Influence of *Oenococcus oeni* and *Brettanomyces bruxellensis* on Wine Microbial Taxonomic and Functional Potential Profiles

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Abstract: The interactions between lactic acid bacteria, yeast, and other wine microbiota impact on wine quality. Some strains of lactic acid bacteria *Oenococcus oeni* possess cinnamoyl esterase activity that releases hydroxycinnamic acids (HCAs) that can subsequently be processed by some strains of the spoilage yeast *Brettanomyces bruxellensis* and lead to off-flavor compounds. Using metagenomic analyses, this study investigated the influence of *O. oeni* and *B. bruxellensis* on the taxonomic and functional potential profiles of the microbial community of Cabernet Sauvignon wine, particularly regarding flavor formation. Metagenomic datasets were generated from inoculations of three strains of *B. bruxellensis*, in combination with two *O. oeni* strains, with and without cinnamoyl esterase activity. Effect on the microbial profiles was found to depend on the *O. oeni* and *B. bruxellensis* strains being combined and on the abundance that the inoculants reach in the final wine, which depends on unidentified conditions (e.g., derived from microbial interactions). This study confirmed that the potential of *B. bruxellensis* to produce off-flavor compounds from HCAs is strain dependent. Interestingly, the samples without microbial inoculants also had this potential, suggesting that microbiota from the native grape could also influence the levels of HCAs. The metagenomic analyses complemented by experimental validation also found that the presence of *B. bruxellensis* did not interfere with the microbial functional potential to transform L-malic acid into L-lactic acid, which typically leads to a less acidic flavor. We show that metagenomic approaches can help uncover the complex wine microbial community traits, such as flavor, impacted by the simultaneous presence of *O. oeni* and *B. bruxellensis*.

Key words: *Brettanomyces*, hydroxycinnamic acids, metagenomics, microbiome, *Oenococcus oeni*

Winemaking and wine microbiome. Although various microbes can affect wine characteristics, studies of wine microbial ecosystem have mostly focused on two key fermentation players, *Saccharomyces cerevisiae*, which performs alcoholic fermentation (AF), and *Oenococcus oeni,* which performs malolactic fermentation (MLF). MLF is a decarboxylation process where dicarboxylic L-malic acid is converted to monocarboxylic L-lactic acid and CO_2, which can raise pH. L-lactic acid is perceived as less acidic than L-malic acid, thus, MLF softens the acid structure of wine. MLF usually starts spontaneously about one to three weeks after completion of AF, and lasts two to 12 weeks. Furthermore, during MLF, some carbohydrates are metabolized, phenolic acids are released, and esters are synthesized, among other compounds, which are important for the wine flavor profile. MLF can be carried out by lactic acid bacteria (LAB) naturally present in wine or using commercial strains isolated from wine. *O. oeni* is the preferable species due to its ethanol and acid tolerance, as well as its impact on the wine flavor profile (Liu 2002).

Factors affecting yeast biodiversity in wine have been widely documented (Longo et al. 1991, Schutz and Gafner 1993, Setati et al. 2012, Jolly et al. 2014), yet determining the effect of a specifically selected starter or mixed-starter culture of yeast and bacteria on the wine profile cannot be effectively accomplished without also characterizing the entire microbial community. However, few studies (Liu et al. 2017) have been performed to gain deeper insights on the latter. In the process of making fermented food and beverage, starting ingredients can include various associated indigenous microbial communities, which might vary depending on the source, consequently affecting final product characteristics. This issue has been investigated in wine, where the grape microbiota is influenced by cultivar, vintage, and climate (Bokulich et al. 2014). Besides yeasts such as *Saccharomyces*, and filamentous fungi such as *Aspergillus* and *Penicillium*, large bacterial diversity has been observed on

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grapes and must (Barata et al. 2012). Some bacteria are plant or environmental microbes, while others have physiological characteristics that allow them to grow in harsh enological conditions (low nutrients, high acidity, ethanol concentrations of up to 15% v/v; Maitre et al. 2014), thus enabling them to be part of the wine microbiome (Barata et al. 2012). Through the winemaking process, the microbial interactions and their succession dynamics affect the hygienic and organoleptic properties of the final wine product. For example, *Botrytis cinerea* influences the microbial taxonomic profile through nutrient release (Barata et al. 2008). Microbial interactions are also known to be highly strain-specific. For example, Branco et al. (2014) has shown that a strain of *S. cerevisiae* under enological conditions produces antimicrobial peptides that can inhibit growth of *B. bruxellensis*. In the wine industry, *B. bruxellensis* is a spoilage yeast difficult to get rid of, and is mostly present in barrel-aged wines (Suarez et al. 2007).

Hydroxycinnamic acids. Hydroxycinnamic acids (HCAs) are a potential source of off-flavor compounds in wine. They are usually found in grapes and wine as esters bound to tartaric acid, and their content depends on the grape variety and growth conditions (Nagel and Wulf 1979). This family of organic acids has been studied in wine and some food systems for its properties, such as antimicrobial activity against some yeast and bacteria (Ou and Kwok 2004). However, the full effect of HCAs in food and wine is not yet fully understood. Some strains of *Oenococcus* and other *Lactobacillus*, as well as some fungi, have been shown to possess a cinnamoyl esterase activity, which releases HCAs from their bound form (Rumbold et al. 2003, Cabrita et al. 2008). HCAs can also be released by chemical hydrolysis because of wine acidity in a slow process that continues through winemaking and storage (Hixson et al. 2012). *B. bruxellensis* is not capable of producing free HCAs; however, some strains possess an HCA decarboxylase gene (*HcD*) and a vinyl reductase activity, which can convert HCAs into the off-flavor volatile phenols 4-ethylphenol and 4-ethylguaiacol, conferring the "*Brettanomyces* aroma" (Hixson et al. 2012). Thus, an increase in free HCAs could increase the risk of spoilage by a *B. bruxellensis* strain with both activities (Kheir et al. 2013). Importantly, Madsen et al. (2017) showed that the concentration of volatile phenols depends more on the strain of *Brettanomyces* than on the HCA esterase activity of *O. oeni*.

While several studies have characterized microbiota on the grape surface and must, characterization of the microbial community in the final wine product is scarce. *Omics* methodologies in the food sciences, particularly in fermented foods, have been applied to deeper and broader analyses of the microbial system relevant to both the fermentation process and characteristics of the final product (De Filippis et al. 2017). In this study, we undertook a metagenomics approach to characterize the impact of inoculating two strains of *O. oeni* (with and without cinnamoyl esterase activity) and three *B. bruxellensis* strains, alone and in combination, on the wine microbial community 114 days postinoculation in a Cabernet Sauvignon wine.

Materials and Methods

Wine production and culture inoculation. Destemmed grape must from Cabernet Sauvignon before AF was transported at 4°C from Bulgaria to Denmark and imported by Chr. Hansen A/S. The must was then inoculated with *S. cerevisiae* strain NI6 (Chr. Hansen A/S) for AF. Wine production and inoculation with cultures were performed as described in Madsen et al. (2017). Briefly, AF was accomplished in 50 L tanks without addition of nutrients or SO_2. The wine was pressed through filter cloths to remove grape seeds and skin. After mixing the wine by stirring, the wine was decanted into 5 L containers and stored at 5°C. The wine was measured on an OenoFoss™ (Foss) and had an alcohol percentage of 12.6%, 0.0 g/L glucose, and 0.0 g/L fructose. The sulfite level was measured with a kit from Megazyme, and found to be 25 ppm.

Three strains of *B. bruxellensis* (Centraalbureau voor Schimmelkultures) were used for inoculation: CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). Two to three colonies of each *B. bruxellensis* strain from yeast extract glucose chloramphenicol (YGC) agar plates (5.0 g yeast extract, 20.0 g glucose, 0.05 g chloramphenicol, 15.0 g agar, 1 L milli-q water, pH 6.6 ± 0.1) were grown in 100 mL yeast extract glucose peptone (YGP) broth (5.0 g yeast extract, 10.0 g peptone, and 11.0 g glucose monohydrate, pH 5.6) in 250 mL Erlenmeyer flasks with loose metal lids for three days at 150 rpm and 25°C before inoculation. Inoculation was performed at a concentration of ~5×10^2 CFU/mL.

Two *O. oeni* strains were used for inoculation (Chr. Hansen). One of the *O. oeni* strains is cinnamoyl esterase negative (OEN), and the other is cinnamoyl esterase positive (OEP). Frozen culture (1.2 g) made for direct inoculation was dissolved in 200 mL sterilized peptone water, and 5 mL was added to 1 L of wine, corresponding to an inoculation level of ~10^6 CFU/mL.

Two wines were used as controls with no inoculations, four wines were inoculated with only an *O. oeni* strain, six wines with only one *B. bruxellensis* strain, and 12 wines were inoculated with an *O. oeni* strain in combination with a *B. bruxellensis* strain. All inoculations were performed in duplicates at 22°C (Figure 1).

Measurement of MLF. Fermentations were performed at 20°C. The pH of the wines was measured with a pH meter (PHM 250, Radiometer) during MLF and was found to increase from 3.47 to 3.5 pH. Samples were taken on day 0, 4, 7, 10, 14, and 114 from every bottle and frozen for later measurement of the malic acid content using a malic acid enzyme test kit (R-Biopharm) and a spectrophotometer. A cuvette with 1.00 cm light path was used at wavelength 340 nm at 20 to 25°C. The cuvettes were prepared according to the kit instructions to calculate the malic acid concentration using the absorbance values.

DNA extraction and sequencing. The wines were sampled on day 114 postinoculation for DNA extraction and sequencing. Cells were pelleted from 50 mL of wine centrifuged at 4500*g* for 10 min and subsequently washed three times with 10 mL of 4°C phosphate buffered saline. The pellet was mixed with G2-DNA enhancer (Ampliqon A/S) in 2 mL tubes and

incubated at room temperature for 5 min. Subsequently, 1 mL of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 40 mg/mL lysozyme) was added to the tube and incubated at 37°C for 1 hr. An additional 1 mL of CTAB/PVP lysis buffer was added to the lysate and incubated at 65°C for 1 hr. DNA was purified from 1 mL of lysate with an equal volume of phenol:chloroform:isoamyl alcohol mixture (49.5:49.5:1); the upper aqueous layer was further purified with a MinElute PCR purification kit and the QIAvac 24 plus (Qiagen), according to the manufacturer's instructions; and finally eluted in 100 µL DNase-free H_2O.

Genomic DNA was then fragmented to an average length of ~400 bp using the Bioruptor XL (Diagenode, Inc.), with a profile of 20 cycles of 15 sec of sonication and 90 sec of rest. Sheared DNA was converted to Illumina-compatible libraries using NEBNext library kit E6070L (New England Biolabs) and blunt-ended library adapters, as described by Meyer and Kircher (2010). The libraries were amplified in 100 µL reactions, with each reaction containing 20 µL of template DNA, 10 U AmpliTaq Gold polymerase (Applied Biosystems), 1× AmpliTaq Gold buffer (Applied Biosystems), 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 µM IS4 forward primer, and 0.2 µM reverse primer with sample-specific 6 bp index. The PCR conditions were 12 min at 95°C to denature DNA and activate the polymerase, 14 cycles of 95°C for 20 sec, 60°C annealing for 30 sec, 72°C extension for 40 sec, and a final extension of 72°C for 5 min. Following amplification, libraries were purified with Agencourt AMPure XP (Beckman Coulter, Inc.) bead purification, following the manufacturer's protocol, and eluted in 50 µL of elution buffer (Qiagen). The quality and quantity of the libraries were measured on the Bioanalyzer 2100 (Agilent Technologies), and the libraries were pooled at equimolar concentration. Sequencing was performed on the Illumina HiSeq 2500 in PE100 mode following the manufacturer's instructions.

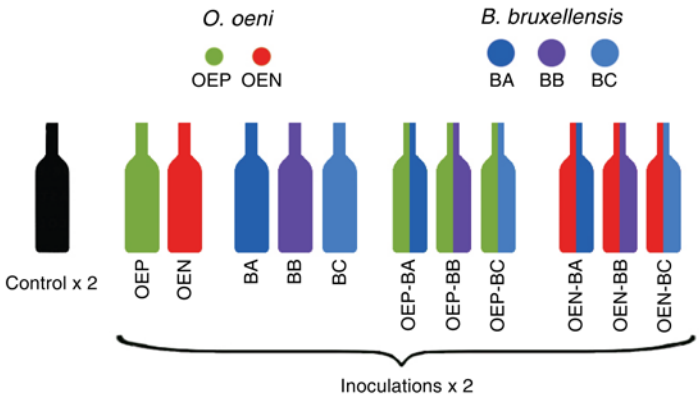

Figure 1 Inoculation scheme. Two strains of *Oenococcus oeni* were used: an esterase positive (OEP) and an esterase negative (OEN). Three strains of *Brettanomyces bruxellensis* were used: CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). Inoculations were performed with the different strains of *O. oeni* and *B. bruxellensis* alone or in combination. A control with no inoculation was used. Inoculations and controls were performed in duplicates. Metagenomic DNA from the 24 samples in this scheme was extracted and sequenced (except from one of the replicates of OEP-BC, where DNA could not be extracted).

Metagenomic taxonomic profiling. The reads were first cleaned with cutadapt (Martin 2011) to remove adapter sequences and low-quality bases (min quality = 33, 3′-end minimum quality = 30, minimum length = 30). To evaluate the presence of the inoculants in the final wine, Burrows-Wheeler Aligner v0.7.10 (Li and Durbin 2009) was used to map the cleaned reads against the genomes of the *O. oeni* strains (in-house genomic sequences) and the published genome of *B. bruxellensis* (Piskur et al. 2012) (CBS 2499 v2.0), and to calculate the depth of coverage using Sequence Alignment/Map tools (SAMtools) v1.3.1 and Browse Extensible Data tools (BEDTools) v.2.26. The coverage statistics of *B. bruxellensis* were calculated excluding scaffolds AHMD01000878.1, AHMD01000885.1, and AHMD01000879.1, which contain rDNA tandem repeats that artificially inflate the coverage due to mapping of reads likely deriving from other yeasts.

The taxonomic microbial characterization was done with MGmapper (Petersen et al. 2017). The clean reads were first mapped against the PhiX genome, and the unmapped reads were then used to map against the next databases extracted from the National Center for Biotechnology Information (NCBI; release 215) in *best mode*: human, plant, vertebrates, invertebrates, virus, fungi, protozoa, plasmid, and bacteria. The taxonomic identifications were filtered by accounting for the next parameters: minimum abundance of 0.01%, minimum ratio of unique mapping reads to total mapping reads of 0.005, maximum edit distance of 0.01, and a minimum of 10 mapping reads. The unmapped reads from the top three samples with highest percentage of taxonomically unassigned reads were further taxonomically evaluated by mapping them with MGmapper against the NCBI nt (nucleotide) database (release 215) and processing the hits, as previously described.

Metagenomic taxonomic comparison. OEN_23 was removed from the taxonomic and functional potential comparisons because it had the lowest number of sequenced reads (1,899,372), so it would not capture the low abundant microbes identified by the other samples, and because the *O. oeni* inoculation did not seem to succeed in this replicate (0.63× coverage to the *O. oeni* genome, Supplemental Figure 1). OEN-BB_18 had the highest number of reads (162,426,646), so it was also removed from the taxonomic comparisons because it would show bias for identifying the very low abundant species that the other samples would not capture; however, it was not removed from the functional comparisons. To compare the microbial populations of the different inoculation types, we first built a matrix with the number of reads mapping to the filtered identifications from all samples and then normalized the counts by percentage of abundance and by depth of coverage. This matrix was used for the comparative analyses.

The taxonomic cores were obtained by identifying the microbes present in the two replicates of each inoculation type. To calculate the microbial diversity distances between and within the inoculation types, the Bray, Jaccard, and Euclidean distances were calculated with the R vegan package and clustered with the ward.D and average methods. The differential abundance was performed using Fisher tests with

alternative hypotheses greater and less. The contingency tables were built with the average of the technical replicates of the inoculation types and used to perform all the possible pairwise comparisons. The *p*-values (*p*) were adjusted by the false discovery rate (FDR) and the significant comparisons were those with FDR ≤ 0.05.

The abundance correlations were performed with the R function cor.test using the Spearman method. The identifications present in less than 10 samples were removed from the normalized count matrix. Two types of significant correlations ($p < 0.05$, rho < -0.4 or rho > 0.7) were defined: (1) unaffected correlations were identified when comparing all the samples, as well as when comparing without each of the inoculation types; and (2) affected correlations were identified when removing only one of the inoculation types.

Metagenomic functional potential profiling. The cleaned reads were *de novo* assembled using Iterative De Bruijn Graph De Novo Assembler with Uneven Sequencing Depth v1.1.1 (IDBA-UD; Peng et al. 2012) with the precorrection parameter. Genes were then predicted on the assemblies with Prodigal v2.6.2 (Prokaryotic Dynamic Programming Gene-finding Algorithm; Hyatt et al. 2010) using the meta mode. Afterward, the predicted genes of each sample were clustered using vsearch v2.1.2 (Edgar 2010) with an identity threshold of 95% and a minimum sequence length of 20. The centroid sequence of each cluster was kept as the representative sequence to form a nonredundant gene set. Next, Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology annotations (KOs) were assigned to the predicted nonredundant genes using blastx with e-value 0.000001 against the KEGG database. The blast hits were filtered by a minimum bit score of 50 and minimum of 30% identity.

The presence of the *HcD* gene (id HQ693758.1) in the *B. bruxellensis* strains was confirmed using the software large-scale genome alignment tool (LASTZ; Harris 2007). To evaluate whether there is an increased potential to release HCAs due to microbes other than *O. oeni*, sequences of the cinnamoyl esterase gene from fungal- and LAB-origin available from NCBI were searched against the nonredundant gene catalog.

Metagenomic functional potential comparison. The reads of each sample were mapped against the nonredundant gene catalog using Burrows-Wheeler Aligner mem (Li and Durbin 2009) to then obtain the coverage of each gene using SAMtools and BEDTools; the coverage was used to build an abundance matrix. Principal component analysis (PCA) was then performed on the normalized matrix using the function prcomp from R v3.2.0 with scaling. As another method to evaluate the variation between and within the inoculation types, the Bray, Jaccard, and Euclidean distances were calculated with the R vegan package using the abundance matrix, and the values were clustered using the average and the ward.D methods.

Pairwise Fisher tests were performed to identify KOs in different abundance, as previously described. The differentially abundant KOs were then grouped by the pathways they belong to, as annotated in KEGG, and a two-sided Fisher test was performed, as previously described for the KOs, using the number of differentially abundant KOs belonging to that pathway as counts. The functional cores of the different inoculation types were defined as those nonredundant genes present only in the replicates of each inoculation type. The most abundant genes were identified as those within the top 5% of genes with highest number of mapping reads.

Results

Taxonomic profiling. *Inoculant abundance.* Although two replicates were produced for each inoculation type and control (Figure 1), extracting DNA from one of the OEP-BC inoculation replicates was not possible. The abundance of *O. oeni* and *B. bruxellensis* in the wines was evaluated 114 days postinoculation (Table 1). The genome of *O. oeni* was present at medium and high coverage in the two BB inoculations. Also, one of the replicates of BC had the genome of *O. oeni* at high coverage, while the other was present in low abundance. To test whether the identified *O. oeni* is able to perform MLF in the wines not inoculated with it, the amount of malic acid in the samples was measured. No malic acid was identified at day 114 in the samples without an *O. oeni* inoculation (Figure 2).

Table 1 Statistical results of sequencing and genome coverage of the inoculants.

Sample[a]	Cleaned reads	Unmapped reads (%)	*Oenococcus oeni*[b] coverage	*Brettanomyces bruxellensis*[c] coverage
Control_1	3,317,232	21.0	2.75	0.35
Control_2	5,195,056	15.23	4.45	0.88
BA_3	4,107,740	8.54	1.15	26.3
BA_4	6,695,169	8.41	18.1	38.7
BB_5	2,761,092	2.08	70.65	0.4
BB_6	10,993,754	2.04	420	0.45
BC_7	13,827,278	3.25	399.8	23.27
BC_8	2,054,860	4.35	14.35	12.97
OEP-BA_9	8,067,295	7.9	43.85	27.2
OEP-BA_10	65,488,400	5.54	769.5	66.26
OEP-BB_11	59,758,655	1.91	2369.1	7.9
OEP-BB_12	19,900,342	1.89	216.25	3.37
OEP-BC_13	39,001,458	3.59	945.75	105.4
OEN-BA_15	43,950,984	4.27	1333	21
OEN-BA_16	6,728,890	10.18	4.3	44.6
OEN-BB_17	37,224,934	2.12	1280.85	2.12
OEN-BB_18	159,467,366	2.08	4621.2	13
OEN-BC_19	6,443,410	6.41	1.25	37.4
OEN-BC_20	6,458,253	6.19	110.5	26.3
OEP_21	65,477,885	1.17	5.8	1.29
OEP_22	6,451,292	9.15	8.785	1.23
OEN_23	1,831,535	51.0	0.635	0.13
OEN_24	7,664,243	3.6	47.25	0.9

[a]*B. bruxellensis* strains CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). *O. oeni* strains esterase positive (OEP) and esterase negative (OEN).
[b]Reported mapping statistics for *O. oeni* derive from the average of mapping to genomes of OEP and OEN.
[c]Reported mapping statistics for *B. bruxellensis* derive from mapping to the publicly available genome of BC. Coverage is the average number of reads mapping per nucleotide in the genome.

OEP-BA_9 had the genome of *O. oeni* at medium coverage with *B. bruxellensis* at abundance similar to the other samples, while OEP-BA_10 had *O. oeni* in high abundance and was the sample with the second most abundant *B. bruxellensis*—even higher than the samples where *B. bruxellensis* was inoculated alone. The OEP-BC inoculation had the highest *B. bruxellensis* coverage. *B. bruxellensis* was also identified among the predominant fungi in OEP-BA_10 and OEP-BC_13.

Bacterial identifications. Sample BC_8 had the least number of bacterial identifications (Supplemental Table 1); however, its sequencing had much less depth than BC_7 (<25%), similar sequencing to other samples with more identifications, and a similar number of identifications to BA_3, which had double the sequencing depth. Similarly, the total number of identifications for other inoculation types was uncorrelated with their sequencing depth. For example, the samples with highest sequencing had a similar number of identifications to the control samples, which had an average sequencing depth.

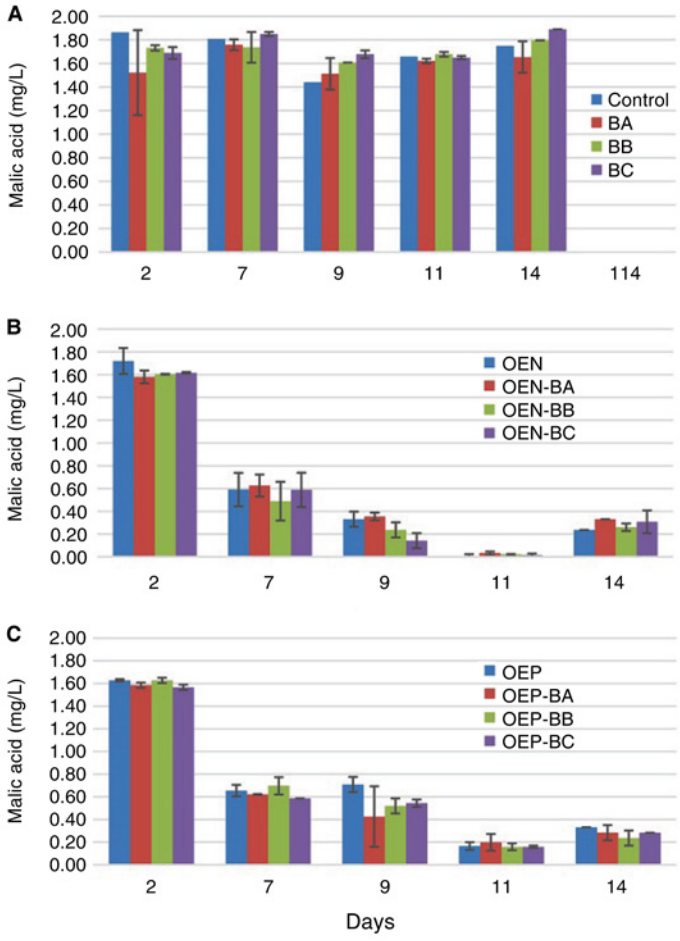

Figure 2 Concentration of malic acid over time. Malic acid concentrations are average values of duplicates. Error bars are standard deviations. *Brettanomyces bruxellensis* strains CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). *Oenococcus oeni* strains esterase positive (OEP) and esterase negative (OEN). (**A**) Malic acid concentration of inoculations with the *B. bruxellensis* strains. The malic acid concentration on day 114 was 0 mg/L in all inoculations, suggesting the presence of *O. oeni* able to perform malolactic fermentation in the samples not inoculated with *O. oeni*, which could derive from native grape flora or another contaminant source, such as the machinery for making grape must. (**B**) Wines inoculated with OEN-*B. bruxellensis*. (**C**) Wines inoculated with OEP-*B. bruxellensis*.

Bacterial differential abundance. OEN inoculation had the lowest number of bacteria present in higher abundance compared with the controls (33 taxa, average = 144.6), BA is in second place (38 taxa), and OEP in third (54 taxa). Regarding the number of species in less abundance compared with the controls, the inoculations with only BA had the average (118 taxa, average = 120.1), while OEN had 141 taxa, and OEP-BC had the maximum (148 taxa) (Figure 3). Interestingly, while the BA inoculation changed the bacterial community the least, it also changed the bacterial community the most when inoculated together with *O. oeni* (Figure 3). As observed, all inoculation types had similar amounts of less abundant bacteria when compared with the control samples, with the greatest difference only in the higher abundance of bacteria in OEN-BA (412 taxa) and OEP-BA (300 taxa). Compared to BA, both OEN-BA and OEP-BA had low numbers of differentially less abundant bacteria (24 and 36 taxa, respectively). When compared with OEP, OEN had 37 species in differentially higher abundance, and 142 in less abundance.

Taxonomic abundance correlations and taxonomic distances. The number of positive and negative taxonomic abundance correlations that were not affected by the inoculation type, thus likely representing the stable wine microbial community, were almost the same (371 and 384, respectively) (Figure 4). The addition of only a *B. bruxellensis* strain is the inoculation type with the highest number of correlations disrupted (791 affected correlations); the number of correlations affected by the OEP-*B. bruxellensis* combinations was the second highest (617 affected correlations), while the number of correlations affected by the inoculation of *O. oeni* was only 187. No correlation was identified as affected by only one specific combination of *O. oeni*-*B. bruxellensis*, but affected correlations were identified by accounting for the three possible combinations of each *O. oeni* strain.

As expected, the distance between the controls was the lowest comparison of all the samples versus the controls (Figure 3C and 3D). Interestingly, the distances of samples inoculated with only *B. bruxellensis* were similar to those of the OEN-*B. bruxellensis* combinations. The distances also show that OEP-*B. bruxellensis* combinations had the highest difference from the controls, with OEP-BA_10 as an outlier.

Plasmid and fungal identifications. Similar to the bacterial profiling, OEP was closest to the control in plasmid profiling (Supplemental Figure 2A). Also, inoculation of *O. oeni*-BA caused most plasmids to be in differentially higher abundance than with *O. oeni*-BB or *O. oeni*-BC. Interestingly, only OEP-BA_10 was an outlier very distant from its pair, with about double the number of identifications than OEP-BA_9; however, it had a similar number of identified plasmids to other samples. BA_3 had the fewest identified plasmids and OEN-BA_15 had the most. However, OEN-BA_15 did not have the highest depth of sequencing; the samples with highest depth of sequencing had a similar number of plasmids as those with average depth. Furthermore, BA_3 and OEN-BA_15 clustered together within the main cluster. Analysis of the fungal profile

showed that most samples cluster tightly together (Supplemental Figure 2B). The only three samples placed outside of the main cluster were one of the replicates of the OEP-*B. bruxellensis* combinations.

Profiling of functional potential. A total of 430,713 genes was predicted, with a mean of 18,726.65 genes per sample (Supplemental Figure 3). A final nonredundant gene catalog with all genes was constructed and contained 5614 different KO annotations on the filtered 40,525 nonredundant genes for the comparative analyses. The sequencing depth of the samples did not affect the number of predicted genes (Pearson correlation = 0.3667). However, sample OEN_23 was excluded because it had the lowest depth of sequencing and its functional potential profile was an extreme outlier (Supplemental Figure 1).

The inoculations with only *O. oeni* clustered close to each other and to the controls, however, considerable functional potential variability between all the samples was identified, even within the controls (Figure 5). Variation in the functional potential was identified between pairs of similar and different amounts of sequencing as well. For example, the OEN-BB replicates were observed to be functionally close despite large differences in sequencing depth, the OEN-BC replicates lay separate in two different tight clusters not containing the control samples, and the OEP-BA replicates were also in separate clusters. This OEP-BA pair was among the replicate pairs most distant from each other. The sample with the least number of assembled nonredundant genes was BB_6 (9184 genes), although its depth of sequencing was not the lowest. The sample with the lowest depth of sequencing

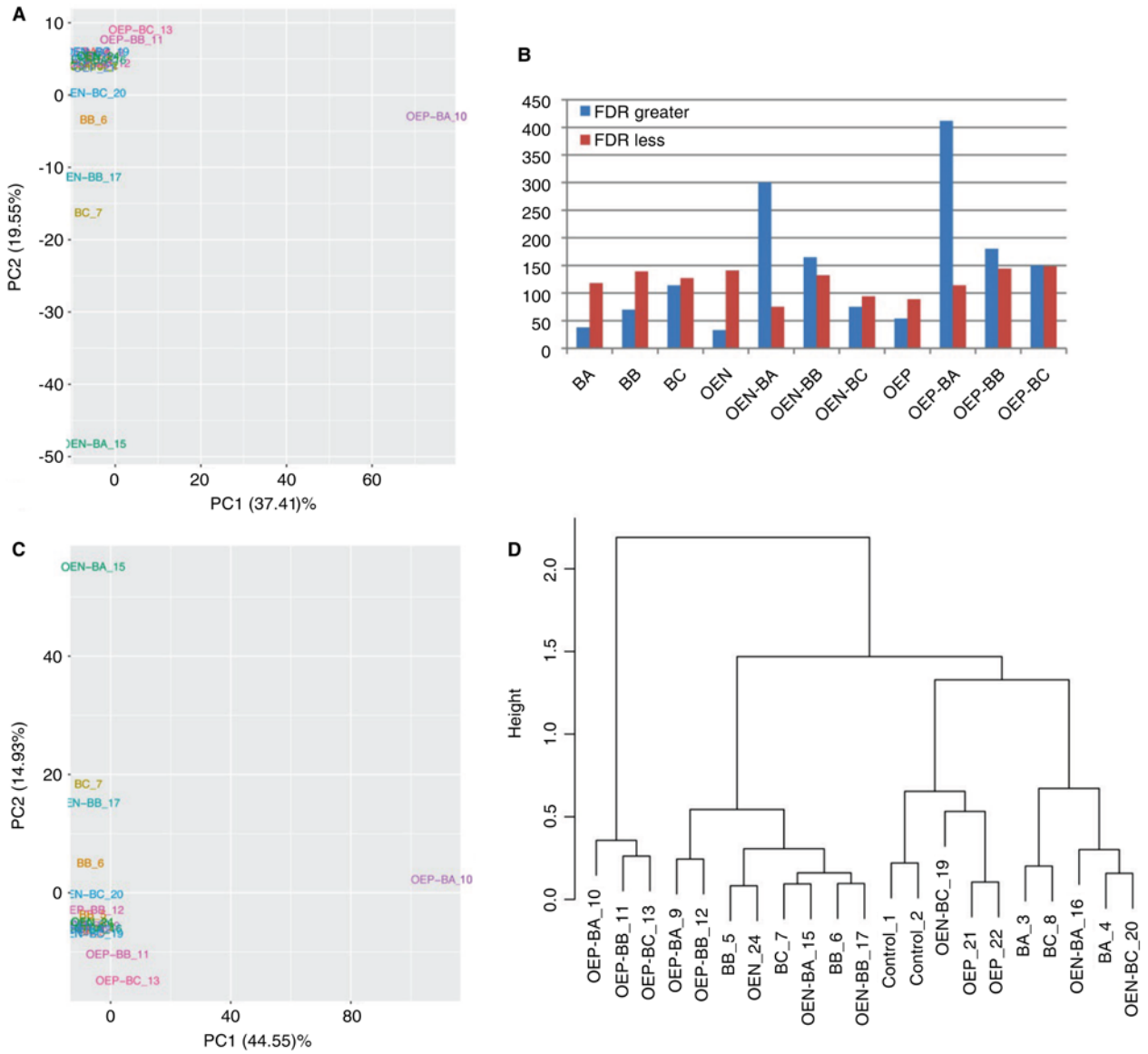

Figure 3 Wine microbiome bacterial profiling. *Brettanomyces bruxellensis* strains CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). *Oenococcus oeni* strains esterase positive (OEP) and esterase negative (OEN). False discovery rate (FDR). (**A**) Principal component analysis (PCA) of all identified wine bacteria at species level. (**B**) Differentially abundant wine bacterial species by inoculation type compared with the control samples. "FDR greater" denotes bacteria that was in statistically significant higher abundance, and "FDR less" are those in statistically significant less abundance. (**C**) PCA of the abundance of all identified microbial taxa. (**D**) Cladogram of ward.D clustered Bray distances of all identified wine microbial taxa.

(OEN_23) and that with the highest (OEN-BB_18) had assembled around the average number of nonredundant genes (14,078 and 19,651 genes, respectively).

Differentially abundant functions. Compared with the control, both replicates inoculated with only *O. oeni* had the lowest total of differentially abundant KOs (OEP = 63, OEN = 1477), while the inoculations with only *B. bruxellensis* had a high number of differentially abundant KOs (BA = 3379, BB = 4340, BC = 2760). The BB and OEN-BB inoculations had the highest numbers of differentially less abundant KOs (3481 and 3582, respectively; average = 1676.7 KOs) (Figure 6), whereas BC had a similar number of differentially abundant KOs (higher abundance = 1229, less abundance = 1531), and OEP had the least number of KOs with higher and less abundance (24 and 39, respectively). However, OEN-BC had the second greatest number of KOs present in higher abundance (2006 KOs), and OEP-BC is the third with the largest number of KOs present in less abundance (3200 KOs). Compared with OEP, OEN had more KOs present in higher abundance (956 KOs) than present in lower abundance (29 KOs).

Compared with controls, more metabolic pathways were affected by KOs in higher abundance (median = 25 pathways) than by those in lower abundance (median = 10 pathways). When analyzing the pathways differentially abundant in only

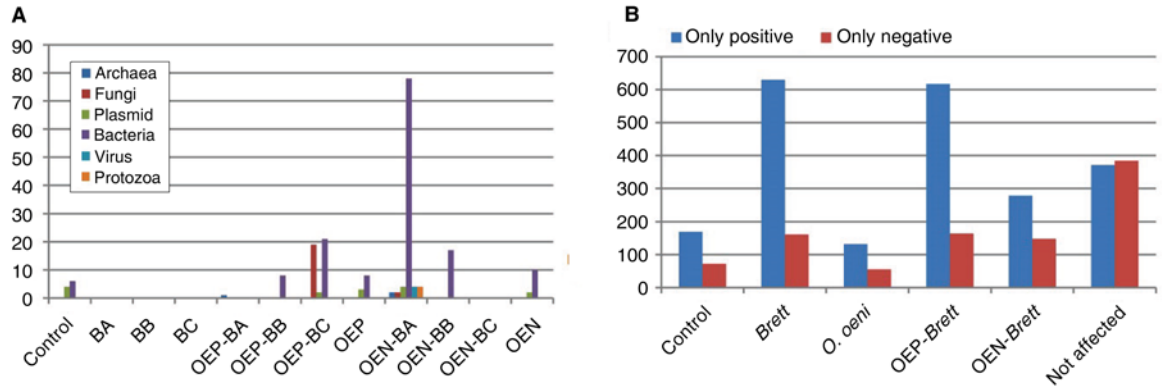

Figure 4 Taxonomic profiling. *Brettanomyces bruxellensis* strains CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). *Oenococcus oeni* strains esterase positive (OEP) and esterase negative (OEN). (**A**) Microbial species taxonomic cores. The general low number of identified cores highlights the high diversity in the wine microbial community, but the high number of taxa identified only in OEN-BA shows the high impact of the inoculation on microbial diversity. (**B**) Microbial abundance correlations. Correlations identified only when removing from the comparisons the values from a given inoculation type are called to be "affected" by that inoculation type. Correlations identified regardless of removing any of the inoculation types are called "unaffected correlations."

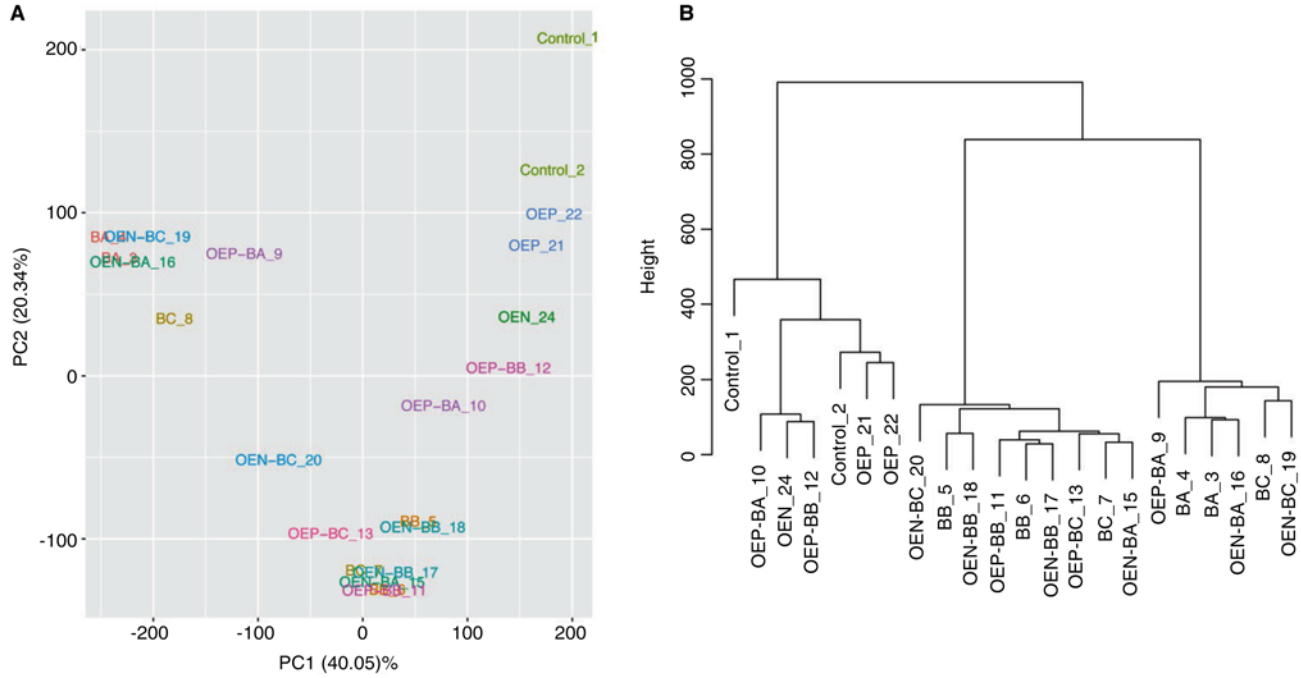

Figure 5 Functional potential profiling. *Brettanomyces bruxellensis* strains CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). *Oenococcus oeni* strains esterase positive (OEP) and esterase negative (OEN). (**A**) Principal component analysis of the normalized gene mapping counts. (**B**) Cladogram of the Euclidean distances clustered by the ward.D method.

a given inoculation type, *O. oeni*-BC was found to have the highest number of differentially less abundant pathways (52 pathways) (Figure 6C, Supplemental Table 2), with most of those differentially abundant KOs coming from OEP-BC (2068), while OEN-BC has 593.

Core and top abundant genes. Out of the 40,525 annotated, filtered, nonredundant genes, only 867 were present in all compared samples. The BA inoculations had only one core gene, annotated as coming from *Candida glabrata*, a common inhabitant of the human flora; thus, this gene was likely derived from human contamination or is a misidentification from *Candida apicola*, which has been previously isolated from wine. Similarly, the BB and BC inoculations had zero genes present in their two replicates. *O. oeni*-BB had zero genes in its core, *O. oeni*-BC had only nine core genes, and *O. oeni*-BA had 15 core genes. OEN had 16 core genes, much fewer core genes than OEP (329 genes). Interestingly, although OEP was closest to the control, it was second for the largest number of genes in its core (329; median = 2, average = 67.15). As expected, the control samples had the largest number of core genes (515 genes).

The percentage of most abundant genes present in the two replicates of each inoculation type was on average 53.49%. OEN-BA had the lowest number of genes present in greatest abundance in the two replicates (5 genes), while both replicates had an average number of genes in greatest abundance (580 and 646 genes, average = 627.67 genes). Notably, OEN-BB had the highest percentage of replicable genes in greatest abundance (80.2%, 355 genes) (Figure 6D).

HCA derivatives. In the search for fungal cinnamoyl esterase genes, only two genes were found, one of which was originally annotated as deriving from the yeast *Pichia stipites*; however, this gene was not annotated as esterase, rather as triacylglycerol lipase, with only 31.8% identity. The other gene was originally annotated from the yeast *Pichia pastoris*; however, it was annotated with only 30.5% identity to an uncharacterized protein.

To evaluate whether the microbial community has the functional potential to produce off-flavor compounds derived from HCAs, genes involved in decarboxylation of HCAs were identified in the nonredundant gene catalog. A gene was identified with 35.29% identity as phenolic acid decarboxylase (K13727) from *Erwinia gerundensis*, a cosmopolitan epiphyte. This phenolic acid decarboxylase has decarboxylation activity on HCAs ferulic, *p*-coumaric, and caffeic acids. This same gene had a hit with 73.86% identity to an unannotated protein from *Nectria haematococca* (*Fusarium solani* subsp. pisi), which is a fungal plant pathogen.

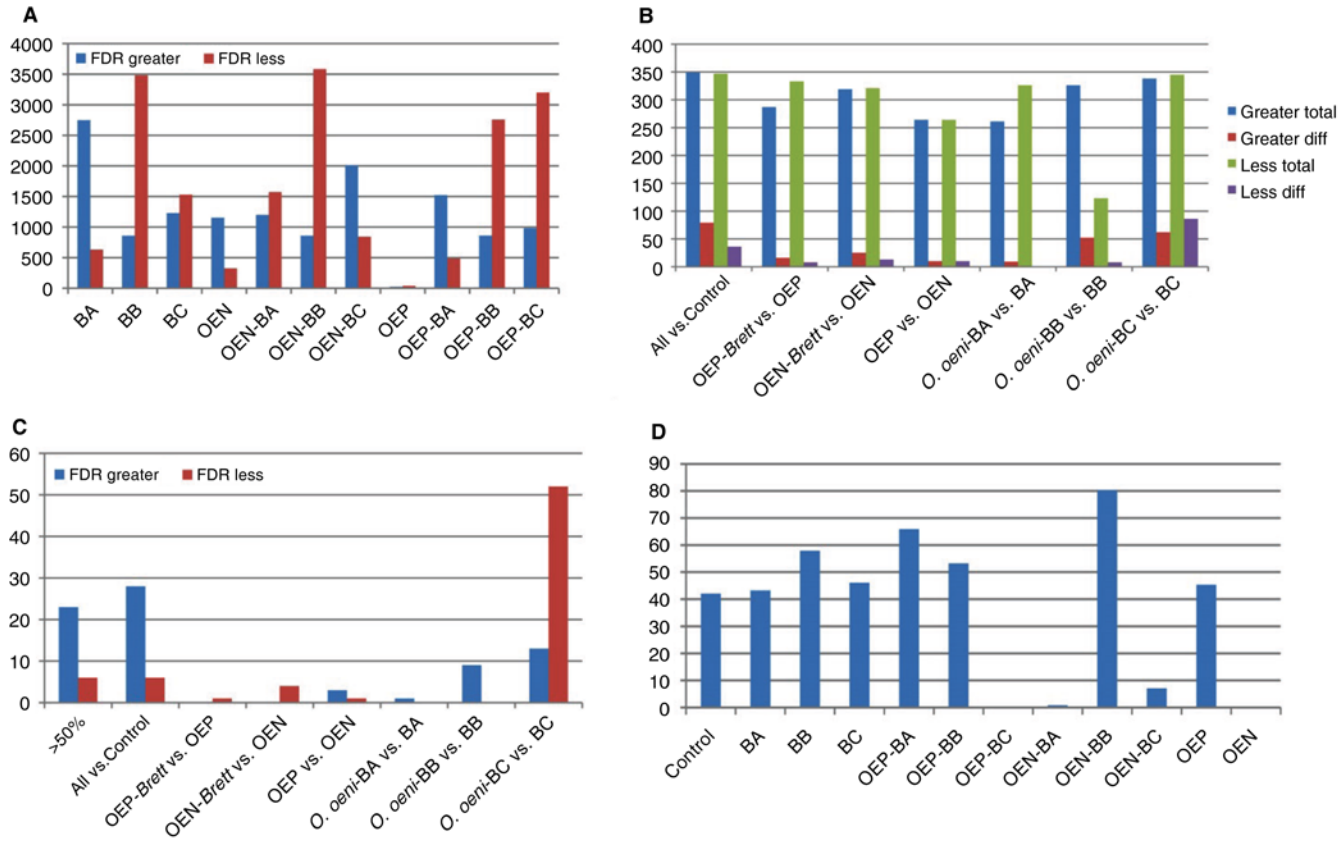

Figure 6 Comparisons of the functional potential profiles of the inoculation types. *Brettanomyces bruxellensis* strains CBS 73 (BA), CBS 2336 (BB), and CBS 2499 (BC). *Oenococcus oeni* strains esterase positive (OEP) and esterase negative (OEN). False discovery rate (FDR). (**A**) Differentially abundant KEGG orthologies (KOs) from the different inoculation types compared with controls. "FDR greater" denotes the KOs in statistically significant higher abundance, and "FDR less" are those in less abundance. (**B**) Pathways from the differentially abundant KOs. (**C**) Core differentially abundant pathways from the differentially abundant KOs. (**D**) Percentage of replicable top 5% of most abundant genes in each inoculation type. OEN and OEP-BC did not have a replicate to consider.

This gene was also found to be significantly more abundant in BA ($FDR_{BAvsBB} = 4.16e^{-10}$, $FDR_{BAvsOEP\text{-}BA} = 0.0138$, $FDR_{BAvsOEN\text{-}BA} = 6.13e^{-5}$, $FDR_{BAvsControl} = 2.91e^{-8}$), OEP-BA ($FDR_{OEP\text{-}BAvsOEP} = 0.0014$, $FDR_{OEP\text{-}BAvsControl} = 0.0012$), BC ($FDR_{BCvsOEP\text{-}BC} = 0.00027$, $FDR_{BCvsControl} = 4.54e^{-7}$), and OEN-BC ($FDR_{OEN\text{-}BCvsOEN} = 1.27e^{-11}$, $FDR_{OEN\text{-}BCvsControl} = 3.22e^{-11}$).

A ferulic acid decarboxylase 1-like gene (K20039) from *S. cerevisiae* was also identified in the nonredundant gene catalog and was in significantly higher abundance in OEP-BA compared with BA ($FDR_{OEP\text{-}BAvsBA} = 0.00014$) and in OEP-BB compared with BB ($FDR_{OEP\text{-}BBvsBB} = 0.02$). Compared with OEP, some genes with this KO were identified as less abundant in OEP-BB ($FDR_{OEP\text{-}BBvsOEP} = 0.01$, $FDR_{OEP\text{-}BBvsControl} = 0.013$) and OEP-BC ($FDR_{OEP\text{-}BCvsOEP} = 3.82e^{-9}$, $FDR_{OEP\text{-}BCvsControl} = 2.10e^{-9}$). Additionally, some genes with this KO were shown to be less abundant in all OEN-*B. bruxellensis* inoculations than in samples inoculated with only OEN ($FDR_{OEN\text{-}BAvsOEN} = 2.1e^{-10}$, $FDR_{OEN\text{-}BAvsControl} = 1.19e^{-7}$, $FDR_{OEN\text{-}BBvsOEN} = 5.25e^{-8}$, $FDR_{OEN\text{-}BBvsControl} = 1.88e^{-5}$; $FDR_{OEN\text{-}BCvsOEN} = 4.17e^{-8}$, $FDR_{OEN\text{-}BCvsControl} = 8.92e^{-6}$), and comparatively less abundant in inoculations with only *B. bruxellensis* than in controls ($FDR_{BAvsControl} = 1.99e^{-6}$, $FDR_{BBvsControl} = 3.55e^{-7}$, $FDR_{BCvsControl} = 1.23e^{-8}$).

Discussion

Microbial taxonomic and functional potential profiling. *Wine-related identifications.* Among the taxonomic identifications, we found expected soil and plant bacteria, such as *Xanthomonas alfalfa*, *Dyella japonica*, and *Micrococcus luteus* (found on the surface of table grapes). Wine spoilage bacteria were also identified, such as *Aeromonas hydrophila*, in various samples. Regarding the number of bacterial identifications, it is interesting to note that *Bodo saltans* was found in greatest abundance in OEP-BB_12, OEN_24, OEN-BA_16, BA_3, and both OEN-BC samples. *B. saltans* is a free-living nonparasitic protozoan that feeds upon bacteria found in freshwater and marine environments. Notably, samples with *B. saltans* in high abundance also had fewer bacterial identifications compared with the rest of the samples or with their respective pair. Although the causal relationship cannot be identified, it deserves further study. This identification highlights the use of metagenomics datasets not only for profiling bacterial and fungal taxa, the most common targets of microbiome studies, but also for discovering the potentially relevant roles that other microbial eukaryotes can have in the microbial community, as previously discussed for the eukaryotic component of the human gut microbiome (Parfrey et al. 2011).

The taxonomic core of OEN-BA contained *Lactobacillus paracollinoides*, isolated from brewery environments, and *Lactobacillus pentosus*, which is the most prevalent LAB in Spanish-style green olive fermentations. Several other LAB were identified among the most abundant bacteria in the samples containing OEP combined with a *B. bruxellensis* strain. For instance, *Lactobacillus collinoides*, previously found in cider, was identified in one of the replicates of each OEP-*B. bruxellensis* combination in higher abundance compared with the respective *B. bruxellensis* strain, OEP, and controls. Similarly, the next bacteria were found among the most abundant taxa: *Lactobacillus crustorum*, isolated from two traditional Belgian wheat sourdoughs; *Lactobacillus herbarum*, a species related to *Lactobacillus plantarum*; *Lactobacillus oeni*, LAB isolated from wine; and *Lactobacillus paucivorans*, isolated from a brewery environment. Additionally, we identified *Lactobacillus nagelii,* isolated from a partially fermented wine, and *Lactobacillus parafarraginis,* a heterofermentative lactobacilli isolated from a compost of distilled shochu residue, among the highly abundant bacteria identified as differentially more abundant in samples with OEP-*B. bruxellensis*, compared with the respective *B. bruxellensis* strain, OEP, and controls. As expected, grape-related fungi were also identified, such as the plant pathogens *Verticillium dahlia* and *Verticillium longisporum,* and *Mucor ambiguus*, present in soil and plants, and the wine common yeast *S. cerevisiae* and *Lachancea kluyveri*.

In regard to AF, *Mucor indicus* was identified in various samples. This fungus has been previously isolated from tempeh, a traditional, fermented, Indonesian food. *M. indicus* is able to produce ethanol in amounts comparable to *S. cerevisiae*. Also, *Candida sorboxylosa*, a yeast common in the winery environment, was identified in OEP-BC_13. Notably, the OEN replicate with lowest sequencing also had the highest percentage of unmapped reads (51%). Mapping of these reads to the nt database identified most reads as derived from *S. cerevisiae* (Supplemental Table 3). The controls are the second and third samples with the highest number of unmapped reads (21% and 15.2%). Mapping these reads to the nt database also identified *S. cerevisiae* as the main source of unassigned reads (Supplemental Table 3). Given that a large diversity of *S. cerevisiae* has been found in wine (Capece et al. 2016), these previously unmapped reads are likely derived from strain-specific sequences from *S. cerevisiae* that are not reported in the whole-genome databases used for the main taxonomic identification.

Concerning identifications of wine functional potential, a previous metagenomics analysis on good- and bad-quality Chinese rice wine samples identified the metabolic pathways of glycolysis, biotin, pyruvate metabolism, and fatty acid elongation as important for making good-quality Chinese rice wine (Hong et al. 2016). Notably, this study also identified such pathways as differentially abundant in the inoculation type comparisons (Supplemental Table 2).

Abundance of the inoculants after MLF. We observed a different abundance of reads derived from *O. oeni* at day 114 postinoculation (Table 1). BA_4 had *O. oeni* at low coverage, while BA_3 does not have it, as expected if *O. oeni* was not inoculated. However, the genome of *O. oeni* is also present at medium and high coverage in the two BB and BC replicates. This finding suggests that *O. oeni* in those replicates derives from the native grape flora or any other contaminant source (e.g., the crusher). Notably, no malic acid was identified on day 114 in the samples not inoculated with our *O. oeni* culture strains (Figure 2), suggesting that *O. oeni* not derived from the inoculant can potentially affect properties of the final wine. This result concurs with previous studies that identified wild *O. oeni*

as the predominant species in wines with spontaneous MLF (López et al. 2007).

A different abundance of reads derived from *B. bruxellensis* was also observed in the inoculations (Table 1). For example, OEP-BA_9 has the genome of *O. oeni* at average coverage compared to the other samples, and *B. bruxellensis* covered at 27.2×, which is close to the median *B. bruxellensis* coverage of all the samples inoculated with *B. bruxellensis* (median = 26.8×). OEP-BA_10 has *O. oeni* in high abundance and is also the sample with the second highest *B. bruxellensis* abundance (66.26×), even higher than the samples inoculated with *B. bruxellensis* alone (average = 17×). DuBois (2017) has observed such different growth capability of the *B. bruxellensis* strains dependent on the presence of *O. oeni*. We suggest that the variable growth capacity depends on certain unidentified conditions derived from microbial community interactions, which has been observed in grape juice where *S. cerevisiae* benefits *L. plantarum* through nitrogen overflow (Ponomarova et al. 2017). Furthermore, regarding samples inoculated with *B. bruxellensis* alone, BB did not seem able to successfully grow in these wines, as all the BB inoculations (alone or with *O. oeni*) showed low *B. bruxellensis* genome coverage (average BB coverage = 4.54×). This finding suggests that some *B. bruxellensis* strains are better at thriving in enological conditions than others, which is consistent with a previous study by Vigentini et al. (2008), where five strains of *B. bruxellensis* differed in their abilities to grow in wine.

These observations of the inoculants' abundance, together with the inability to precisely predict the activity of a spoilage yeast and its effect on the entire microbial community, highlights the importance in winemaking of inoculating with sufficient numbers of strong and viable yeast and bacteria to ensure the presence of the desired microbial community (Gerbaux et al. 2009).

Microbial community identification comparisons. We observed that inoculations with only OEN or OEP were the closest to control samples, and that most of the impact occurred when *O. oeni* was inoculated together with *B. bruxellensis*. These results are similar to the *B. bruxellensis* effect on taxonomic identifications, supporting the suggestion that primary changes in the wine microbial community occur when *B. bruxellensis* is present. Notably, while the number of sample reads moderately influenced the number of identified taxa (Pearson correlation = 0.6832), it did not affect the number of identified genes (Pearson correlation = 0.3667). Moreover, the number of identified taxa moderately correlated with the number of identified genes (Pearson correlation = 0.6854) (Supplemental Figure 3). This suggests that the functional potential space of the wine microbiome is more defined than the taxonomic profile; however, we found functional potential variability such that even controls did not cluster tightly together as in the taxonomic PCAs.

Furthermore, only the control and OEP had a large number of core genes, but the OEP and control samples were functionally the closest types of inoculation, suggesting that the main effect on the functional profiles of the different inoculations is not in the integration of new functions, but in changes in their abundance. Contrasting with the taxonomic profiling, where most of the taxa of the inoculations were in statistically significant higher abundance than the control, at the functional level, most of the differentially abundant KOs were in less abundance compared with the control. Interestingly, when relating KOs to their corresponding pathways, we found that more pathways were affected by KOs in higher abundance than by those in less abundance. This observation suggests that the wine microbiome requires a minimum set of present pathways to survive in the enological conditions, as well as to drive the winemaking process.

This variation in the microbial functional potential has been previously observed in other better studied environments, such as the human gut microbiome. For example, the gut microbiota of unweaned infants is a simple community with high taxonomic and functional potential variation, but the gut microbiota in adults and weaned children is more taxonomically complex, with a higher uniformity of functional potential. Importantly, the genes found in the cores of each sample type are enriched in functions that allow the microbes to adapt to the gut environment (Kurokawa et al. 2007).

O. oeni-B. bruxellensis strain-specific dependent effect. In identifying bacterial species from plasmids, all inoculation types seem to affect the community compared to the controls (Supplemental Figure 2), but the effect on the bacterial community was only for certain inoculation types (Figure 3A). This result suggests that the presence of plasmids is inherently variable, although to a low extent.

It was observed that, in general, the samples inoculated with only *B. bruxellensis* had fewer bacterial identifications (average samples with *B. bruxellensis* = 71, average all samples = 152.4) than the ones inoculated with only *O. oeni* (average samples with *O. oeni* = 135.25) (Supplemental Table 1). The observation that the inoculations of both *O. oeni* strains together with BA had low numbers of differentially less abundant bacteria suggests that the *O. oeni*-BA inoculations impacted the community by allowing more bacterial taxa to grow instead of repressing growth. The large variability within OEP-BA was likely because BA was able to grow either poorly or successfully with OEP. However, this same effect was not observed in OEN-BA, where BA grew more in OEN-BA_16 than in OEN-BA_15, and where the taxonomic core was the largest (Figure 4A). Furthermore, clustering patterns on the PCA of the taxonomic identifications also suggest that the specific combination of *O. oeni-B. bruxellensis* strains affected bacterial composition. The abundance correlation analyses support this observation. Correlations were unaffected when accounting for only one specific combination of *O. oeni-B. bruxellensis*, but they were affected when considering all the possible *O. oeni-B. bruxellensis* combinations. In other words, the effect of OEP-BA was not the same as that of OEP-BB and OEP-BC; similarly, the effect of OEN-BA was not the same as OEN-BB and OEN-BC.

Regarding the inoculants' effect on the functional potential profile, the inoculations with only *O. oeni* were observed to cluster together with the controls and had the lowest total of differentially abundant KOs, while the inoculations with

only *B. bruxellensis* strains had higher numbers of differentially abundant KOs. When looking at the functional potential profiles, it was found that BA and OEP-BA had similar numbers of differentially less abundant KOs, and more KOs in higher abundance. However, OEN-BA did not follow the same pattern of BA and OEP-BA, as it had more than double the amount of less abundant KOs and less KOs in higher abundance (Figure 6A). Furthermore, the inoculations of BB and *O. oeni*-BB follow the same pattern of being among the samples with the highest numbers of differentially less abundant KOs compared with the control, and a lower number of KOs in higher abundance. On the other hand, BC had a similar number of greater and less abundant KOs compared with the control, whereas OEN-BC had the second largest number of KOs present in greater abundance, and OEP-BC had the third largest number of KOs present in less abundance. Moreover, although OEN-BA and OEN-BC had the least number of replicable most abundant genes, OEN-BB had the highest percentage of replicable genes in greatest abundance, suggesting that the OEN-BB functional potential profile was less variable than that of the other inoculation types. These observations suggest that the effect on the functional potential of the system does not depend on only the *O. oeni* strain or only the *B. bruxellensis* strain, but rather on their specific strain combinations.

O. oeni strain-specific effect. OEN had the lowest number of bacteria present in higher abundance compared with the control, whereas OEP was the closest to the control and was among the inoculation types with the least bacteria present in higher abundance. This suggests that inoculating with an *O. oeni* strain alone least affects the wine microbial profile after 114 days of inoculation. This suggestion is supported by the microbial abundance correlation analyses, where the number of correlations affected by an *O. oeni* inoculation is close to the number of correlations identified as affected by removing the control samples.

Regarding the microbial functional potential effect of the *O. oeni* inoculation, we observed that OEP had the smallest number of greater and less abundant KOs compared with the control, and its functional potential diversity clustered together with the controls (Figure 5B and 6A). This suggests that OEP causes the least change in the functional potential of the studied inoculation types. Compared to OEP, OEN has more KOs present in higher abundance than present in less abundance; however, when looking at the pathways those KOs belong to (Figure 6B), only 10 pathways in higher and 10 in less abundance are identified. These observations suggest that the degree of functional impact of those *O. oeni* strains alone is similar. A low number of genes were present in the top 5% abundant genes in both replicates of OEN-BA (Figure 6D), though they had roughly the average number of highly abundant genes, this was likely due to the difference in abundance of *O. oeni*; one of the replicates had *O. oeni* in high abundance, while the other replicate had *O. oeni* in very low abundance and *B. bruxellensis* in moderate abundance.

The effect of B. bruxellensis abundance. Interestingly, although identified in low abundance, the *S. cerevisiae* killer virus M1 was found in OEN-BA (22 mapping reads) and in OEN-BB (24 mapping reads). Also, the *Phytophthora infestans* RNA virus 1 was identified in OEN-BB (36 mapping reads). These viruses could be contributing to reduction of fungal diversity in these OEN-*B. bruxellensis* combinations compared with those combined with OEP; however, this hypothesis would need further experimental validation. As with identifying protozoan *B. saltans*, identifying these viruses highlights a need to characterize all organisms with an underexplored yet potentially important effect on the microbial community. These studies would be particularly relevant for winemaking, as the viral community in wine is still poorly characterized despite potential effects on the MLF (Costantini et al. 2017).

Analysis of the pathways of the differentially abundant KOs showed that the comparisons of *O. oeni*-BC versus BC had the highest number of differentially less abundant pathways (Figure 6C, Supplemental Table 2). Notably, most of those differentially abundant KOs came from OEP-BC_13, which was the sample where *B. bruxellensis* grew the best. Among the less abundant pathways in OEP-BC_13 versus BC was the regulation of mitophagy in yeast. This finding was interesting, as well-growing *B. bruxellensis* strains have been shown to have an impact on the abundance of other non-*Brettanomyces* yeast in wine (Renouf et al. 2006).

Flavor potential. *Taxonomic and functional potential identifications.* Regarding taxonomic identifications related to flavor formation in wine, *Lactobacillus diolivorans* (176 mapping reads) was found only in the OEP-BC sample. This bacterium has the potential to degrade 1,2-propanediol, a compound that is nearly odorless but possesses a faintly sweet taste. Also, *Clavispora lusitaniae* (596 mapping reads) was found only in this sample. This fungus has been found to produce a good balance between concentrations of ethyl acetate (sweet smell) and high alcohols (Mingorance-Cazorla et al. 2003). Furthermore, the taxonomic core of OEN-BA contained *Staphylococcus equorum*, which is frequently isolated from fermented food products and contributes to the formation of aroma compounds during ripening, especially in cheeses and sausages. However, the putative presence of these flavor compounds derived from identified bacteria requires further experimental validation.

Regarding potential functions, 53 genes in the nonredundant gene catalog were annotated with KO K05349 (β-glucosidase) from many different bacteria and non-*Saccharomyces* yeast. One gene was predominant in various samples and was annotated as coming from *O. oeni*, which is interesting because β-glucosidase activity is involved in hydrolysis of several important compounds for developing the wine flavor profile, and microbial β-glucosidases have been used for enhancing wine aroma. Furthermore, glycosidases not encoded by *S. cerevisiae* have been shown to impact the flavor profile in wine (Rosi et al. 1994). However, sensorial evaluation is required to assess the impact of these identified genes on wine flavor.

MLF. To evaluate whether the presence of *B. bruxellensis* affects the microbial functional potential to transform L-malic

acid into L-lactic acid, thus reducing acidity of the wine, we mined the nonredundant gene catalog and found genes annotated as KO K00027 (encompassing the genes, malate dehydrogenase and malolactic enzyme). The genes annotated as K00027 were identified in differential abundance in various comparisons and as coming from various species, including *O. oeni*. As expected, the two gene sequences annotated as K00027 from *O. oeni* were in greatest abundance in all samples inoculated with *O. oeni* with and without *B. bruxellensis.* Thus, the analyzed *B. bruxellensis* strains did not affect the functional potential of the microbiome to transform L-malic acid into L-lactic acid, which most likely occurs through the MLF activity of the evaluated *O. oeni* strains. However, sensorial analyses would be necessary to evaluate whether the presence of these genes correlates with the less acidic flavor expected from MLF.

HCA production. A previous study by Madsen et al. (2017) measured the amount of off-flavor volatile organic compounds using gas chromatography-mass spectrometry in wines inoculated with cinnamoyl esterase positive and negative *O. oeni* strains together with BA and BC. That study found that while the cinnamoyl esterase positive *O. oeni* degraded more of the tartaric ester-bound forms of HCAs into free HCAs, there was no significant difference in the production of volatile phenols compared with wines inoculated with the cinnamoyl esterase negative *O. oeni*. However, on day 180, a significant difference was observed in the level of volatile phenols and HCAs between wines with the two strains of *B. bruxellensis*. Thus, the level of volatile phenols in wine depends more on strain differences of *B. bruxellensis* than on the cinnamoyl esterase activity of *O. oeni*.

Given that cinnamoyl esterase activity can also be present in different fungi and contribute to increased production of HCAs, this study also examined whether the observed higher variability of fungi, in combination with OEP and a well-growing *B. bruxellensis*, could possess this esterase activity and contribute to the increase in HCAs production. However, only two yeast genes were found belonging to *P. stipites* and *P. pastoris* with inconclusive functional annotations. Thus, it cannot be concluded that there is a higher production of HCAs due to the potential activity of other fungi. Further experimental functional characterization should be performed on these identifications to validate whether they confer the cinnamoyl esterase activity.

To evaluate the spoilage potential of other wine community members, non-*Brettanomyces* decarboxylating HCA genes were also examined. A higher abundance of reads mapping to genes annotated as phenolic acid decarboxylase (K13727) was observed in the BA and BC samples, compared to the OEP-BA and OEP-BC inoculations. Also, OEP-BA and OEN-BC were found to have a higher abundance of reads mapping to K13727 than the controls and wines inoculated with only the respective *O. oeni* strains. This observation also supports the suggestion by Madsen et al. (2017) that the presence of genes with putative decarboxylase activity on HCAs is not dependent on the presence of an *O. oeni* strain with or without the esterase activity, but on the *B. bruxellensis* strain. Notably, the ferulic acid decarboxylase 1-like gene (K20039) was also identified in the controls, suggesting that genes with decarboxylase activity on HCAs may not only come from *B. bruxellensis*, but also from the native grape microbiota or from any other contaminating source (e.g., machinery used for making must).

Conclusions

This metagenomic study characterized the effect of inoculating two different strains of *O. oeni* (with and without cinnamoyl esterase activity) and three *B. bruxellensis* strains, alone and in combination, on the microbial community of a Cabernet Sauvignon wine 114 days postinoculation. We found that the *O. oeni*-*B. bruxellensis* combinations affect the taxonomic and functional potential profiles of the wine microbiota depending on (1) the specific *O. oeni* and *B. bruxellensis* strains being combined, and (2) the abundance reached by the inoculants. Regarding the flavor profile in the final wine, we found that the functional potential for decarboxylation of HCAs is not dependent on the *O. oeni* strain, but rather on the *B. bruxellensis* strain and other wine flora, which could derive from the indigenous grape flora or from the machinery used for making must.

This study showcases the usefulness of metagenomic analyses to obtain deeper insight into general microbial profile characteristics and the effect of specific inoculants, not only in the taxonomy, but also in the functional potential of the wine microbiome. However, experimental validation is needed in future studies to obtain details on the mechanisms of traits identified with metagenomics. Also, sensorial analyses of wines are needed to evaluate the flavor profile affected by the microbial functional potential unveiled by metagenomic results.

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