changes during spontaneous fermentations of sound and *Botrytis*-affected grapes. The results included the detection of some bacterial genera not detected before in sour rot or botrytized musts like *Enterobacter*, *Bacillus* and *Staphylococcus*, some of them capable to survive in fermenting musts (Nisiotou et al., 2011).

Among molecular methods, massive sequencing (MS) technologies are becoming a widely used methodology to characterize more precisely the microbial community of complex environmental ecosystems, including food samples (Ercolini, 2013). For example, MS technologies have allowed metagenomic analysis of vineyard and wine microbiome deciphering which microorganisms are present with higher sensitivity than previous techniques and how their communities are affected by several magnitude factors (reviewed in Morgan et al., 2017).

In this study, we aim to establish the relationship between the sour rot and *Botrytis* infection affecting Macabeo grapes with specific changes on the grape microbiota. In order to achieve this objective, sound and damaged grapes were harvested and their microbial diversity

monitored during subsequent spontaneous alcoholic fermentations by both culture dependent and independent methods including PCR-DGGE, qPCR and MS to weigh the biases introduced by the techniques in an effort to estimate the community changes introduced by sour rot and *Botrytis* infection.

2. MATERIALS AND METHODS

Grape samples and experimental wines

During 2016 vintage, grape clusters from the experimental vineyard of the Faculty of Enology (Mas dels Frares, Tarragona Spain) were collected. The sampled vineyard plot produced Macabeo cultivars. Samples were collected using gloves, ethanol, sterilized scissors and sterile plastic bags. Between 10 and 12 replicate grape clusters from different plants within the plot were collected from each grape state in order to capture the heterogeneity present in the sampled lot. Samples without damaged grapes or infection signals were denominated healthy or “H”, grape clusters presenting brown, damaged grapes (typical from sour rot) were denominated rotten or “R” and grape clusters with gray mold in the surface (typical from the *Botrytis* affected grapes) were denominated botrytized or “B”. H and R grapes were collected just before normal harvest, at the beginning of September and the B ones were collected two weeks later. Samples were immediately transported to the experimental cellar located 100 m away from the sampled plot and were crushed by a manual press, skins and seeds were removed by using a sieve resulting in approximately 3 liters of each grape health state. 50 mL of grape juice was directly sampled corresponding to “Must” samples. Afterwards, as a normal procedure in the cellar, 80 mg L-1 potassium metabisulphite (40 ppm SO_2) was added to the rest of the juice. Must samples and the rest of the juice were transported refrigerated to the laboratory within the next hour. Part of the must samples was directly used for microbiological culture and the rest of the must was stored at -80ºC until DNA extraction. The sulfited juice was incubated during 24h at 4ºC to allow clarification. From each health status juice, triplicates of 400 mL clarified juice were

incubated at 23ºC under agitation of 120 rpm in 500 mL flask and allowed to ferment spontaneously without inoculation.

Sampling and monitoring during spontaneous fermentations

The fermentation kinetics was followed considering the time needed to consume the 50% (t_{50}) and the 90% (t_{90}) of sugars. In order to easily monitor the fermentations, the density was measured daily with Densito 30PX Portable Density Meter (Mettler Toledo, Spain). Glucose and fructose concentration was daily measured by Miura One Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A. (Barcelona, Spain). Acetic acid and ethanol were just evaluated during late fermentation, in the last juice sampling point (when the juice density was below 1000 g/L and stable for two consecutive days). Acetic acid content was analyzed by Miura One Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A. (Barcelona, Spain). In the case of ethanol, due to volume limitation, it was measured on the last sampling point by enzymatic method using Ethanol Boehringer Mannheim kit (R-biopharm).

Samples for plating, qPCR, PCR-DGGE and massive sequencing were taken from the must, the beginning of the fermentation (24 hours after the incubation), middle fermentation (juice density between 1050-1040 g/L) and, finally, late fermentation when the juice density was below 1000 g/L and stable for two consecutive days.

Plate culturing

Samples for plating were serially diluted in sterile MilliQ water (Millipore Q-PODTM Advantage A10), plated on (i) YPD medium (Glucose 2%, Peptone 2%, Yeast Extract 1%, Agar 1.7%) and (ii) lysine agar medium (Oxoid, England) plates incubated at 28ºC for 48h; (iii) MRS Agar medium (De Man et al., 1960) supplemented with 4 g/L L-malic acid, 5 g/L fructose, 0.5 g/L L-cysteine, 100 mg/L nystatin and 25 mg/l sodium azide adjusted to pH 5.0 and incubated at 28 ºC in a 10% CO_2 atmosphere and (iv) GYC Agar (glucose 5%, yeast extract 1%, $CaCO_3$ and agar 2%, pH 6.3) supplemented with 100 mg/L natamycin and incubated at 28 ºC for 3–5 days under aerobic conditions. Appropriate dilution plates were

153 counted. The YPD medium provided the total yeast counts, whereas the lysine agar medium
154 is considered to provide the non-*Saccharomyces* cell counts since most *S. cerevisiae* strains
155 have limited growth using lysine as a unique nitrogen source (De Angelo and Siebert, 1987;
156 Heard and Fleet, 1986). However, it has to be considered that probably not all the non-
157 Saccharomyces yeast related to wine environment are able to use lysine as nitrogen source
158 (Jolly et al., 2006). MRS medium and GYC-Ca provided LAB and AAB counts, respectively.
159 **DNA extraction, qPCR and PCR-DGGE**
160 Genomic DNA was extracted from grape must and spontaneous fermentation stages using
161 the recommended procedure for the DNeasy Plant Mini kit (Qiagen, Hilden, Germany),
162 including three bead-beating steps for 3 min in a FastPrep-24 bead beater (MP Bio, Solon,
163 OH) to homogenize the samples. Extracted DNA concentration was measured by nanodrop,
164 adjusted with molecular grade water to a concentration of 50 ng/µl and stored at −20 °C until
165 further processing.
166 Quantitative PCR (qPCR) was performed in an Applied Biosystems 7300 Fast Real-Time
167 PCR System (Applied Biosystems). SYBR Premix Ex Taq (Tli RNase H Plus) was used
168 according to the manufacturer's instructions (Takara). An ABI PRISM96 well optical plate
169 was used for the reaction. This instrument automatically determined the Ct. Yeast
170 quantification was performed using the primers YEASTF/YEASTR for total yeast (Hierro et
171 al., 2006), CESPF/SCERR for *Saccharomyces genus*, generic CESPF/HUVR for
172 *Hanseniaspora* genus (Hierro et al., 2007), AF/200R for *Starmerella bacillaris* (Andorrà et
173 al., 2010), TodsL2/TodsR2 for *Torulaspora delbrueckii* (Zott et al., 2010), Mp5-fw / Mp3-bw
174 for *Metschnikowia* spp. (Díaz et al., 2013) and Bc3F/Bc3R (Suarez et al., 2005) for *B.*
175 *cinerea*. Bacterial quantification was performed using AQ1F/AQ2R primers for general AAB
176 (González et al., 2006) and WLAB1/ WLAB2 for general LAB (Neeley et al., 2005). All the
177 primers anneal the ribosomal gene region. Standard curves were calculated for each type of
178 microorganism in triplicate samples using serial dilutions of purified DNA (Supplementary
179 Table 1).

For the PCR-DGGE analysis, the primer pairs U1GC/U2 and 341fGC/518r were used to amplify the specific U1/U2 of the 28S ribosomal region of yeast (Meroth et al., 2003) and the 16S ribosomal region of bacteria (Muyzer et al., 1993), respectively. The DGGE procedure followed the description in Lleixà et al. (2016). DNA from excised bands was re-amplified with the same primer pair without the GC-clamp and sequenced by Macrogen Company (South Korea). The BLASTN algorithm was applied to the GenBank database to the identify the closest relative at species level. However, the accuracy of the taxonomic identification at species level is not accurate due to the length of the sequences.

Sequencing library construction

The library construction was done with the amplification of 1 sample for each of the musts and 2 samples for each of the fermenting points in the case of bacterial library. In the case of fungal library, 1 sample for each of the must and 1 sample of each of the fermented points were taken. The universal primer pairs 515F/806R (Caporaso et al., 2011) and FR1/FF390 (Prévost-Bouré et al., 2011) with adapters for the sequencing by the equipment PMG from Ion Torrent with chips 318 were used to amplify a region of the 16S and 18S ribosomal gene of bacteria and fungi, respectively. The use of 18S as taxonomic marker for eukaryotic genera is considered limited because many yeast species have no 18S sequence available in the databases, thus we used SILVA (v119) database as described later on because it is more updated and includes more eukaryotic genera than other databases. The universal forward primers included a 10-bp barcode unique to each amplified sample. PCR reactions contained 5–100 ng DNA template, 1x GoTaq Green Master Mix (Promega), 1 mM $MgCl_2$, and 2 pmol of each primer. Reaction conditions consisted of an initial 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 50 °C (for Bacteria) or 52 °C (Fungi) for 60 s, and 72 °C for 90 s, and a final extension of 72 °C for 10 min. PCR reactions were performed in triplicate for each sample replicate, pooled by sample and cleaned using a GeneRead Size Selection kit (Qiagen, Hilden, Germany). Cleaned PCR products were submitted to Centre for Omic Sciences (Reus, Spain) where their quality was checked by a Bionalyzer and their quantity adjusted for sequencing.

208 **Data analysis**

209 Raw sequences were demultiplexed and quality filtered using QIIME v1.9.1 (Caporaso et al.,
210 2010b). Reads were discarded if the length of the read was <200 or >1000 and if any read
211 contained one or more ambiguous base calls. Additionally, reads were truncated at any site
212 containing 3 or more consecutive bases receiving quality score below 10 and reevaluating
213 the remaining length with the aforementioned length rule. After quality filtering, 3,672,972
214 sequences remained with an average of 306,081 sequences per sample (Supplementary
215 table 2). Operational taxonomic units (OTUs) were picked by using QIIME's open-reference
216 pipeline, where Greengenes (13_8) and SILVA (v119) were used as reference databases for
217 16S and 18S rRNA sequences correspondingly, at a 99% similarity threshold. The same
218 databases and threshold have also been used for sequence alignment using PYNAST
219 (Caporaso et al., 2010a) and OTU taxonomy assignment (Quast et al., 2013). The
220 taxonomic assignment up to level species is not accurate for such a small fragment of DNA
221 so the genera level was indicated except when the species was confirmed by qPCR and
222 PCR-DGGE analysis. A final OTU table was created, excluding singletons (sequences
223 observed just once), sequences detected by less of 0.001 abundance and sequences
224 matching plant mitochondria or chloroplast. To avoid biases generated by differences in
225 sequencing depth, bacterial and eukaryotic reads were rarefied to an even depth of 790 and
226 84000 sequences per sample, respectively.
227 Alpha diversity (within-sample species richness) estimates were calculated by analyzing the
228 observed OTUs, the phylogenetic diversity by the PD whole tree index, and Shannon and
229 Simpson diversity indexes.
230 **Statistical analysis**
231 Beta-diversity (between-sample microbial community dissimilarity) estimates were calculated
232 within QIIME using weighted UniFrac distances (Lozupone and Knight, 2005) between
233 samples for bacterial sequences and eukaryotic sequences. Principal coordinate analysis
234 (PCoA) was used to summarize and visualize patterns in species composition. ANOSIM (an
235 analogue of univariate ANOVA which tests for differences between groups of samples) was

performed in QIIME to determine significant differences in phylogenetic or species diversity among experimental factors (grape health state and fermentation stage). Kruskal-Wallis test was used to determine which taxa differed between sample groups. Taxonomic groups were considered to present significant differences in abundances across samples when False discovery rate (FDR)-corrected *P* values were lower than 0.05 for bacteria and P<0.05 for fungi with no FDR correction due to the lack of replicated samples.

3. RESULTS

3.1. Effect of grape health state on fermentation kinetics

Clarified musts from each health state, healthy (H), rotten (R) and botrytized (B), were divided into three biological replicates and allowed to ferment spontaneously (with no yeast or bacteria inoculation, Figure 1). Sugar concentration in R and B initial musts was higher than in the H one (Table 1). Despite the higher sugar content, R fermentations were the faster to consume the 50% (t_{50}) and the 90% (t_{90}) (Table 1). Both fermentations from H and B grapes consumed the 50% of the sugars in 5 days. However, H fermentations reached t_{90} in 11 days, 5 days earlier than the B ones that resulted in the slowest fermentations.

We also evaluated the sugar, ethanol and acetic acid concentration of the last sampling point, when density reached 1000 mg/L. In the case of H grapes, the fermentation was not complete on this time point considering the high sugar and low ethanol concentration. On the other hand, the low sugar and amount of ethanol suggested that R and B fermentations were almost finished at the last measured point. Interestingly, H and damaged grape juices presented similar acetic acid content (Table 1).

3.2. Fungal and bacterial taxonomic composition of Healthy, Rotten and Botrytized musts and fermentations.

Changes in microbial population were monitored along the alcoholic fermentation (must, beginning, middle fermentation and, finally, late fermentation (density was below 1000 g/L for two consecutive days) of H, R and B Macabeo grapes.

As we have previously mentioned, culture dependent (plate culturing in specific media) and independent techniques (qPCR, PCR-DGGE and MS) were applied and compared.

3.2.1. Plate culturing

The yeast population quantification was based on the colony growth in YPD (total yeast population) and lysine agar medium (most non-*Saccharomyces* yeasts) while LAB and AAB populations were quantified using MRS and GYC media, respectively. Total yeast, non-*Saccharomyces* yeast, and AAB counts were higher in the musts and the beginning of the fermentation from R and B grapes compared with the same stages from H grapes (Table 2). However, during the mid and late fermentation, yeast populations were comparable for both damaged and healthy grapes (Table 2).

LAB population was also higher in R must than in the H one. Nevertheless, LAB colonies count increased slightly through the end of H must fermentation while decreased to undetectable levels in R samples. In *Botrytis*-affected samples no LAB colonies were detected at any stage of the fermentation (Table 2).

Even if the AAB populations were higher in R and B musts samples, the number of colonies decreased through the fermentation to undetectable levels while it remained low but constant in H samples (Table 2).

3.2.2. Quantitative PCR (qPCR)

The population levels of total yeast, total LAB, total AAB, *Saccharomyces* spp., *Hanseniaspora* spp., *Torulaspora delbrueckii*, *Metschnikowia* spp., *Starmerella bacillaris* and *Botrytis cinerea* were separately quantified by qPCR with specific primers (Table 2). The total yeast population determined by qPCR was higher in R and B musts than in H one. Apparently, the increase of total yeast in R and B musts was due to an increase in the genera *Hanseniaspora* and *S. bacillaris* while *Saccharomyces* remained at the same level than in H must. Moreover, *Saccharomyces* spp. population in R and B did not increase as much as in H during the fermentation (Table 2). The quantification of *B. cinerea* was only positive for damaged grape samples and it was considerably higher in the B ones that were obtained from grapes visibly affected by this filamentous fungus. However, *B. cinerea*

population gradually decreased through the end of fermentation (Table 2). The anaerobic conditions during alcoholic fermentation would explain the sharp decrease of *B. cinerea* in the last fermentation stages and, probably, the quantification could correspond to DNA from dead cells as no viable fungi was recovered on YPD from the mid fermentation stage onwards.

T. delbrueckii was detected in low proportion in the three musts just increasing through the mid and end of alcoholic fermentation of H samples. The last yeast species quantified, *Metschnikowia* spp., was only detected in low proportion in B samples (Table 2).

In the case of bacteria, the quantification of AAB was at least three orders of magnitude higher in musts from damaged grapes than in the healthy ones. Finally, it was remarkable the increase of LAB population on the late fermentation of H samples (Table 2).

3.2.3. PCR-DGGE

Microbial communities from H, R and B grapes were analyzed at different fermentation stages by PCR-DGGE for Eukarya. The excised DGGE bands were re-amplified and identified by sequencing. Occasionally, some bands migrating differently were identified as the same taxon. Though the identification to species level from the short sequences obtained by PCR-DGGE analysis was not reliable, we were able to detect eight different yeast species (closest relatives: *S. cerevisiae, Hanseniaspora uvarum, S. bacillaris, Candida* spp., *Issatchenkia* spp., *Kazachstania* spp., *Zygosaccharomyces* spp. and *Aureobasidium pullulans*) and two filamentous fungi (*Rhizopus* spp. and *B. cinerea*). *Saccharomyces cerevisiae* was not detected with this technique in any grape must (Table 2). However, it was detected during mid and late fermentation in H, R and B fermentations. Moreover, the highest intensity of *S. cerevisiae* was reached at late fermentations regardless of grape health status. *Hanseniaspora uvarum* and *S. bacillaris* exhibited a similar behavior being present along all grape fermentations and showing higher band intensity from mid to late fermentation (Table 2).

Kazachstania spp., *Zygosaccharomyces* spp., *B. cinerea* and *A. pullulans* were just identified in damaged grape samples. Concretely, *Kazachstania* was detected just in the

must and the beginning of the fermentation while *Zygosaccharomyces* was present in all stages. Besides, *B. cinerea* was observed during all B fermentation phases while it was just detected in the must and the beginning of R fermentations. As previously mentioned, the detection of *B. cinerea* in the last fermentation phases could correspond to DNA from dead cells. In the case of *A. pullulans*, this yeast like fungus was only identified in the first part of B grape fermentation (Table 2).

Apart from *B. cinerea*, we observed another filamentous fungus identified as *Rhizopus* spp. This fungus was present in all grape musts and it was detected until the middle of the R fermentation and late fermentation of B (Table 2).

The PCR for the DGGE analysis with bacterial specific primers did not result in strong amplifications indicating less proportion of bacteria in comparison with yeast population (results not shown). The different DGGE bands from bacterial profiles were excised and amplified for their identification, but most of the resulting sequences did not have a match on the NCBI database probably due to co-migration of bands from similar species and thus, cloning of the excised bands should have been done in order to have single sequences from co-migrating bands. Just two bands recovered from must and the beginning of the fermentation of R grapes were identified as *Gluconoacetobacter* and *Gluconobacter* (Table 2).

3.2.4. Massive sequencing

Barcode amplicon sequencing was used to analyze the bacterial and fungal communities of the different grapes through their fermentation. A total of 382,990 bacterial sequences and 1,954,049 eukaryotic sequences were used to build the OTU tables with an average of 31,916 and 162,837 sequences per sample, respectively (Supplementary table 2). The massive sequencing analysis detected a higher diversity of fungal and bacterial genera than the other techniques (Table 2 and supplementary Fig. 1). However, considering those genera more abundant than 1% on average, 9 fungal and 6 bacterial genera were detected (Table 2).

346 The most abundant yeast on average across all samples was *Hanseniaspora* (38.2%),
347 detected mainly in the beginning and mid fermentation (Figure 2). Interestingly, two different
348 abundant OTUs within *Hanseniaspora* were identified and the closest relatives were *H.*
349 *uvarum* (23.1%) and *H. osmophila* (15.1%). *Hanseniaspora uvarum* was more abundant in H
350 than in R or B samples, while *H. osmophila* was more abundant in mid fermentations of R
351 and B (Figure 2). Other non-*Saccharomyces* yeast were detected in less proportion on
352 average, for example, *Starmerella* (3.3%), and *Zygosaccharomyces* (5.3%) (Figure 2).
353 *Saccharomyces* (19.8% on average) was detected in all musts and every stage of
354 fermentations, being the predominant yeast (between 50.2 and 59.9% of sequences) during
355 late fermentations samples. Yeast like *Hanseniaspora* or *Saccharomyces* quickly replaced to
356 filamentous fungi or molds detected by this technique in the first stages of the fermentation.
357 Within these molds, *Rhizopus* was abundant (13.6% on average) in H and R, while *B.*
358 *cinerea*, (6.1% on average) predominated in must and beginning of the fermentation of B
359 grapes (ranging between 36.4-40.6%), *Aspergillus* (6.9% on average) was more abundant in
360 R must at the beginning of the fermentation (23 and 22.4%, respectively) than in the rest of
361 the samples, *Penicillium* was just detected in damaged samples (ranging between 3.1 and
362 5.2% and *Cladosporium* (1.1%) slightly more abundant on H and B musts and the beginning
363 of fermentation (ranging between 2.2 and 4.4%) than in the respective R samples (0.5-
364 0.8%). Other fungal genera detected in lower proportion than 1% but higher than 0.1% on
365 average across all samples are indicated on the heatmap (Supplementary Fig. 1). Some of
366 these low abundance genera were present just in samples from damaged grapes, like
367 *Saccharomycopsis*. On the other hand, *Fusarium* was detected just in H samples. All these
368 taxa, except the fermentative yeast *Saccharomycodes,* disappeared at the late fermentation,
369 indicating a low implication during wine fermentation.
370 In the case of bacteria detected by MS, the 6 most abundant genera were the AAB genera
371 *Gluconobacter, Gluconoacetobacter*, *Acetobacter*, *Tantiocharoenia*, and *Ameyamaea*
372 (accounting for 82.4 % on average across all samples) and the LAB genus *Oenococcus*.
373 The abundance of these bacterial genera varied among the samples with different health

states (Figure 2B). *Oenococcus* was predominant during late fermentation of H (90.9%) and also represented an important proportion of the sequences during the rest of H fermentation stages while it was scarcely detected in damaged grapes samples (Figure 2). R and B samples harbored higher proportion of *Gluconoacetobacter* than H samples and the genus *Gluconobacter* was clearly the most abundant from the must to mid fermentation of H grapes (52.7-88.6%). In addition, sequences related to *Tantiocharoenia* were more abundant in damaged samples than in H ones. Finally, R samples harbored higher proportions of the genera *Acetobacter* and *Ameyamaea* than H or B. Other bacterial genera detected in lower abundance than 1% but higher than 0.1% are listed on Supplemetary Fig. 1. Within these genera, some LAB like *Aerococcus*, *Lactococcus* or *Streptococcus* were also identified. All these genera disappeared during late fermentation of H grapes while some of them remained in damaged grapes (Supplementary Fig. 1). In addition, some of the genera detected just at late fermentation of R and B grapes increased their abundance with respect to the must and beginning of fermentation samples, for example, *Acinetobacter*, *Bacillus*, *Staphylococcus* and *Tatumella*.

3.3. Bacterial and fungal alpha diversity

The highest microbial diversity as determined by the number of different genera identified by the PCR-DGGE analysis was observed in the must and at the beginning of the fermentation of each health type grape, with higher diversity in the must samples and also more diversity in R and B samples than in H ones (Table 2). The diversity was lower through the end of H fermentation that for the damaged ones (Table 2).

According to MS analysis, fungal diversity ranged from 1 to 1.6 for the PD whole tree index and from 42 to 68 observed OTUs (Figure 3A, Supplementary Table 3). Higher diversities were reached for all samples in the musts and during the first stages of fermentation with similar values for damaged and H samples in those stages. However, diversity decreased sharply for H samples during the second half of the fermentation while, in the case R samples, diversity remained high and relatively constant along the fermentation and in the case of B samples, diversity decreased just a little during late fermentation stage (Figure

3A). The lowest fungal diversity belonged to late fermentation of H grapes. Simpson and Shannon indexes pointed to H samples during the mid-fermentation as the ones with the lowest diversity values (Supplementary Table 3)

This scenario was slightly different for bacterial diversity. The PD whole tree index for bacterial sequences ranged from 1 and 3.1 while observed OTUs ranged from 15 to 49 (Figure 3B, Supplementary Table 4). The bacterial taxonomic diversity increased through the end of fermentations for damaged samples while decreased sharply from the first 24 hours to late fermentation for H samples. The same tendency was observed for the number of OTUs with the exception of R samples harbored a relatively constant number of OTUs through the fermentation (Figure 3B). Other indexes like Simpson or Shannon also revealed that the lower bacterial diversity was observed for Healthy samples from mid to late fermentation while the values for the other samples remained quite constant (Supplementary Table 4).

3.4. Health status of the grapes influences must and fermentation communities

Fungal and bacterial communities changed across the different fermentation stages and between the different health statuses of the grapes used for the alcoholic fermentation (Figure 4). Unifrac distance matrices (Lozupone and Knight, 2005) were calculated with the taxonomic composition and abundance data from samples analyzed by MS in order to be used for the analysis of similarities (ANOSIM) of the microbial communities from the different samples. Fungal communities resulted significantly different and clustered by the different fermentation stages (Table 3, Figure 4A). According to statistical analysis, *Aspergillus*, *Rhizopus* and *Saccharomyces* were the fungal genera that varied significantly across all fermentation stages, thought other additional genera showed variation in their proportions across the fermentation stages and different health statuses (Figure 2). Bacterial populations from the different samples of H, R and B were significantly different and clustered by health status (Table 3 and Figure 4B). The bacterial genera that varied significantly in abundance between the H, R and B samples were *Acetobacter, Aeyamaea*, *Gluconoacetobacter*, *Gluconobacter*, *Oenococcus* and *Tanticharoenia* (Figure 2).

4. DISCUSSION

Grape health status is a primordial fact during winemaking and it can negatively impact on the fermentation process and the composition and quality of wine (Riberéau-Gayon et al., 2006). In the present work, we described the ecological changes along the fermentation of Macabeo grapes with different health status, H, R and B, using various techniques. Analyzing the influence of grape health state on fermentation kinetics, *Botrytis* infection had the strongest effect on the delay of the fermentation evolution since fermentations affected by this fungus were the slowest to consume 90% of the sugars. Nevertheless, undamaged grape fermentation presented the highest amount of sugar and ethanol in the last sampling point (density below 1000 g/L for two consecutive days). Previous studies have reported higher residual sugar when non-*Saccharomyces* yeasts were abundant during alcoholic fermentation (Ciani et al., 2006; Maygar and Tóth, 2011), which can occur in spontaneous fermentations (Andorrá et al., 2008; Llauradó et al., 2002).

In the present study, the techniques of plate culturing, qPCR, PCR-DGGE and MS have been used to monitor the changes of microbial community on grapes with three different health statuses. All these techniques allowed for the differentiation of the microbial communities in musts and fermentations of the three types of grapes, but differences in the results were observed depending on the technique.

Most of the studies on sour rot and *Botrytis*-affected grape ecology have been based on plate counts (Barata et al., 2008, 2012b; Mateo et al., 2014; Nisiotou et al., 2007, 2011). However, the inability of some microorganisms to grow in some media and/or under certain conditions (Amann et al., 1995) can give a biased result of the microbial diversity (Rantsiou et al., 2005). Considering these facts, we additionally used molecular methods since they have shown to be more informative about environmental microbial diversity.

One of the most used molecular techniques to quantify microbial populations is the qPCR. Nevertheless, the specific primer design limits the quantification to the targeted groups or

457 species. The PCR-DGGE using general primers is a good molecular technique to obtain a
458 fingerprint of the microbial community in a sample, but hardly detects populations with lower
459 density than 10^3 CFU/ml or two orders of magnitude lower than the most abundant members
460 (Andorrà et al., 2008; Muyzer and Smalla, 1998; Prakitchaiwattana et al., 2004). Recently,
461 high-throughput sequencing or MS techniques can be used to obtain a more detailed image
462 of the microbial communities of various ecosystems, including food processing (Ercolini,
463 2013). To our knowledge, this is the first study analyzing microbial populations in sour rot or
464 *Botrytis*-affected grape musts and fermentations by qPCR and MS. It is important to
465 consider that the used molecular methods detect both viable and non-viable cells. Thus, it is
466 possible that DNA of dead or non viable cells lead at some point to the overestimation of a
467 taxonomic group. However, an increase in the proportion of DNA probably will correspond to
468 an increase of the population.
469 In general, our plate counts agreed with similar studies analyzing sound and damaged
470 grapes with a higher fungal and bacterial population in the affected grapes (Barata et al.,
471 2008; Fleet, 2003; Wang et al., 2015).
472 Total yeast and AAB bacteria quantification by qPCR was higher than the counts detected
473 by plating probably due to the quantification of viable but non culturable and dead cells by
474 qPCR (Hierro et al., 2007; Torija et al., 2010). In addition, the primers used to quantify total
475 yeast have been described to also detect many filamentous fungi apart from yeast (Hierro et
476 al., 2006).
477 Fungal communities varied significantly across the different fermentation stages as shown in
478 this study by the used techniques. As in previous studies, the yeast population number and
479 diversity resulted higher in damaged grape musts than in H one (Barata et al., 2008, 2012a;
480 Barbe et al., 2001; Nisiotou et al., 2011). The higher yeast number might have been induced
481 by physically damaged grapes (Barata et al., 2008; Barbe et al., 2001) together with the
482 release of nutrients from the berry that encourage their growth (Fleet, 2003).
483 The high proportion of non-*Saccharomyces* in damaged musts, determined by plate counts,
484 qPCR and MS, could interfere with *Saccharomyces* imposition along the fermentation as a

485 consequence of interactions between both populations. Among others, these interactions
486 involve the competition for substrate, yeast-yeast cell contact or the release of antimicrobial
487 compounds (Ciani and Comitini, 2015; Wang et al., 2016). However, R must presented
488 higher difference between total yeast and non-*Saccharomyces*, indicating a higher initial
489 concentration of *Saccharomyces*, which could explain why the R microbial population was
490 the fastest to consume the 90% of the sugars.
491 Higher populations of *Hanseniaspora* and *Candida* (or *Starmerella*) observed by qPCR in
492 damaged grapes coincided with previous ecological studies on damaged grape berries
493 (Loureiro and Malfeito-Ferreira, 2003; Mills et al., 2002; Prakitchaiwattana et al., 2004).
494 Nevertheless, these species are also predominant worldwide in healthy grapes and during
495 the first stages of fermentation (Constantí et al., 1997; Jolly et al., 2014; Loureiro and
496 Malfeito-Ferreira, 2003; Torija et al., 2001,). In our study, independently on the grape status,
497 *H. uvarum* and *S. bacillaris* were detected in high proportions across the alcoholic
498 fermentations by qPCR and PCR-DGGE. A previous study using PCR-DGGE to monitor
499 yeast populations during sound and *Botrytis*-affected fermentations (Nisiotou et al., 2007)
500 found a similar behavior of *H. uvarum* to what we describe by PCR-DGGE but they did not
501 found *Saccharomyces*. In our study, *Saccharomyces* was not detected in any must sample
502 by PCR-DGGE but was detected from mid fermentation of damaged and H grapes
503 fermentation. MS together with qPCR allowed *Saccharomyces* detection and quantification,
504 respectively, in all samples from the must onwards.
505 MS also enabled us to identify abundantly *H. uvarum* sequences in all fermentations, but
506 above all, during the middle of H fermentations. Despite the low proportion of *S. bacillaris*
507 identified by MS, its quantification by qPCR was proportional to the values obtained for *H.*
508 *uvarum*. Other sequences related to *H. osmophila* were more abundant than *H. uvarum* in
509 damaged grapes but there were not detected or differentiated by PCR-DGGE or qPCR
510 techniques. A previous study on Dolce wine fermentation was able to differentiate *H.*
511 *osmophila* from *H. uvarum* by PCR-DGGE analysis (Mills et al., 2002), indicating that their

512 bands migrated differently. However, differentiation of different species of *Hanseniaspora* is
513 not possible by qPCR with the used primers.
514 Some key yeast species previously associated with damaged grapes as *Botrytis*,
515 *Kazachstania* and *Zygosaccharomyces* (Barata et al., 2012a; Nisiotou et al., 2007) were
516 observed by PCR-DGGE just in R and B samples. Barata et al. (2012b) proposed as
517 biomarkers for sour rot the presence of the yeast *Zygoascus hellenicus* and *Issatchenkia.*
518 However, in our case, *Zygoascus* was not detected by PCR-DGGE in any sample while
519 *Issatchenkia* was detected in both damaged and H samples by both PCR-DGGE and MS
520 techniques.
521 *B. cinerea* was detected by qPCR and PCR-DGGE in damaged samples, although its
522 quantification in R samples was low and constant. In contrast, MS analysis revealed a very
523 small proportion of *Botrytis* in H must and even lower proportion in R grapes. In B samples
524 though, *Botrytis* represented more than 30% of the sequences analyzed by MS in the must
525 and beginning of the fermentation. This proportion lowered close to the end of the
526 fermentation evidencing the sensibility of this fungus to the semi anaerobic conditions and
527 the increasing concentration of ethanol along the fermentation (Steel et al., 2013).
528 *Rhizopus* and *A. pullulans* were detected also by both DGGE and MS techniques. *Rhizopus*
529 was present in all must and beginning stages. This fungus has been described as a
530 saprophytic organism that can be a secondary bunch rot invader infecting grape berries
531 (Steel et al., 2013) and lead to organoleptic defects in grapes and wines when is associated
532 with *B. cinerea* (La Guerche et al., 2006). Furthermore, *A. pullulans* was identified in must
533 and initial fermentations of H and damaged grapes by MS analysis. This coincides with
534 previous studies where it was isolated from both sound and damaged grapes (Barata et al.,
535 2012a; Padilla et al., 2016; Prakitchaiwattana et al., 2004).
536 In general, MS analysis revealed a higher number of yeast genera than PCR-DGGE. The
537 higher proportion of some yeasts could inhibit or impede the detection of other less
538 abundant yeasts or microorganisms by PCR-DGGE (Prakitchaiwattana et al., 2004).
539 However, some of the genera that PCR-DGGE failed to detect were more abundant than 1%

on average as determined by MS (for example, *Aspergillus*, *Penicillium* and *Cladosporium*). On the other hand, PCR-DGGE analysis detected additional genera that were not abundant or even not detected by MS, like *Kazachstania*, *Issatchenkia* or *Candida*. These differences in the detection of genera by both techniques might be due to PCR amplification preferences as the primers used for DGGE and MS were not the same. Recent studies using MS technique to analyze the wine fermentation process of different grape varieties have detected the fungal genera *Hanseniaspora*, *Issatchenkia*, *Rhodotorula*, *Penicillium*, *Cladosporium*, *Botrytis*, *Sporobolomyces*, *Aspergillus*, *Cryptococcus* and *Pichia* (Bokulich et al., 2014; Pinto et al., 2015; Setati et al., 2012), all of them also detected in the present study and making our fungal community results solid (Table 2, Supplementary Figure 1).

LAB and AAB are the most relevant bacterial groups related to grapes and wine fermentation. In this study, GYC and MRS media were employed to count AAB and LAB populations, respectively. As in previous studies, our plate counts revealed an evident increase of AAB population in musts and beginning of the fermentations of R and B grapes (Barata et al., 2008, 2012a; Barbe et al., 2001; Nisiotou et al., 2007, 2011). As explained above, the reason could be the release of nutrients from the berry that encourages AAB and yeast growth (Fleet, 2003). The evaluation of AAB population by plate culture is usually complicated (Bartowsky and Henschke, 2008; Torija et al., 2010) mainly for its ability to enter in VBNC (viable but non-culturable state) (Millet and Lonvaud-Funel, 2000) or because they die under inappropriate conditions. Thus, the use of specific primers to quantify AAB by qPCR (González et al., 2006) allowed us to detect higher populations of AAB in all fermentations than the plate culturing, indicating the capacity of qPCR to detect VBNC and dead bacteria (Table 2). In order to identify the AAB genera, PCR-DGGE and MS techniques were applied. Unfortunately, PCR-DGGE allowed the identification of just *Gluconoacetobacter* and *Acetobacter* in the must and beginning of R fermentation. This might be due to the limitation of PCR-DGGE to detect populations two orders of magnitude lower than the most abundant members (Prakitchaiwattana et al., 2004), and, as noticed by qPCR results, yeast population was mostly two or even three orders above the bacterial

568 one. On the other hand, MS technique allowed the identification of up to 21 bacterial genera,
569 most of them related to AAB genera. Clear differences in bacterial composition were
570 detected between H and damaged grapes. *Gluconobacter* followed by *Gluconoacetobacter*
571 were the most abundant until the mid-fermentation of H grapes. In R grapes though,
572 *Gluconoacetobacter* represented the most abundant genus in all fermentation stages and it
573 was also more abundant in Botrytized samples than in H ones. Thus, the abundance ratio
574 between *Gluconobacter* and *Gluconoacetobacter* was higher in H samples than in R and B
575 ones. This fact is really aligned with previous observations where the health status of the
576 grapes indicated that *Gluconobacter* is more abaundant in healthy grapes, whereas
577 *Gluconoacetobacter* (or even *Acetobacter*) are more abundant in damaged grapes
578 (Guillamón and Mas, 2017).
579 In a wine fermentation study in a Grenache variety using MS (Portillo and Mas, 2016), we
580 showed that AAB and LAB were more abundant during fermentation than previously
581 thought, with a dominance of *Gluconobacter* during the mid-fermentation. The latter finding
582 contradicts the previous notion that *Gluconobacter*, being alcohol sensitive, usually declines
583 during the alcoholic fermentation (Du Toit and Lambrechts, 2002; González et al., 2005;
584 Joyeux et al., 1984). Similar results have also been reported in other studies using MS
585 analysis on low-sulfited or unsulfited wine fermentations (Bokulich et al., 2015). The same
586 authors found *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* as dominant bacteria
587 during winemaking processes (Bokulich et al., 2012).
588 Plate culturing also allowed the quantification of considerable LAB populations in H and R
589 musts, contrasting with previous studies where LAB populations were not detected or
590 detected in low concentrations (Barata et al., 2012a; Nisiotou et al., 2011). In fact, our MRS
591 counts overestimated LAB population respect to those of qPCR analysis using LAB specific
592 primers, probably due to non-LAB species that may grow in MRS media (Barata et al.,
593 2012a). However, both techniques detected a LAB population increase at the H late
594 fermentation.

595 MS analysis also supported qPCR results, with low percentages of LAB taxa in comparison
596 with those of AAB populations, except during the last sampled point of H fermentation,
597 making solid this tendency. This LAB population increase at late H fermentation suggests
598 that the spontaneous evolution of malolactic fermentation might not occur spontaneously in
599 damaged grapes fermentations. No LAB genus was identified by the PCR-DGGE technique
600 but MS analysis deciphered the LAB community composition in our samples and the main
601 player was *Oenococcus oeni*. Thus, to our knowledge, this is the first study relating the
602 presence of *Oenococcus oeni* to the grape health status.
603 The diversity of fungal and bacterial communities as determined by the quantification of the
604 identified genera by DGGE gels resulted in higher diversity in the musts and beginning of
605 every type of fermentations and, in general, higher diversity for damaged grapes. However,
606 according to MS results, similar diversity indexes were observed for the different types of
607 grapes in the musts and the health status of the grapes influenced on fungal and bacterial
608 diversities in a different way. For the fungal communities, H fermentation decreased the
609 diversity through the last fermentation point while R and B samples remain almost constant.
610 In the case of bacterial communities, the diversity declined sharply along H fermentation
611 while increased (PD whole index) or remained relatively constant (number of OTUs) for
612 damaged grapes fermentations. Both DGGE and MS analysis suggest that the microbial
613 diversity of must obtained from H grapes decreased along the alcoholic fermentation while
614 musts from damaged grapes maintain or increase their diversity. The higher diversity during
615 the mid and late damaged fermentations may result from the additional metabolisms present
616 in the infected grapes and musts, making possible the survival of non-conventional yeast for
617 longer time respect to H fermentations but also, compromising the success of the alcoholic
618 fermentation or including off-flavours to the final wine.
619

620 **5. CONCLUSIONS**

621 The present study is the first to include the molecular techniques qPCR and MS to evaluate
622 the population evolution along spontaneous fermentation of sour rot and *Botrytis* affected

623 grapes in comparison with healthy grapes. Both culture and molecular based analyses
624 showed differences in fungal and bacterial communities of Macabeo grapes depending on
625 its health status. However, MS analysis provided higher diversity at each stage than the
626 other compared techniques and detected *Saccharomyces* and *Oenococcus* even in the
627 initial must samples. The main differences in the fermentations revealed by MS were that *H.*
628 *osmophila* was predominant during mid-fermentation of damaged samples instead of *H.*
629 *uvarum*. Besides, *Oenococcus oeni* and *Gluconobacter* were more abundant in healthy
630 samples than in damaged ones, while the later had higher proportion of *Gluconoacetobacter*
631 with respect to the healthy samples. The microbial diversity of healthy fermentations
632 decreased from the middle to the end. Similarly to other studies that used MS to describe
633 the microbial population, in this work MS was the technique that contributed the most in the
634 deciphering of the community microbiome and for the first time, the health status of the
635 grape was related to the relative abundance of *Oenococcus oeni* during the alcoholic
636 fermentation.
637

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Figure Legends.

Figure 1. Fermentation density and population dynamics in YPD, Lysine Agar, MRS and GYC medium of (A) healthy, (B) rotten and (C) botrytized grapes fermentations.

Figure 2. Relative abundance of fungal (A) and bacterial (B) taxa detected at > 1% by MS. Taxa that differed significantly (*P* value <0.05) by fermentation stage (for fungi) or by health status (for bacteria) are indicated by an asterisk.

Figure 3. Alpha diversity graphs showing the PD whole tree index (left) and number of different OTUs (right) for the fungal (a) and bacterial (B) communities determined by MS.

Figure 4. Weighted Unifrac distance PCoA plots for fungal (A) and bacterial (B) communities from Macabeo must and fermentations.

Supplementary Figures

Figure S1: Heatmaps of the relative abundance of the fungal (A) and bacterial (B) taxonomy groups represented by less than 1% on average across all the samples. “US” represent the healthy samples, “UX” the rotten samples and “UP” the botrytized samples taken during grape must (t0), beginning (D1), middle (MF) or final (FF) fermentation stages.

Table 1: Fermentation kinetics of healthy, rotten and botrytized grapes. The values indicate initial sugar content, sugar (residual sugars), ethanol and acetic acid concentration of the last sampling point of healthy, rotten and botrytized grapes. *t50* and *t90* are the time used to consume the 50% and 90% of initial sugars, respectively.

Fermentation	**Initial sugar content (g/L)**	**t_{50} (days)**	**t_{90} (days)**	**Residual sugars (g/L)**	**Ethanol (% v/v)**	**Acetic acid (g/L)**
Healthy	205,26 ± 0,59	5	11	11,40 ± 1,36	11,30 ± 0,09	0,79 ± 0,07
Rotten	225,45 ± 4,12	4	7	2,97 ± 1,51	12,83 ± 0,65	0,52 ± 0,03
Botrytized	226,21 ± 1,12	5	16	5,21 ± 2,63	12,74 ± 0,54	0,79 ± 0,04

Table 2

Table 2: Abundance and detection of the fungal (upper) and bacterial (lower) groups determined by the different compared techniques. Results are the mean of three different biological replicates evaluated from the must (Must), the beginning of the fermentation (IF), the middle of the fermentation (MF) and the final sampling point (FP) taken when juice density was below 1000 g/L for two consecutive days. Nd for not detected.

	Healthy				Rotten				Botrytized			
	Must	**IF**	**MF**	**FF**	**Must**	**IF**	**MF**	**FF**	**Must**	**IF**	**MF**	**FF**
Plate culture (CFU/mL)												
YPD	1.3E+05	1.3E+05	5.7E+07	1.7E+07	4.4E+06	5.0E+06	9.0E+07	9.2E+06	1.4E+07	5.7E+06	3.0E+07	3.2E+06
Lysine Agar	2.0E+05	8.3E+04	3.3E+07	1.1E+07	2.6E+06	2.0E+06	2.1E+07	4.1E+06	1.3E+07	1.7E+06	3.3E+07	1.7E+06
MRS	4.1E+03	2.5E+03	5.5E+03	1.1E+04	1.0E+06	7.9E+05	1.6E+04	Nd	Nd	Nd	Nd	Nd
GYC-Ca	7.0E+03	2.5E+03	1.0E+03	4.8E+03	1.4E+05	4.2E+05	2.5E+03	Nd	1.8E+05	2.6E+05	1.3E+03	Nd
qPCR (cells/mL)												
Total yeast	1.3E+06	1.9E+07	4.8E+08	4.8E+08	2.6E+08	6.4E+07	4.8E+08	1.0E+08	3.7E+07	6.2E+07	2.2E+08	2.0E+07
Hanseniaspora spp.	4.0E+05	1.8E+05	1.5E+08	5.6E+07	2.1E+07	1.1E+07	1.3E+08	1.2E+07	6.6E+06	8.8E+06	3.3E+07	1.4E+06
Sacharomyces spp.	1.6E+04	8.4E+03	2.3E+06	3.5E+07	2.7E+04	1.3E+04	7.3E+05	6.0E+06	2.6E+03	4.6E+03	1.1E+05	2.2E+06
Starmerella bacillaris	8.4E+05	7.2E+05	2.2E+07	1.0E+08	1.8E+07	9.3E+06	8.7E+07	2.5E+07	5.7E+06	6.6E+06	4.5E+07	4.3E+06
Torulaspora delbrueckii	1.2E+03	7.3E+02	5.2E+04	1.3E+05	4.7E+03	2.6E+03	3.7E+04	9.9E+03	1.5E+03	2.8E+03	2.0E+04	7.1E+02
Metschnikovia spp.	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	5.1E+03	8.0E+03	3.2E+03	2.2E+02
Botrytis cinerea	Nd	Nd	Nd	Nd	7.5E+04	2.1E+04	4.9E+03	1.4E+04	1.6E+07	1.8E+07	5.3E+07	1.1E+06
Total LAB	3.9E+03	1.5E+03	7.7E+01	7.5E+05	1.1E+04	2.9E+03	3.5E+02	7.6E+01	6.6E+03	1.1E+04	9.6E+02	1.5E+02
Total AAB	4.7E+04	1.3E+04	2.0E+04	4.2E+03	1.5E+07	5.9E+06	1.5E+06	8.3E+04	1.5E+08	5.3E+07	8.2E+07	1.9E+07
PCR-DGGE (-/+/++)												
Kazachstania africana[a] (4)[b]	-	-	-	-	+	+	-	-	+	+	-	-
Rizhopus stolonifer (0)	++	+	-	-	++	++	+	+	+	+	+	-
Botrytis cinerea (2)	-	-	-	-	+	+	-	-	+	+	+	+
Zygosaccharomyces bisporus (5)	-	-	-	-	+	+	+	+	+	+	+	+
Aerobasidium pullulans (0)	-	-	-	-	-	-	-	-	+	+	-	-
Issatchenkia hanoiensis (2)	+	+	-	-	+	+	-	-	+	+	-	-
Candida californica (1)	+	+	-	-	+	+	-	-	+	+	-	-
Starmerella bacillaris (0)	+	+	+	++	+	+	++	++	+	+	++	++
Hanseniaspora uvarum (0)	+	+	++	++	+	-	++	+	+	+	+	+
Saccharomyces cerevisiae (0)	-	-	+	++	-	-	+	++	-	-	-	++
Acetobacter pastorianus (4)	-	-	-	-	+	+	-	-	-	-	-	-
Gluconoacetobacter xylinus (0)	-	-	-	-	+	+	-	-	-	-	-	-
NGS (>1% on average)												
Botrytis cinerea	0	0.3	0	0	0	0	0	0	40.6	36.4	2.3	0
Cladosporium	4.4	3.3	0	0	0.5	0.8	0	0	3	2.2	0.2	0
Hanseniaspora osmophila	3.8	3.8	1.2	2.5	2.3	5.1	47	25.7	6.4	7.9	53.1	3.1
Hanseniaspora uvarum	10	36.9	95.4	25.8	6.9	12.1	36.7	8.5	14.3	16.4	30.4	1.9
Aspergillus	14.9	3.9	0	0	23	22.4	1.6	0.8	10.3	9.8	1.4	0
Rhizopus	52.3	21.6	0	0	51.1	39.2	2.1	0.6	4.9	4.5	0.6	0.1
Saccharomyces cerevisiae	1.1	4.7	2.1	59.9	0.5	0.8	1.5	50.2	0.5	0.4	0.6	86.6
Starmerella spp.	1.5	14.2	1	6.2	1.6	2.1	5.4	4.7	0.7	0.8	3	0.5
Penicillium	0	0	0	0	3.3	3.1	0.2	0.1	5.2	4.4	0.5	0
Zygosaccharomyces	0.2	0.7	0.1	2	6	9.2	5	8.9	6.5	9.8	6.9	7.5
Acetobacter	1.5	2.9	0.3	3.4	3.6	4	4.2	4.7	1.5	1.2	2	2.6
Ameyamaea	0.4	0.3	0	0	4.5	4.8	5.4	5.1	1.3	1.6	1.8	0.5
Gluconacetobacter	9.5	11.9	2.2	0.2	45.1	44.7	48.7	42.3	34.4	36	28.9	14.6
Gluconobacter	67.2	52.7	88.6	5.3	42.9	41.8	34.6	41.4	52.3	49.8	44.5	52.3
Tanticharoenia	0.4	0.8	0	0	3.1	3.3	5.5	0.6	8.1	8.3	8.6	0.2
Oenococcus	15.1	19.2	4.1	90.9	0	0.1	0.2	2.3	0.1	0.1	9.6	17.8

[a] Closest relative according to BLAST analysis; [b] Sequence difference (number of substitutions/indels) from type strain.

Table 3: ANOSIM results showing the analysis of similarities of the different fungal and bacterial communities calculated from the weighted Unifrac distances matrices for the factors health status of the grape and the fermentation stage.

	ANOSIM Bacterial		**ANOSIM Fungal**	
Factor	**R**	***p***	**R**	***p***
Health	**0.355**	**0.001**	0.013	0.356
Ferm. Stage	0.005	0.455	**0.598**	**0.003**

Supplementary Table 1

Supplementary Table 1. Correlation coefficient (R^2), slope, intercept and efficiency of standard curves obtained for the different primer pairs with serial dilutions of the corresponding microorganism's DNA. Efficiency was calculated by the formula E = ((10-1/slope) - 1) x 100.

Target	R^2	Slope	Intercept	Efficiency (%)	Primers	Ribosomal gene region
Total yeast	0,9926	-3,4236	38,751	95,9252	200F/324R	26S rRNA
Saccharomyces spp.	0,9953	-3,4987	37,283	93,1169	CESPF/SCERR	ITS2 and 5.8S rRNA spanning region
Hanseniaspora spp.	0,9959	-3,5347	39,837	91,8269	CESPF/HUVR	ITS2 and 5.8S rRNA spanning region
S. bacillaris	0,9938	-3,7675	43,082	84,2587	AF/200R	D1/D2 (26S rRNA)
T. delbrueckii	0,9974	-3,506	39,282	92,8525	TODSL2/TODSR2	ITS (between 18S rRNA and 26S rRNA)
Metschnikovia spp.	0,9998	-3,5226	35,181	92,2566	MP5FW/MP3BW	26S rRNA
B. cinerea	0,9976	-3,4934	41,912	93,3099	BC3F/BC3R	ITS (between 18S rRNA and 28S rRNA)
LAB	0,9986	-3,6645	41,338	87,4513	WLAB1/WLAB2	16S rRNA
AAB	0,9992	-3,292	46,767	101,2643	AQ1F/AQ2R	16S rRNA

Supplementary Table 2

Supplementary table 2: Number of sequences obtained by Massive sequencing analysis **Before** and **After** quality filtering. The number of sequences used to build the **OTU table** is also indicated.

	Bacteria			Eukaryotes			Total		
Sample	Before	After	OTU table	Before	After	OTU table	Before	After	OTU table
Healthyt0	187058	101891	1057	149561	116171	101236	336619	218062	102293
HealthyD1	156795	87314	1630	122932	105324	91316	279727	192638	92946
HealthyMF	115106	87536	39101	155963	128718	113336	271069	216254	152437
HealthyFF	176803	140748	114457	204918	159046	137148	381721	299794	251605
Rottent0	247598	175776	32402	427632	311732	275651	675230	487508	308053
RottenD1	197261	143346	28006	348188	277696	249161	545449	421042	277167
RottenMF	118589	88681	19267	333245	267481	243875	451834	356162	263142
RottenFF	146690	107150	2994	275136	238860	222353	421826	346010	225347
Botrytizedt0	215983	139773	25024	209529	160971	136889	425512	300744	161913
BotrytizedD1	211867	139867	28455	176344	134603	113884	388211	274470	142339
BotrytizedMF	217797	141060	53010	237642	181350	156396	455439	322410	209406
BotrytizedFF	150132	109777	37587	157124	128101	112804	307256	237878	150391
Total	**2141678**	**1462919**	**382990**	**2798214**	**2210053**	**1954049**	**4939892**	**3672972**	**2337039**
% Filtering	**35,23%**			**23,69%**			**29,21%**		
Average/sample	**178473**	**121910**	**31916**	**233185**	**184171**	**162837**	**411658**	**306081**	**194753**

Supplementary table 3: Diversity indexes of fungal communities calculated from the different OTUs obtained in the MS analysis.

	PD_whole_tree	observed_otus	simpson	shannon
Healthyt0	1.5	65	0.83	3.49
HealthyD1	1.6	67	0.92	4.31
HealthyMF	1.3	51	0.76	2.70
HealthyFF	1.0	42	0.90	3.82
Rottent0	1.5	67	0.83	3.51
RottenD1	1.5	66	0.87	3.97
RottenMF	1.6	68	0.86	3.65
RottenFF	1.6	66	0.90	3.90
Botrytizedt0	1.5	64	0.89	4.20
BotrytizedD1	1.5	65	0.91	4.30
BotrytizedMF	1.6	68	0.83	3.41
BotrytizedFF	1.5	59	0.80	2.94

Supplementary table 4: Diversity indexes of bacterial communities calculated from the different OTUs obtained in the MS analysis.

	PD_whole_tree	observed_otus	simpson	shannon
Healthy t0	2.97	43	0.93	4.34
Healthy BF	3.04	43	0.93	4.38
Healthy MF	2.58	32	0.83	3.41
Healthy FF	1.37	18	0.63	1.95
Rotten t0	1.93	40	0.92	4.30
Rotten BF	2.01	39	0.92	4.31
Rotten MF	2.51	40	0.92	4.18
Rotten FF	2.86	40	0.92	4.30
Botrytized t0	2.85	42	0.92	4.15
Botrytized BF	2.42	41	0.93	4.24
Botrytized MF	3.05	47	0.93	4.35
Botrytized FF	3.09	45	0.92	4.26

Figure

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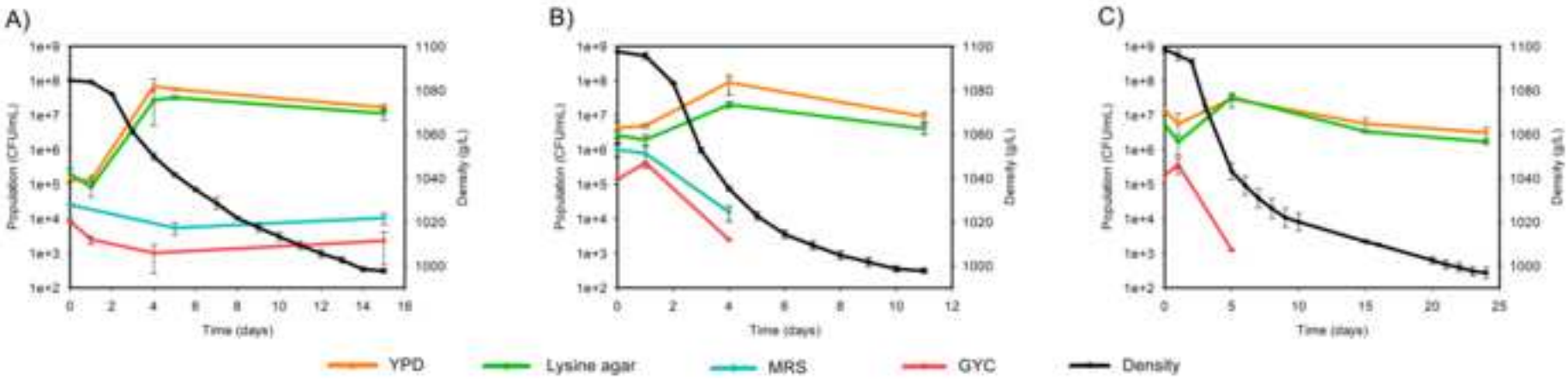

Figure 2

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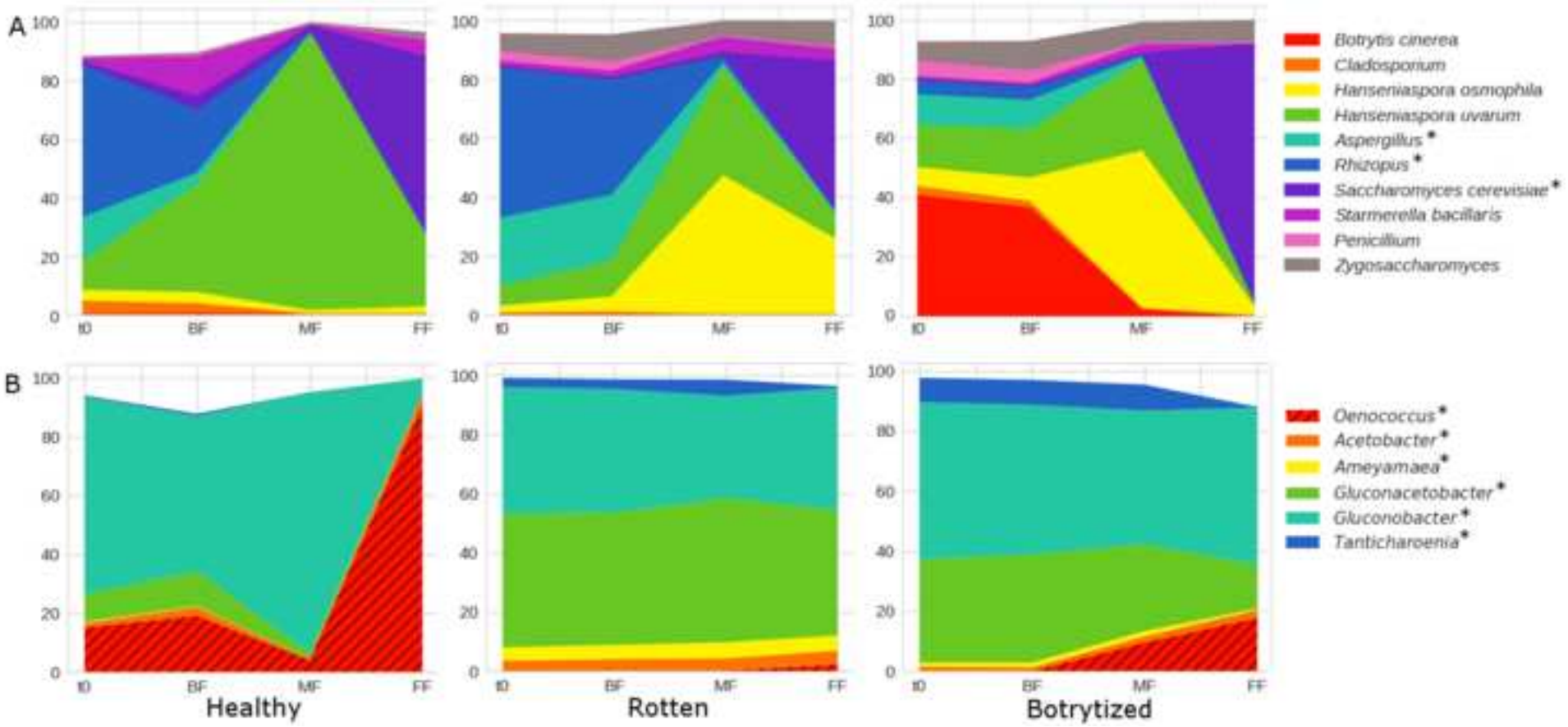

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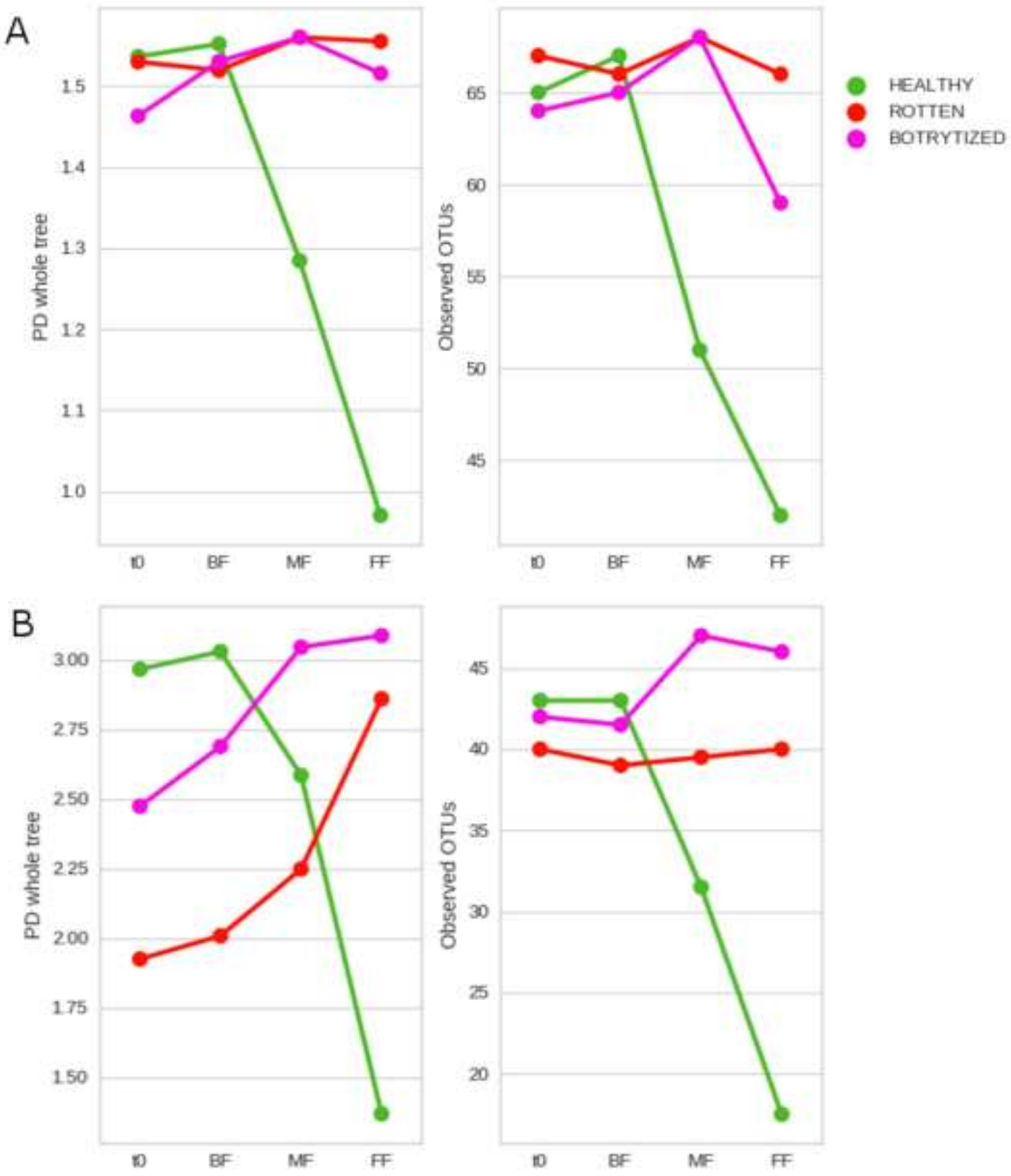

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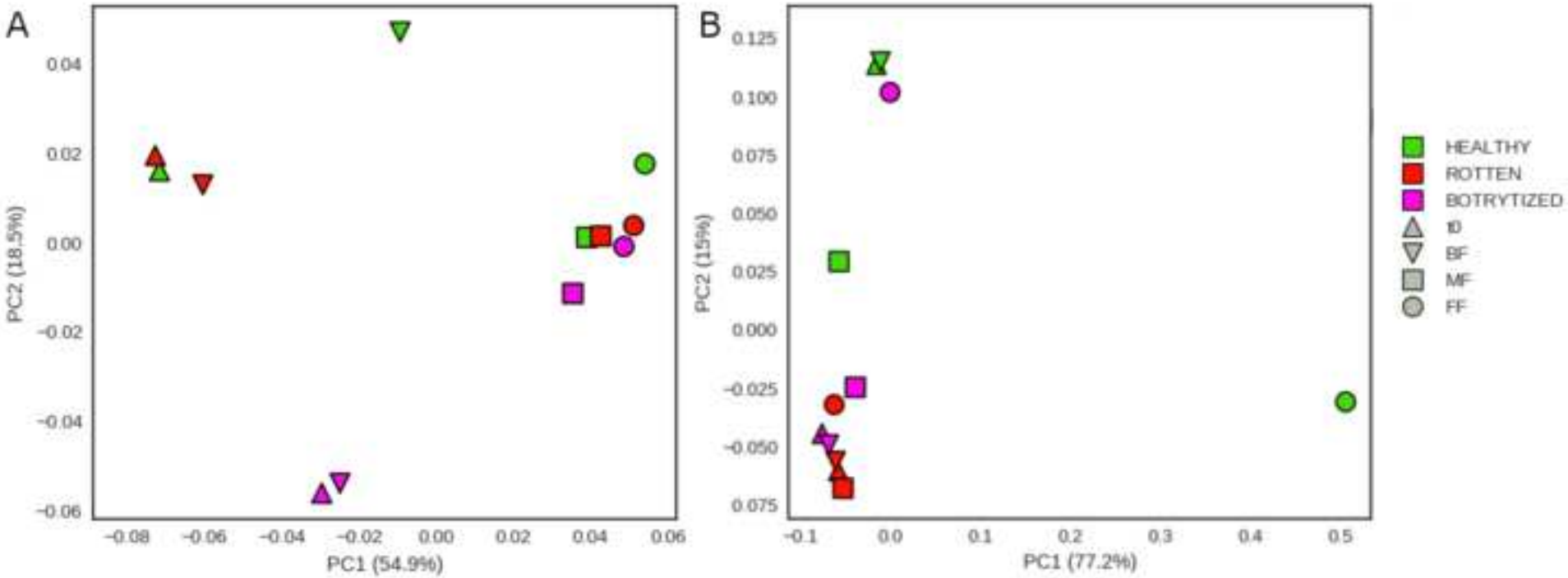

Supplementary Figure 1

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