Bacterial Diversity of Grenache and Carignan Grapes Surface from Different Vineyards at Priorat Wine Region (Catalonia, Spain)

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Abstract

Epiphytic bacteria on grape berries play a critical role in grape health and quality, which decisively influence the winemaking process. Despite their importance, the bacteria related with grape berries surface remains understudied and most previous work has been based on culture-dependent methods, which offer a limited view of the actual diversity. Herein, we used high-throughput sequencing to investigate the bacterial diversity on the surface from two grape varieties, Grenache and Carignan, and compared them across five vineyards included within Priorat region (Spain). We could detect up to 14 bacterial phyla with *Firmicutes* (37.6% *Bacillales* and 14% *Lactobacillales*), *Proteobacteria* (16.8% *Pseumonodales* and 11.6% *Enterobacteriales*) and *Actinobacteria* (3,4% *Actinomycetales*) being the most abundant. Bacterial community was different at each vineyard being grape varietal, geographical situation and orientation related with changes in bacterial populations. The most abundant bacterial taxa and those driving differences between the vineyards and grape varietals were identified. This study indicates that bacterial community heterogeneities can be influenced by geographic factors like orientation.

Keywords: next generation sequencing, bacterial diversity, grape surface, vineyard orientation

1. Introduction

Bacteria can readily colonize any surface including parts of the plant above the ground affecting the health of their hosts in diverse ways. The surface of grape berries represents a complex natural reservoir of bacterial microbiota originating from the surrounding environment (Zarraonaindia et al., 2015). Grapevine bacteria play a key role not only in plant health, but also in crop quality and yields which can influence the winemaking process (Nisiotou et al., 2011; Verginer et al., 2010). Many bacteria associated with grape surface cannot survive the extreme conditions of wine fermentation but their metabolic activity on the grape surface can have long-ranging consequences and they are undoubtedly included in the initial fermentation steps. Recently, Splivallo et al., (2014) demonstrated that bacteria associated with truffle-fruiting bodies contribute to truffle aroma. Thus, grape surface bacteria may play a significant role influencing the flavor, color, and quality of the final product but this aspect still remains to be studied.

Despite their importance, the diversity of epiphytic bacteria on grape berries remains poorly described. Most research has concentrated on bacteria of oenological interest, like acetic acid and lactic acid bacteria (LAB) present in the microbiota on grape berries (Bae et al., 2006; Nisiotou et al., 2011). Acetic acid bacteria are usually related with spoilage of wine and *Oenococcus oeni* and some other LAB species are known to perform the malolactic fermentation or to promote spoilage of wine depending on the species or strain. Nevertheless, the role of other bacteria in wine fermentation has been mostly ignored (reviewed in Barata et al., 2012). Furthermore, previous studies of grape-associated microbiota have been limited by methodological biases of culture-based techniques (Nisiotou and Nychas, 2007; Renouf et al., 2005, 2007) and low resolution of early molecular techniques (Martins et al., 2012). It is well reported that only a fraction of most environmental bacteria have been cultivated (Amann et al., 1995). Advances in massive parallel short-amplicon (100-600bp) sequencing technologies have revealed a bacterial diversity of grape berries much more elevated than previously thought and important ecological questions on the grapevine microbiome are being answered (Bokulich et al., 2014; Leveau and Tech, 2011; Perazzolli et al., 2014; Pinto et al., 2014; Taylor et al., 2014; Zarraonaindia et al., 2015). For example, from recent high-throughput studies we know that the bacterial community on leaves differed, both in size and structure, from that on berries (Leveau and Tech, 2011) and that soil serves as a key source of vine-associated bacteria with edaphic factors influencing the native grapevine microbiome, being the microbial community of soils from the same viticultural region quite heterogeneous (Zarraonaindia et al., 2015). Furthermore, grape-associated microbial biogeography is nonrandomly associated with regional, varietal, and climatic factors across multiscale viticultural zones (Bokulich et al., 2014), fungi communities from the same vineyard can be highly variable (Setati et al., 2012) and the most abundant yeast at the beginning and the middle of the fermentation of grapes cultivated under different treatments were detected (David et al., 2014). However, so far no high-throughput study has investigated bacterial diversity of grape varietal communities within the same grapevine growing region where climatic and regional factors are expected to be similar and produce wines of similar characteristics. Thus, it is ecologically relevant to know if bacterial communities of a single viticulture region are different and which factor influences the population changes.

Our goal was to test for heterogeneity of the bacterial community associated with different varietal at Priorat grapes and characterize the observed communities. For that reason, the present study characterizes the bacterial communities of Grenache and Carignan grape varieties across seven vineyards within the same viticultural zone, the Priorat region, by using 16S rRNA amplicon sequencing.

2. Material and methods

2.1. Sample collection. Grape samples were collected at 7 vineyards on Priorat region near Tarragona (Catalonia, Spain) located within 15 km^2 (Table 1, Supplementary Fig.1). These vineyards were denominated Ferrer Bobet (FB), Mas Martinet (MM), Jaume Sabaté (JS), Roca de les Dotze (RD), and Mas del Botó (MB) and produced mainly Carignan and/or Grenache cultivars with a unique rootstock of each varietal and managed under similar ecologic conditions. Priorat vineyards are characterized by high temperatures in summer (maximum temperature 35ºC), cold winters (minimum temperature -4ºC) and low levels of rainfall (400-600mm/year), which yielded wines with high alcohol content (13-16%). The vineyards are planted on the slopes on terraces at altitudes of between 100 m and 700 m above sea level with a marked contrast between the valleys and the higher areas and there are both freezing winds from the North-North West and also warm and humid one from the East-South East. Five replicate grape clusters from different plants from equidistant intervals were collected from each cultivar and vineyard in order to capture the heterogeneity present in each vineyard lot (Setati et al., 2012). Vineyards were sampled one, two or three times depending on their size. The date of sampling was at maturation of grapes at each vineyard, just before the harvest of 2013 and preserved at 4ºC on sterile plastic bags, resulting in 19 samples (Table 1; 10 from Grenache and 9 from Carignan). The grape clusters of each variety and vineyard were destemmed and crushed to obtain grape must upon arrival to the laboratory within the next hour of recollection. The grape must (including seeds and skin) of each sample was centrifuged at 4ºC and 4,000 x g during 10 minutes and the pellet immediately frozen at -80ºC until DNA extraction. In addition, four

50ml samples of grape must fermented at final malolactic fermentation were collected. These wine samples consisted of mixed Carignan and Grenache grapes must harvested and fermented at “Mas Martinet” cellar.

2.2. DNA extraction and sequencing. Genomic DNA was extracted from grape must and wine samples (19grape must and 4 wine) using the recommended procedure for the DNeasy Plant Mini kit (Qiagen, Hilden, Germany), including three bead-beating steps for 3 min in a FastPrep-24 bead beater (MP Bio, Solon, OH) to homogenize the samples. Extracted DNA concentration was measured by nanodrop, adjusted with molecular grade water to a concentration of 50ng/µl and stored at -20°C until further processing. The V4 region of the 16S rRNA gene was amplified in triplicate for each sample replicate using the primer pair 515F/799R with adapters for the sequencing by the equipment PMG from Ion Torrent with chips 318. The universal primer 515F (GTGCCAGCMGCCGCGGTAA) included a 10-bp barcode unique to each amplified sample. The reverse primer was a modification of the universal primer 799R (CVGGGTATCTAATCCBGTT, Chelius and Triplett, 2001). We used this primer pair because it has been found to be particularly suited for short read sequencing studies (Ghyselinck et al., 2013) and because the primer 799R had been previously used to amplify bacteria from plants avoiding the amplification of host chloroplast sequences (Chelius and Triplett, 2001). Primers sequences used in this study with their adapters and barcodes are listed in Supplementary Table 1.

PCR reactions contained 5-100 ng DNA template, 1× GoTaq Green Master Mix (Promega), 1 mM MgCl2, 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 60 s, and 72 °C for 90 s, and a final extension of 72 °C for 10 min. PCR products were pooled by sample and cleaned using a GeneRead Size Selection lit (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.

2.3. Data analysis. Raw sequences were demultiplexed and quality filtered using QIIME v1.8.0 (Caporaso et al., 2010a). Reads were discarded if the average quality score of the read was <25, if the length of the read was <200 or >400 and any read containing one or more ambiguous base calls. Operational taxonomic units (OTUs) were assigned using QIIME's uclust-based (Edgar, 2010) open-reference OTU-picking workflow, with a threshold of 97% pairwise identity. OTU sequences were aligned using PYNAST (Caporaso et al., 2010b) against a template alignment of the Greengenes core set filtered at 97% similarity. OTU taxonomy was determined using the RDP classifier retrained toward the GreenGenes bacterial 16S rRNA database (13_8 release) (DeSantis et al., 2006) at 97% similarity (Wang et al., 2007). Chimeric sequences were identified and removed using ChimeraSlayer (Haas et al., 2011) and a phylogenic tree was generated from the filtered alignment using FastTree (Price et al., 2009). A final OTU table was created, excluding unaligned sequences, singletons (sequences observed just once), and sequences matching plant mitochondria. To avoid biases generated by differences in sequencing depth, the OTU table was rarified to an even depth of 850 sequences per sample in comparisons of all sample types in this study. Samples represented by less than 850 sequences following all quality filtering steps were discarded.

2.4. Statistical analysis

Alpha diversity (within-sample species richness) estimates were calculated by analyzing the observed species, Shannon, Simpson and Chao1 indexes and Good´s coverage. Comparisons of alpha diversity between categories or sample clusters were run on QIIME.

Beta-diversity (between-sample microbial community dissimilarity) estimates were calculated within QIIME using weighted UniFrac distance (Lozupone and Knight, 2005) between samples. Ordination by non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) were used to summarize and visualize patterns in species composition. ANOSIM (an analogue of univariate ANOVA which tests for differences between groups of samples) were run in PRIMER v6 (Clarke and Gorley, 2006) to determine significant differences in phylogenetic or species diversity among experimental factors (vineyard origin, vineyard orientation and variety). The identification of the taxa explaining the similarity between samples type and the strongest variation of bacterial communities in each vineyard, geographical orientation and grape varietal was done with SIMPER (similarity percentage) run in PRIMER v6 (Clarke and Gorley, 2006). Comparative Mantel test on distance matrices were run in PRIMER v6 (Clarke and Gorley, 2006) with the tool RELATE to examine the correlations among geographic coordinates (latitude, longitude and altitude) and microbial community structures (based on UniFrac distances). Wine fermented samples were considered apart for statistical analysis as they were non representative from must samples.

3. Results and Discussion

3.1. Sequence analysis

The bacterial community of the most commonly grown grape varieties in Priorat, Grenache and Carignan, were analyzed independently at 5 different vineyards from the same region (Supplementary Fig. 1). Must samples consisting of destemmed, crushed grapes were analyzed with a short-amplicon sequencing approach to characterize bacterial community composition. After removal of low quality sequences, those failing alignment or annotated as host sequences, and singleton sequences, 405,668 16S rRNA V4 amplicon sequences were generated from 19 must and 4 fermented wine samples, with an average of 17,637 sequences per sample. These sequences had an average of 299bp (ranging from 200 to 400bp) and clustered into 6,556 operational taxonomic units (OTUs; 97% nucleotide identity).

3.2. Bacterial diversity of Grenache and Carignan grapes from Priorat

The ecological diversity of grape must bacterial communities was estimated using various diversity indexes (Table 2). The observed OTUs of Grenache and Carignan musts samples ranged from 94 to 172. Alpha diversity of Grenache and Carignan must samples was not significantly different between the selected vineyards, their grape variety or geographical location. Wine fermented samples harbored a significantly higher diversity of OTUs (195) than Grenache or Carignan must samples (R=0468, P=0.001). However, the elevated diversity of sequences from wine samples was found within the same genera, as most of the different OTUs of these samples could be assigned to just 21 genera while grape must samples ranged from 30 to 137 genera (Table 2). In fact, 99% of the wine fermented sequences were just related to the genus *Oenococcus*. This fact is justified as the wine samples were taken at the end of the malolactic fermentation where *Oenococcus oeni* has been described as the most abundant genera (Lonvaud-Funel, 1999) and previous studies have described an elevated microdiversity of autochthonous *Oenococcus oeni* in wines conducting malolactic fermentation (Garofalo et al., 2015). It is difficult to ascertain to what extent the observed microdiversity of *Oenococcus* represents ecologically differentiated populations.

Rarefaction plots for observed OTUs at 850 sequences deep were close to reach a plateau for most samples except wine fermented ones (Supplementary Fig.2). Good’s coverage for the samples, which provides an estimate of sampling completeness using a probability calculation with randomly selected sequences, was an average of 90% (±2.8) when calculated with 97% species level phylotypes (Table 2). These results suggest that the level of selected sequences (850 sequences per sample) would identify the majority of bacterial phylotypes present in the Priorat must samples and could be used to compare sample type.

3.3. Phylogenetic composition of the bacterial community of Grenache and Carignan grapes

Altogether, 14 bacterial phyla were detected in the Grenache and Carignan grape must samples, of which 2 phyla had no cultured representatives (Fig. 1A, Supplementary Table 2). The 6,556 different OTUs from this study were included in 19 predominant genera and more than 100 genera at abundance of less than 0.5% on average (Fig. 2) revealing an elevated bacterial diversity unprecedented from culture-dependent studies that barely detected a dozen genera within *Proteobacteria*, *Firmicutes* and *Actinobacteria* phyla (Barata et al., 2012; Martins et al., 2012). This is not surprising as it is well reported that only a fraction of most environmental bacteria have been cultivated (Amann et al., 1995), at least on standard culture media. Taxonomic composition of the bacterial communities of grape must varied greatly across the selected vineyards (Fig. 1A) but predominantly consisted of the orders *Bacillales* (37.6%), *Pseumonodales* (16.8%), *Lactobacillales* (14%), *Enterobacteriales* (11.6%) and *Actinomycetales* (3.4%). Previous culture based studies on grape bacteria mainly detected *Pseudomonadales* (31-51%) and *Micrococcales* (14-21%) (Martins et al., 2012). Our results are in agreement with the dominant taxonomic groups found by HTS techniques on Chardonnay, Cabernet and Zinfandel grapes at California (Bokulich et al., 2014) thought proportions differed from the results obtained for Merlot grapes at New York were *Proteobacteria*, mainly belonging to *Sphingomonadales* and *Pseudomonadales*, represented up to 80.7% of community at the grape samples

(Zarraonaindia et al., 2015). Organisms from the detected families are found in a wide range of environments, including soil and air, that have been previously been proposed as sources and reservoir for potential plant-associated bacteria (Bowers et al., 2011) included grapevine microbiota (Gilbert et al., 2014; Zarraonaindia et al., 2015). Bacillus, Enterobacter and Acinetobacter were abundant genera in the studied must samples (Fig. 2) and have been frequently isolated from grapes but were usually considered innocuous contaminants as they do not have the ability to grow during the wine fermentation process (Barata et al., 2012). Other genera like *Streptococcus* and *Erwinia* also represented an important fraction of the grape must in this study (Fig. 2) and have been occasionally detected by culture in vineyard environments (Barata et al., 2012). Recently, Perazzolli et al. (2014) detected *Pseudomonas*, *Erwinia* and *Acetobacter* in all grapevine plants they surveyed by pyrosequencing which mean these phyllosphere genera could be transferred or shared with grape berries easily. *Pseudomonas* and *Bacillus* sp., can act as biological disease suppression agents, stimulating plant growth and health (Bulgari et al., 2009; Compant et al., 2011; West et al., 2010).

Acetic bacteria, related to spoilage of the wine, represented a minimal proportion of the sequences of this study; and the LAB, related with malolactic fermentation of wine, are usually considered as minor partners of grape microbiota according to microbiological culture methods. Next generation techniques are recently changing that view with percentages of LAB ranging from 15 to 30% of total bacterial communities in grapes (This study; Bokulich et al., 2014; Pinto et al., 2014).

Within LAB, the genus *Oenococcus*, and specifically the species *O. oeni,* is the main agent of malolactic fermentation of wines conferring unique organoleptic properties (Bartowsky, 2005; Davis et al., 1988; Liu, 2002; Lonvaud-Funel, 1999). This genus has been seldom isolated from grapes in the vineyard (Garijo et al., 2009) and its DNA has been detected just once, in a sample of grapes from Bordeaux (Renouf et al., 2005, 2007). Anyway, to our knowledge, *Oenococcus* has never been detected through massive sequencing from grapes berries, must or grape vine thought other genera from the same family like *Leuconostoc* have been previously detected. This study found members of *Oenococcus* in most samples, accounting for 5.5% (on average) of the bacterial communities in grape musts from Priorat region. These results were contrasted by the isolation from the same grape samples on LAB specific medium (MRS medium) of 174 LAB isolates identified as *Oenoccocus oeni* (28%) *Lactobacillus* (66%) and *Pediococcus* (6%) (Franquès et al., unpublished data).

The bacterial community of wine samples at final malolactic fermentation from Mas Martinet cellar were mostly composed by the genus *Oenococcus* (99%) but genera like *Acinetobacter*, *Pseudomonas*, *Methylobacterium*, *Paracoccus* and others were detected at minor proportions (Fig. 2). It has been previously described that *Sphingomonas* and *Methylobacterium* can survive the wine fermentation process (Bokulich et al., 2012) but the role and impact of these and other detected genera on quality and organoleptic properties of wine remain unknown. The general percentage of *Oenococcus* in the grapes from MM vineyard used for the making of wine fermented samples of this study was low (0.1% of total bacterial community). However, the most abundant OTU related to *Oenococcus* in wine fermented samples (OTU ID 32722) was also present at MM grape must samples (specifically at Grenache grapes) and represented the most abundant OTU related to *Oenococcus* in grape must samples from the rest of vineyards. This result indicates that OTU 32722 is the best adapted for the conditions of wine fermented at MM cellar and was highly selected during malolactic fermentation.

3.4. Vineyard origin, grape varietal and geographical situation defines grape must bacterial communities

Based on results from previous studies describing differences between different varietals and viticultural regions (Bokulich et al., 2014), we hypothesized that bacterial communities would cluster according to the analyzed grape varieties (Grenache and Carignan) and that vineyards sampled at shorter spatial distance should have a bacterial community more similar in composition than those vineyards sampled at higher distances.

Community structure varied widely across different selected vineyards within Priorat region (Table 3, Fig. 3), exerting the origin of the samples a significant impact on bacterial genetic diversity (weighted UniFrac $R_{ANOSIM} = 0.367$ $P < 0.001$). However, other factors as geographical orientation and grape variety also influenced deeply on the bacterial composition of Grenache and Carignan grapes (Table 3, Fig. 3). Previous studies have shown intra-vineyard heterogeneity to be high in aboveground tissues (Setati et al., 2012, Zarraonaindia 2015). In our study, bacterial communities on grapes surface from the same vineyard were, on average, more similar than communities from different vineyards as replicates of samples belonging to the same grape variety and same vineyard cluster closer at the NMDS (non-metric multidimensional scaling) than samples from different vineyards, which means that intra-vineyard community differences were smaller than the inter-vineyard ones (Fig. 3A). We consider that the sampling procedure was adequate to capture heterogeneity of each vineyard as various plants evenly distributed were sampled along each plot and some plots were sampled two or three times, depending on the size of the plot.

Bokulich et al. (2014) found that similarities in microbial communities from different Californian regions separated by more than 500 km followed the coastline indicating an environmental trend. The vineyards from the present study followed a similar, ecologic management of the grapevines and they were relatively closely located (15 km maximum distance) so that changes in bacterial populations due to differences on climatological parameters could be ruled out. However, it is possible that the irregular topography generates differences in altitude and geographical orientation of the vineyards could also influence locally in environmental parameters such as insolation-shading or humidity. Some of the sampled vineyards had weak or no orientation because they were in a fairly flat area but some vineyards were sampled on a hillside facing either East or South. These three categories (East, South and Flat) were used to group samples and we found that musts bacterial community similarities were significantly influenced by vineyard geographical orientation ($R_{ANOSIM} = 0.84$, $P = 0.001$) suggesting that environmental differences between hillsides probably underlie the observed community changes (Table 3).

Bacterial communities from Grenache and Carignan grapes across selected vineyards from Priorat region resulted globally different ($R_{ANOSIM}= 0.191$; $P=0.005$) (Fig. 3A). This result has been previously observed for other grapes varieties (Bokulich et al., 2014; Zarraonaindia et al., 2015). Also, must samples within each grape variety harbored significantly different communities at each vineyard and clustered by vineyard orientation (Table 3, Fig 3). We found that Carignan must bacterial community was significantly related with geographical coordinates (latitude, longitude, altitude) ($R_{RELATE}= 0.682$, $P= 0.001$). Thus, bacterial communities associated to Carignan grapes from closer vineyards were more similar than those from distant vineyards. However, we could not observe a clear relationship between Grenache bacterial communities and location (R_{RELATE}= 0.037, P= 0.32) indicating that others factors (i.e. vineyard origin or orientation) showed more influence on bacterial community composition than the geographical situation.

Bacterial community of wine fermented samples was very stable within replicates and resulted clearly different from Grenache or Carignan grape must (Supplementary Fig. 3) thought on the PCoA representation wine samples migrated closer to MB and MM vineyard (the grapes used for the wine making come from MM vineyard).

3.5. Taxonomic groups driving differences between bacterial communities of grape from Priorat vineyards

SIMPER analysis revealed which taxa contributed the most to dissimilarity of samples and similarity between samples according to their orientation or grape varietal (Table 4). According to geographical orientation, the differential relative abundances of *Bacillus*, *Erwinia, Acinetobacter* and *Oenococcus* contributed the most to dissimilarities between grapes from East-, South-oriented and Flat (no orientation considered) vineyards (Table 4A). *Oenococcus, Acinetobacter* and *Streptococcus* were the genera contributing the most to similarities within East-oriented vineyard musts bacterial communities; *Bacillus* in South-oriented vineyards; *Erwinia* and *Acinetobacter* in Flat or not-oriented vineyards (Table 4B). According to grape varietal, *Bacillus*, *Acinetobacter* and *Erwinia* accounted for the higher contribution to dissimilarities between Grenache and Carignan grape must bacterial communities, being also the proportion of these genera together with *Oenococcus* and *Streptococcus* the mayor responsible of the similarities within Grenache or Carignan samples (Table 4C, D).

These results show that the vineyard origin, its orientation and grape varietal determine the presence and proportion of these bacterial genera and could influence ultimately the composition of fermentative populations, which could determine regional wine characteristics. It may be interesting to relate the observed changes in bacterial communities with differences in wine physicochemical properties and the potential use of this knowledge to enhance organoleptic and chemosensory perception of wines.

3.6. Core bacterial community of Grenache and Carignan grapes at Priorat

We found just one OTU shared by all must samples which was related to *Bacillus* (Table 5, Fig. 4) and various OTUs were shared within pairs of vineyards (Fig. 4). Besides, we examined the bacterial community grouped according to each orientation category and resulted in several OTUs shared by samples clustered as East-oriented and Flat or not oriented vineyards, while 1 OTU related to *Bacillus* was present at 100% of South-oriented samples (Table 5). Five and one OTUs were shared by all Carignan and Grenache must samples, respectively. Most of the genera comprising the core OTUs of the samples were related to plant and soil environments with the exception of *Oenococcus* that was related to wine fermented and could be found at every East and South oriented samples (table 5). This *Oenococcus* related OTU (ID 32722) was the most abundant both in grape must samples and wine fermented samples.

These results confirm the nonrandom distribution of bacterial taxa present in different grape musts across selected vineyards and point to the possible consequences of the metabolic activity of these specific taxonomic groups over wine quality and its characterization, even within vineyards from the same growing region, which had not been previously described. Whether these populations' patterns actively produce discriminative chemosensory characteristics of wines within Priorat vineyards must be experimentally demonstrated.

4. Conclusions

The most abundant bacterial taxa of Grenache and Carignan grapes were investigated for the first time by NGS. Regional patterns in grape berries bacterial communities suggest that local environmental conditions and grape varietal are responsible for driving bacterial diversity within a single viticultural zone. We were able to relate bacterial communities with vineyard geographical orientation, vineyard origin and grape variety within the same growing region. This study showed for the first time nonrandom distribution of grape bacteria across differently oriented vineyards, specifically within the Priorat region, which allows to propose that these characteristic bacterial communities could be used to drive specific wine properties and naturally enforce distinctive terroir characteristics in local wine blends

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Captions

Figure 1: Average relative abundance of dominant bacterial phyla at each sample type. “Other Phyla” include Fusobacteria, Acidobacteria, Deinococcus-Thermus, Chloroflexi, Verrucomicrobia, Planctomycetes, Armatimonadetes, FBP, SR1 and Spirochaetes.

Figure 2: Heatmap displaying relative abundances of the most abundant genera detected in grape musts from Priorat averaged by sample type. “Other genera” is represented by phylogenetic groups detected by less than 0.5% on average of all sample types. The color scale is from intense red for the most abundant taxonomic groups to intense blue for less abundant.

“**Other genera**” include members of the genera *Deinococcus, Micrococcus, Rothia, Mycobacterium, Rhodococcus, Friedmanniella, Nocardioides, Propionibacterium, Actinomycetospora, Pseudonocardia, Saccharomonospora, Bifidobacterium, Atopobium, Collinsella, Rubrobacter, Fimbriimonas, Flavisolibacter, Sediminibacterium, Prevotella, Bacteroides, Dysgonomonas, Porphyromonas, Adhaeribacter, Hymenobacter, Pontibacter, Spirosoma, Chryseobacterium, Cloacibacterium, Wautersiella, Capnocytophaga, Flavobacterium, Pedobacter, Alicyclobacillus, Geobacillus, Aneurinibacillus, Paenibacillus, Lysinibacillus, Sporosarcina, Gemella, Granulicatella, Enterococcus, Pediococcus, Leuconostoc, Lactococcus, Streptococcus, Turicibacter, Jan-68, Anaerococcus, Finegoldia, Peptoniphilus, WAL, Clostridium, Ruminococcus, Blautia, Moryella, Oribacterium;* and other unidentified genera belonging to orders Ellin 6075, iii1-15, *Actinomycetales, Solirubrobacterales, Sphingobacteriales, JG30-KF-CM45, BSA2B-08, Clostridiales, Rhizobiales and Rhodospirillales; and to families Geodermatophilaceae, Intrasporangiaceae, Kineosporiaceae, Microbacteriaceae, Moraxellaceae, Halomonadaceae,Methylocystaceae, Micromonosporaceae, Gaiellaceae, Nocardioidaceae, Chitinophagaceae, Porphyromonadaceae, Cytophagaceae, Sphingobacteriaceae, Planococcaceae, Thermoactinomycetaceae, Aerococcaceae, Leuconostocaceae, Clostridiaceae, Lachnospiraceae, Peptostreptococcaceae, Ruminococcaceae, Caulobacteraceae, Bradyrhizobiaceae, Methylobacteriaceae, Idiomarinaceae, Rhizobiaceae, Hyphomonadaceae, Rhodobacteraceae, Acetobacteraceae, Sphingomonadaceae, Alcaligenaceae, Comamonadaceae, Oxalobacteraceae, Rhodocyclaceae, Aeromonadaceae*.

Figure 3: Non-metric multidimensional scaling (NMDS) plots of the weighted pairwise UniFrac distances between Grenache and Carignan musts samples from five Priorat vineyards, FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó. (A) Clustering of bacterial communities by grape varietal with replicates of samples circled by a black loop (R_{ANOSIM}= 0.191; P=0.005); (B) Clustering of Grenache and Carignan musts samples by geographical orientation (R_{ANOSIM}= 0.84; P=0.001).

Figure 4: Venn diagram showing the OTUs shared by the five vineyards. Overlapping colored areas indicated the number of shared OTUs between pairs of vineyards.

Supplementary figures

S1: Map of the sampled vineyards at the Priorat. FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó.

S2: Rarefaction plot for observed OTUs calculated at 850 sequences per sample.

S3: Weighted UniFrac distance PCoA of bacterial communities at Priorat Vineyards including Grenache and Carignan must samples and wine fermentation samples. FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó.

Table 1

Table 1 Description of the collected samples and their location.

Sample ID	Vineyard	Variety	Nº Samples	Coordinates	Elevation (m)	Orientation[1]
FB-Gx	Ferrer Bobet	Grenache	2	N41.1767, E0.8607	464.8	E
FB-Cy	Ferrer Bobet	Carignan	2	N41.1797, E0.8604	439.8	F
MM-Gx	Mas Martinet	Grenache	2	N41.1781, E0.7937	199.8	S
MM-Cy	Mas Martinet	Carignan	3	N41.177403, E0.794808	205.1	S
JS-Gx	Jaume Sabaté	Grenache	2	N41.218875, E0.749972	233.5	E
JS-Cy	Jaume Sabaté	Carignan	1	N41.2662, E0.8784	229.6	F
RD-Gx	Roca de les Dotze	Grenache	1	N41.265672, E0.879789	625.7	E
RD-Cy	Roca de les Dotze	Carignan	2	N41.218564, E0.749825	636.1	F
MB-Gx	Mas del Botó	Grenache	3	N41.196468, F0.919531	553.4	S
MB-Cy	Mas del Botó	Carignan	1	N41.196944, E0.920636	570.6	E
Wine	Ferrer Bobet	Mix	4	-	-	-

[1] E: East, S: South, F: Flat

Table 2

Table 2 Alpha diversity obtained by the average of the different samples replicates using 850 sequences by sample.

	Chao1	Simpson	Shannon	Observed OTUs	Assigned genera	Good´s coverage
FB-Gx[1]	328.5	0.93	5.40	172	119	0.88
FB-Cy	232.1	0.38	2.08	98	41	0.92
MM-Gx	303.4	0.59	3.13	119	58	0.90
MM-Cy	374.5	0.40	2.19	112	117	0.90
JS-Gx	367.7	0.94	5.35	142	137	0.90
Js-Cy	272.8	0.36	1.96	94	32	0.92
MB-Gx	215.5	0.44	2.28	96	67	0.92
MB-Cy	341.0	0.71	3.52	132	30	0.89
RD-Gx	320.6	0.97	6.02	166	61	0.90
RD-Cy	324.3	0.80	4.11	128	72	0.90
Wine	703.4	0.62	3.70	195	21	0.82

[1] Gx: Grenache; Cy: Carignan; FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabate; MB: Mas Boto; RD: Roca de les Dotze

Table 3: ANOSIM of category effects on microbial diversity pattern based on UniFrac distance matrix excluding fermentation wine samples and calculated in PRIMER v6.

Group	Factor	R	*P*
All	Vineyard	0.367	0.001
Grenache	Vineyard	0.677	0.006
Carignan	Vineyard	1	0.002
All	Variety	0.191	0.005
All	Orientation	0.84	0.001
Grenache	Orientation	0.8	0.01
Carignan	Orientation	0.897	0.01

Table 4

Table 4: SIMPER analysis results for those OTUs contributing more than 5% to the similarity between samples (A and C) or similarity within samples (B and D) according to vineyard orientation (A and B) or grape varietal (C and D).

A.

	Dissimilarity %	Genus	Contribution %
East & Flat	89.6	*Erwinia*	24.2
		Acinetobacter	18.8
		Bacillus	6
		Oenococcus	5.7
East & South	85.6	*Bacillus*	45.6
		Acinetobacter	7.8
		Oenococcus	5.3
South & Flat	93.34	*Bacillus*	45.6
		Erwinia	22

B.

	Similarity %	Genus	Contribution %
East	35	*Acinetobacter*	30
		Oenococcus	11
		Streptococcus	9.5
South	21.3	*Bacillus*	88
Flat	12.3	*Erwinia*	65.1
		Acinetobacter	5.1

C.

	Dissimilarity %	Genus	Contribution %
Grenache & Carignan	89.5	*Bacillus*	41.2
		Acinetobacter	12
		Erwinia	12

D.

	Similarity %	Genus	Contribution %
Grenache	21.54	*Bacillus*	27
		Acinetobacter	26
		Oenococcus	6.6
		Streptococcus	6.3

Carignan	11.16	*Bacillus*	46.4
		Erwinia	24.4
		Acinetobacter	17.7

Table 5

Table 5 List of OTUs shared by the samples within each factor or group category.

Factor	Group	Shared Percentage[1]	OTU ID	Taxonomic Affiliation
Vineyard	All	100%	40910	*Bacillus* (Bacillales)
Orientation	East	100%	1114	*Oxalobacteraceae* (Burkholderiales)
			1903, 37262	*Acinetobacter* (Pseudomonadales)
			11667	*Haemophilus* (Pasteurellales)
			12900	*Methylobacterium* (Rhizobiales)
			18038	*Sphingomonas* (Sphingomonadales)
			28121	*Erwinia* (Enterobacteriales)
			32722	*Oenococcus* (Lactobacillales)
			40910	*Bacillus* (Bacillales)
			43218	*Pseudomonas* (Pseudomonadales)
	South	100%	40910	*Bacillus* (Bacillales)
	Flat	100%	830, 40910	*Bacillus* (Bacillales)
			1903, 37262	*Acinetobacter* (Pseudomonadales)
			4511	*Aeromonadaceae* (Aeromonadales)
			12900	*Methylobacterium* (Rhizobiales)
			11173, 18425	Micrococcaceae (Actinomyctales)
			16121	*Streptococcus* (Lactobacillales)
			32722	*Oenococcus* (Lactobacillales)
			28121	*Erwinia* (Enterobacteriales)
			26940	*Staphylococcus* (Bacillales)
			6565	*Enhydrobacter* (Pseudomonadales)
Varietal	Grenache	100%	1114	*Oxalobacteraceae* (Burkholderiales)
			1903, 37262	*Acinetobacter* (Pseudomonadales)
			12900	*Methylobacterium* (Rhizobiales)
			40910	*Bacillus (Bacillales)*
	Carignan	100%	40910	*Bacillus* (Bacillales)

[1] Percentage of samples within each group sharing a specific OTU

Table Sup 1

Supplementary Table 1

REVERSE PRIMER

ADAPTER	*KEY*	*BARCODE*	*LINKER*	*TARGET 16S PRIMER*
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CTAAGGTAAC	GAT	GTGCCAGCMGCCGCGGTAA
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CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TACCAAGATC	GAT	GTGCCAGCMGCCGCGGTAA
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CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	CTGCAAGTTC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTCGTGATTC	GAT	GTGCCAGCMGCCGCGGTAA
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CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCGCAATTAC	GAT	GTGCCAGCMGCCGCGGTAA
CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTCGAGACGC	GAT	GTGCCAGCMGCCGCGGTAA

FORWARD PRIMER

ADAPTER	*LINKER*	*TARGET 16S PRIMER*
CCTCTCTATGGGCAGTCGGTGAT	CC	CVGGGTATCTAATCCBGTT

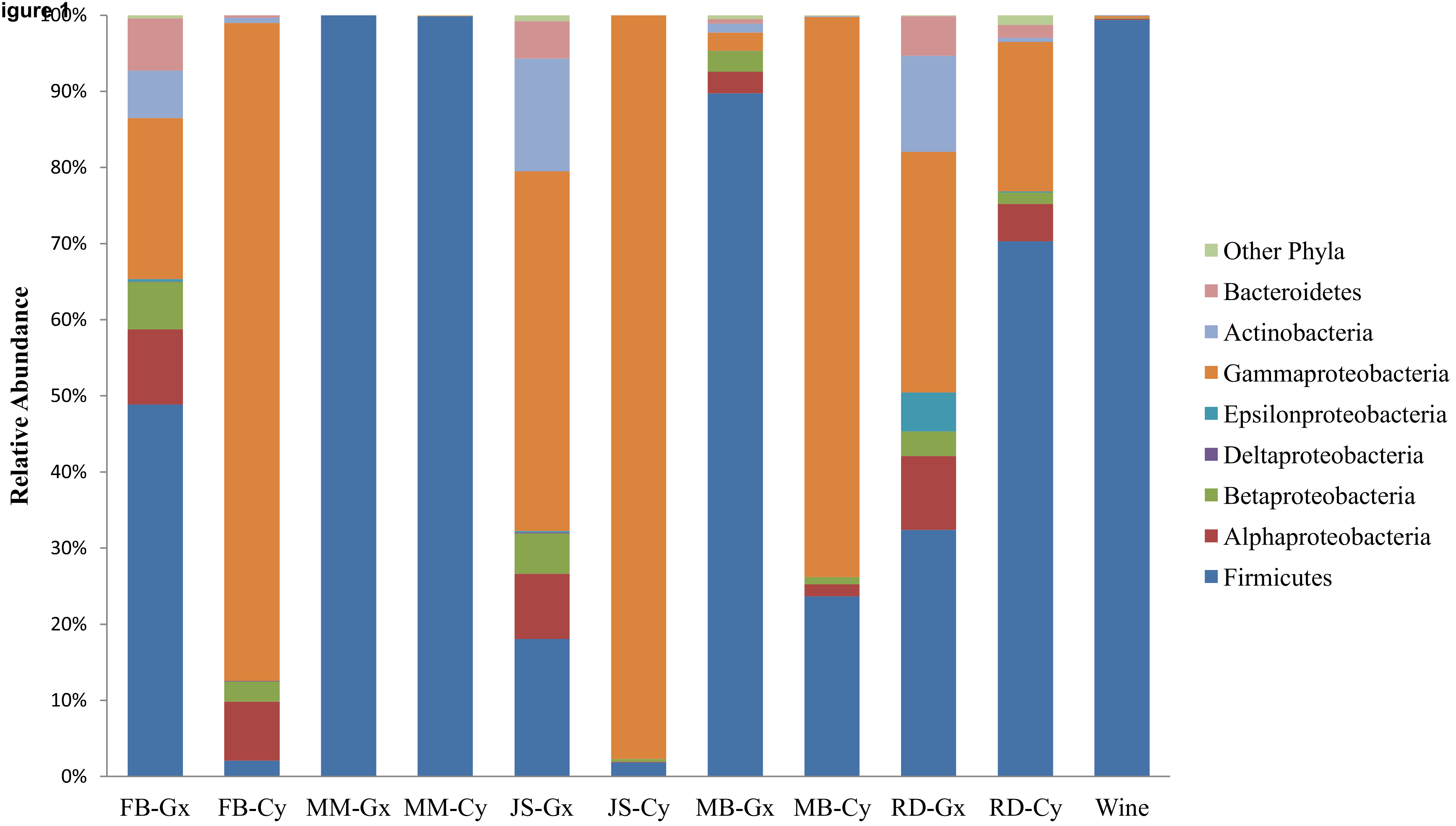
Table Sup 2

Table S2: Relative abundance of main phyla (subphyla for Proteobacteria) at Priorat vineyards. "Other Phyla" include: Fusobacteria, Acidobacteria, Deinococcus-Thermus, Chloroflexi, Verrucomicrobia, Planctomycetes, Armatimonadetes, Spirochaetes and the candidate phyla FBP and SR1.

	FB-Gx	FB-Cy	MM-Gx	MM-Cy	JS-Gx	JS-Cy	RD-Gx	RD-Cy	MB-Gx	MB-Cy	Wine
Firmicutes	48.8	2.1	100.0	99.9	18.1	1.8	89.7	23.6	32.4	70.3	99.4
Proteobacteria	9.9	7.8	0.0	0.0	8.5	0.1	2.9	1.6	9.7	4.9	0.2
Alphaproteobacteria	6.2	2.7	0.0	0.0	5.3	0.4	2.7	1.0	3.3	1.5	0.1
Betaproteobacteria	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Deltaproteobacteria	0.3	0.0	0.0	0.0	0.2	0.0	0.0	0.0	5.1	0.1	0.0
Epsilonproteobacteria	21.2	86.4	0.0	0.1	47.3	97.7	2.4	73.6	31.6	19.6	0.3
Gammaproteobacteria	6.2	0.7	0.0	0.0	14.8	0.0	1.2	0.1	12.6	0.5	0.1
Actinobacteria	6.8	0.3	0.0	0.0	4.9	0.0	0.6	0.0	5.2	1.7	0.0
Bacteroidetes	0.4	0.0	0.0	0.0	0.8	0.0	0.5	0.1	0.1	1.3	0.0
Other Phyla	48.8	2.1	100.0	99.9	18.1	1.8	89.7	23.6	32.4	70.3	99.4

Gx: Grenache; Cy: Carignan; FB: Ferrer Bobet; MM: Mas Martinet; JS: Jaume Sabaté; RD: Roca las Dotze; MB: Mas Botó

Figure 1


Relative Abundance
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70%
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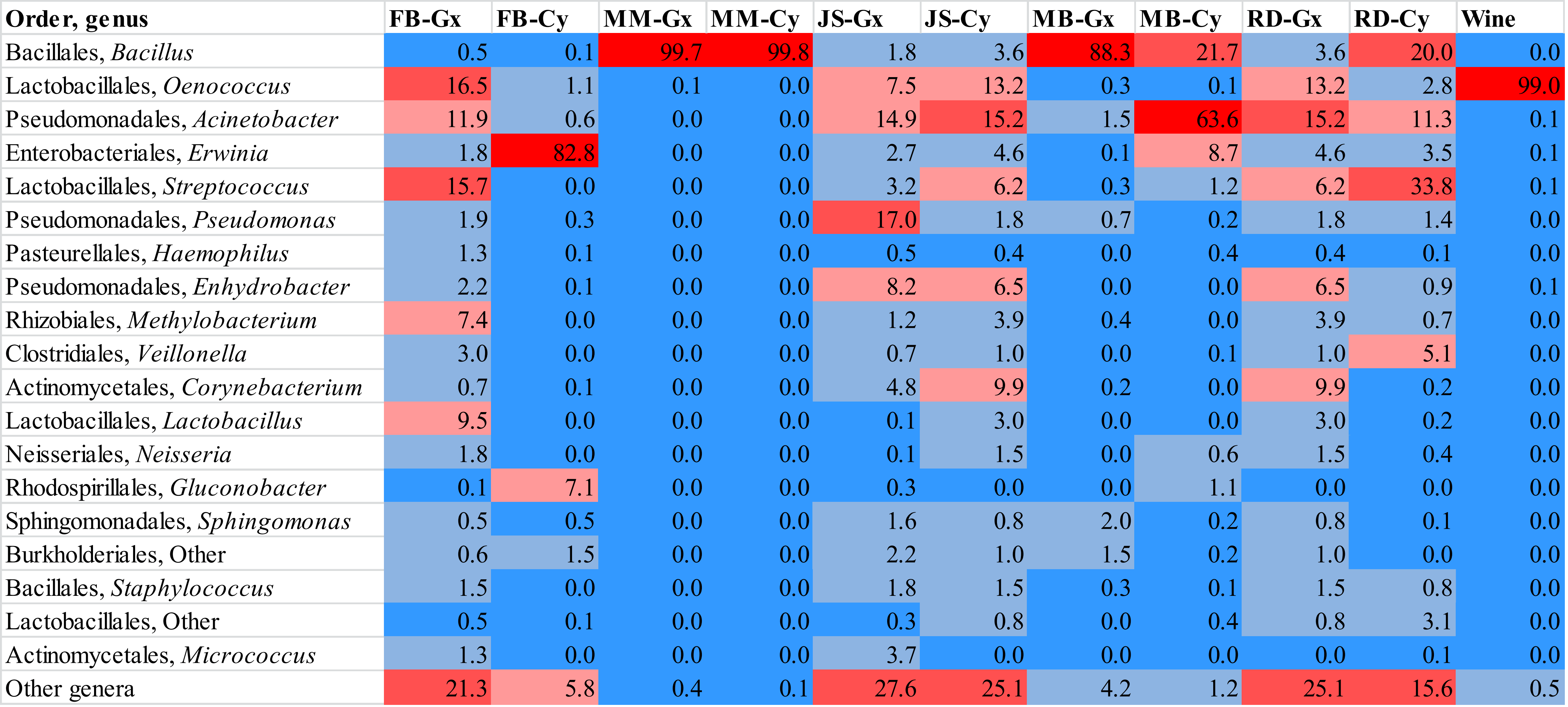
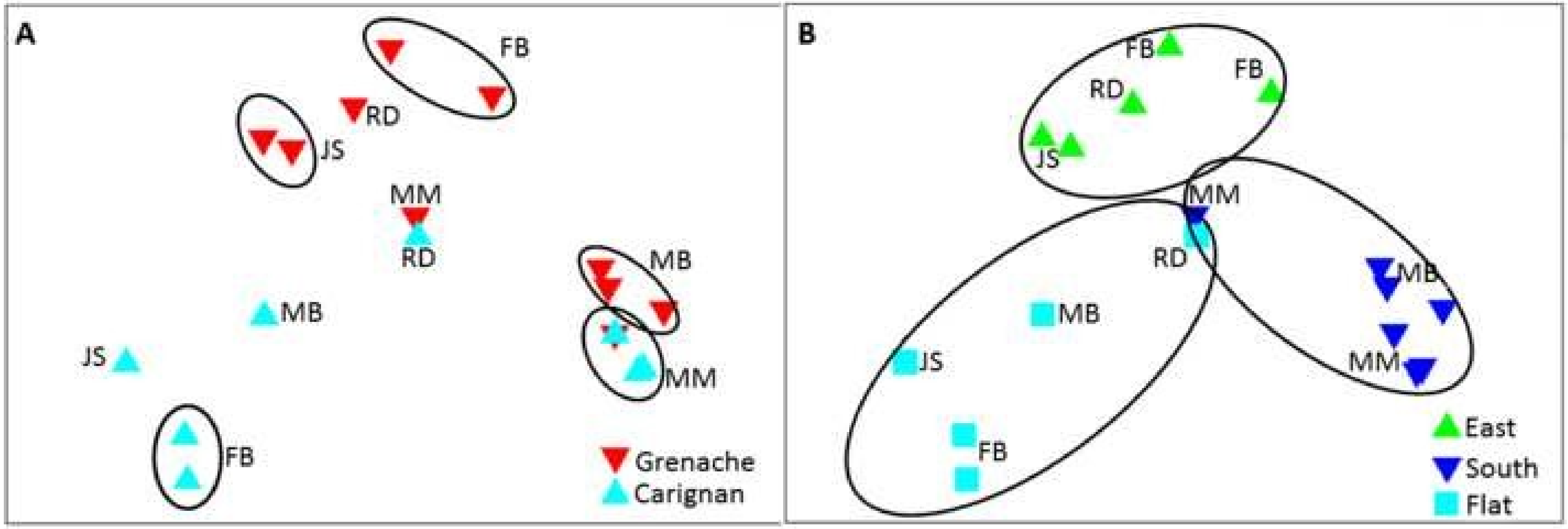
50%
40%
30%
20%
10%
0%
FB-Gx
FB-Cy
MM-Gx
MM-Cy
JS-Gx
JS-Cy
MB-Gx
MB-Cy
RD-Gx
RD-Cy
Wine
Other Phyla
Bacteroidetes
Actinobacteria
Gammaproteobacteria
Epsilonproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Firmicutes

Figure 2



Order, genus	FB-Gx	FB-Cy	MM-Gx	MM-Cy	JS-Gx	JS-Cy	MB-Gx	MB-Cy	RD-Gx	RD-Cy	Wine
Bacillales, *Bacillus*	0.5	0.1	99.7	99.8	1.8	3.6	88.3	21.7	3.6	20.0	0.0
Lactobacillales, *Oenococcus*	16.5	1.1	0.1	0.0	7.5	13.2	0.3	0.1	13.2	2.8	99.0
Pseudomonadales, *Acinetobacter*	11.9	0.6	0.0	0.0	14.9	15.2	1.5	63.6	15.2	11.3	0.1
Enterobacteriales, *Erwinia*	1.8	82.8	0.0	0.0	2.7	4.6	0.1	8.7	4.6	3.5	0.1
Lactobacillales, *Streptococcus*	15.7	0.0	0.0	0.0	3.2	6.2	0.3	1.2	6.2	33.8	0.1
Pseudomonadales, *Pseudomonas*	1.9	0.3	0.0	0.0	17.0	1.8	0.7	0.2	1.8	1.4	0.0
Pasteurellales, *Haemophilus*	1.3	0.1	0.0	0.0	0.5	0.4	0.0	0.4	0.4	0.1	0.0
Pseudomonadales, *Enhydrobacter*	2.2	0.1	0.0	0.0	8.2	6.5	0.0	0.0	6.5	0.9	0.1
Rhizobiales, *Methylobacterium*	7.4	0.0	0.0	0.0	1.2	3.9	0.4	0.0	3.9	0.7	0.0
Clostridiales, *Veillonella*	3.0	0.0	0.0	0.0	0.7	1.0	0.0	0.1	1.0	5.1	0.0
Actinomycetales, *Corynebacterium*	0.7	0.1	0.0	0.0	4.8	9.9	0.2	0.0	9.9	0.2	0.0
Lactobacillales, *Lactobacillus*	9.5	0.0	0.0	0.0	0.1	3.0	0.0	0.0	3.0	0.2	0.0
Neisseriales, *Neisseria*	1.8	0.0	0.0	0.0	0.1	1.5	0.0	0.6	1.5	0.4	0.0
Rhodospirillales, *Gluconobacter*	0.1	7.1	0.0	0.0	0.3	0.0	0.0	1.1	0.0	0.0	0.0
Sphingomonadales, *Sphingomonas*	0.5	0.5	0.0	0.0	1.6	0.8	2.0	0.2	0.8	0.1	0.0
Burkholderiales, Other	0.6	1.5	0.0	0.0	2.2	1.0	1.5	0.2	1.0	0.0	0.0
Bacillales, *Staphylococcus*	1.5	0.0	0.0	0.0	1.8	1.5	0.3	0.1	1.5	0.8	0.0
Lactobacillales, Other	0.5	0.1	0.0	0.0	0.3	0.8	0.0	0.4	0.8	3.1	0.0
Actinomycetales, *Micrococcus*	1.3	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.1	0.0
Other genera	21.3	5.8	0.4	0.1	27.6	25.1	4.2	1.2	25.1	15.6	0.5

Figure 3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FB
RD
JS
MM
RD
MB
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FB
🔻 Grenache
🔺 Carignan
B
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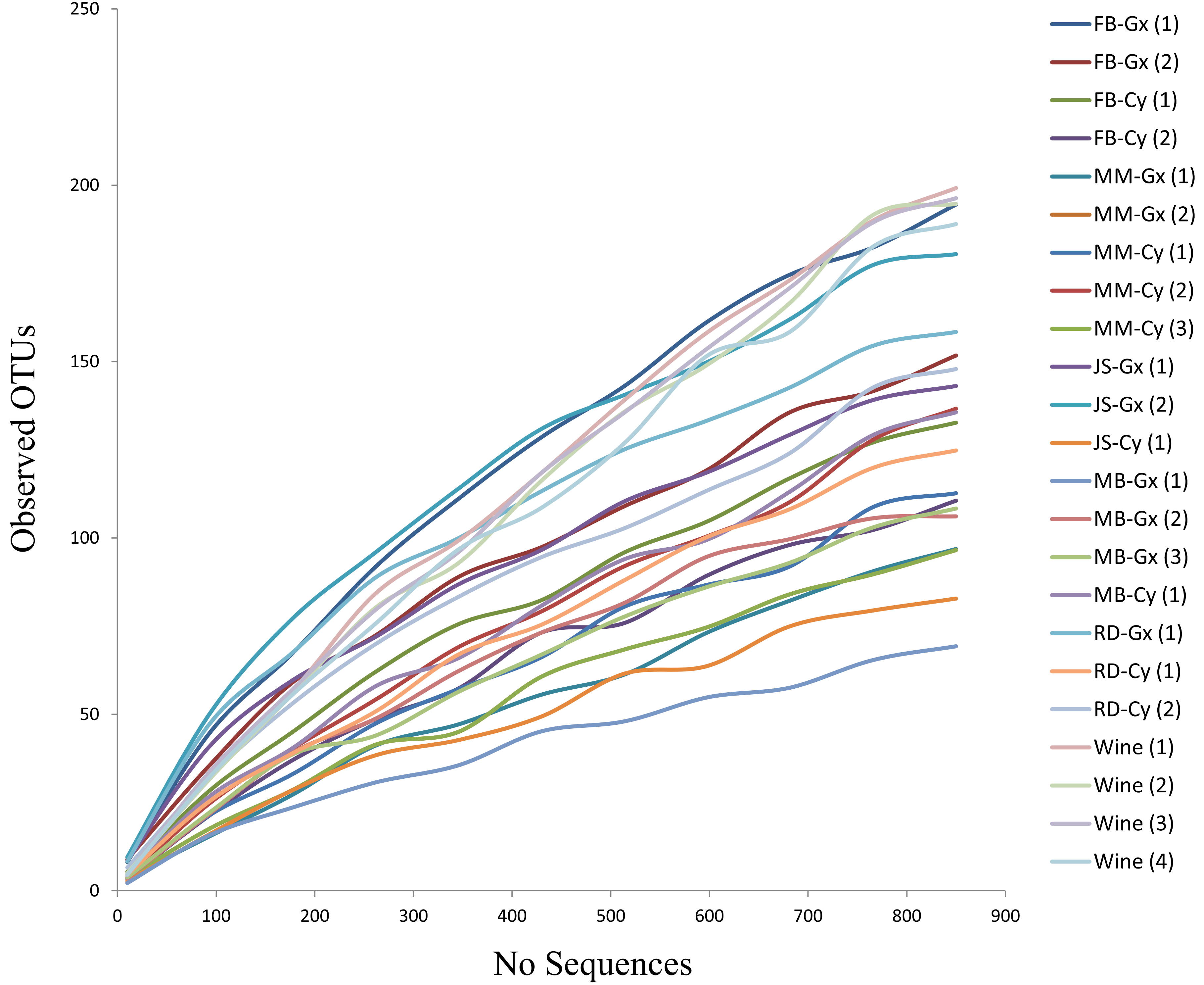
MB
MB
JS
MM
FB
🔺 East
🔻 South
■ Flat

Figure sup 2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400
500
600
700
800
900
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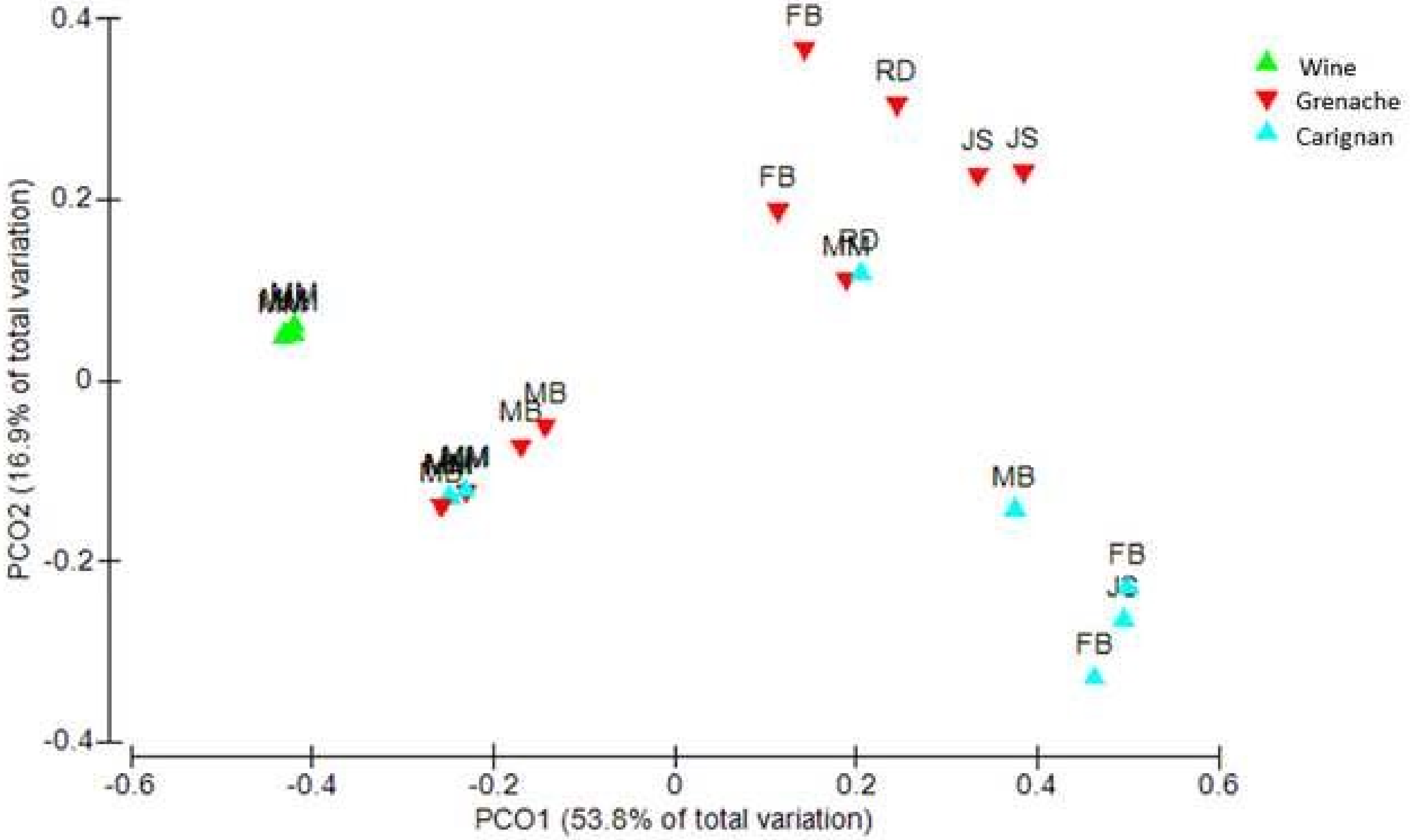
FB-Gx (2)
FB-Cy (1)
FB-Cy (2)
MM-Gx (1)
MM-Gx (2)
MM-Cy (1)
MM-Cy (2)
MM-Cy (3)
JS-Gx (1)
JS-Gx (2)
JS-Cy (1)
MB-Gx (1)
MB-Gx (2)
MB-Gx (3)
MB-Cy (1)
RD-Gx (1)
RD-Cy (1)
RD-Cy (2)
Wine (1)
Wine (2)
Wine (3)
Wine (4)

Figure sup 3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PCO1 (53.8% of total variation)
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0
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-0.4
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-0.2
0
0.2
0.4
0.6
▲ Wine
▼ Grenache
▲ Carignan
FB
RD
JS
JS
FB
MM
RD
MM
MB
MB
MM
MB
MB
FB
JS
FB