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A.M., M.C.P., I. M.-P. and W.A. contributed to the experimental design, funding of the study and writing of the paper. J.Ll., M.M.-S., and M.M. performed the experimentation and data analysis, and contributed to the writing of the paper.

Genetic and phenotypic diversity of *Brettanomyces bruxellensis* isolates from ageing wines

Running title: Diversity of *B. bruxellensis* from ageing wines

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ABSTRACT

Brettanomyces bruxellensis is the most reported spoilage yeast in aged wines mainly due to the production of phenolic off-flavors. In the present study, 64 *B. bruxellensis* strains isolated from Catalonian ageing wines were genetically and physiologically evaluated. The *B. bruxellensis* strains had high intraspecific diversity and were distributed genetically using the polymerase chain reaction of the intron splice site (ISS-PCR) into 8 clusters, mostly depending on their origin, and into 22 clones using microsatellite analysis. Wine-like conditions resulted in poor growth of several strains but, those growing, increased their tolerance to sulfur dioxide (SO_2) with incubation time. However, tolerance to SO_2 was not related to the genetic clusters as defined using ISS-PCR. Furthermore, some of the strains were resistant and grew in 60 mg/L of total SO_2 (2.67 mg/L molecular SO_2) in wine-like conditions. Additionally, the spoilage potential of the isolated strains was evaluated using precursors of 4-ethylphenol and 4-ethylguaiacol. All the strains were able to produce these compounds above their detection threshold even if the cells were losing culturability with incubation time. Thus, both the resistance to SO_2 and the production of volatile phenols varied within the strains. This complexity must be taken into account to optimize both monitoring and corrective interventions, suggesting the importance of an early detection.

Keywords: *Brettanomyces bruxellensis*; wine spoilage; genetic diversity; SO_2 tolerance; phenol production

1. Introduction

One of the main problems for the wine industry is the biological alteration and spoilage of wine by the activity of certain bacteria and yeast. *B. bruxellensis* (teleomorph *Dekkera bruxellensis*) is considered the most important spoilage microorganism in the wine industry (Loureiro & Malfeito-Ferreira, 2003). This yeast has been detected at low frequency in grapes or must (Renouf & Lonvaud-Funel, 2007) but is typically found during wine ageing in barrels and in bottles due to its tolerance for both high ethanol and low oxygen concentrations (Wedral et al., 2010).

Several studies have shown both high genetic and phenotypic diversity of *B. bruxellensis* in different wine regions. (Agnolucci et al., 2009; Albertin et al., 2014; Avramova et al., 2018; Curtin et al., 2007; Di Toro et al., 2015; Guzzon et al., 2018; Oelofse et al., 2009). Also, the ploidy level, substrate used for isolation and even the geographical origin of the *B. bruxellensis* strains seem to influence the structure of this yeast population (Avramova et al., 2018). Nevertheless, the understanding of the *B. bruxellensis* population structure and the factors that modulate it remains limited. There is little information about the biodiversity of this yeast species in Spain, one of the most important wine producers together with Italy and France.

The spoilage effect of this contaminant in wine is mainly associated with the production of volatile phenols which are related to unpleasant aromas described as barnyard, horse sweat or medicinal (Chatonnet et al., 1992; Oelofse et al., 2009). Therefore, the presence of *B. bruxellensis* in wine often provokes rejection by consumers and consequent economic losses for the wine industry (Wedral et al., 2010). It has been shown that the volatile phenol production by *B. bruxellensis* depends on the concentration of their precursors in the must (mainly *p*-coumaric and ferulic acids) and the specific strain implicated (Crauwels et al., 2017; Di Toro et al., 2015). However, from previous studies it is not clear if the production of volatile phenols is associated with the physiological state, the growth rate of *B. bruxellensis*, or both and often results have been controversial (Agnolucci et al., 2010; Di Toro et al., 2015; Sturm et al., 2015).

The most common method to prevent *B. bruxellensis* spoilage is the addition of sulfur dioxide (SO_2) mainly through the addition of a potassium bisulfite solution to must and wine (Ribéreau-Gayon et al., 2006). Unfortunately, the high phenotypic diversity of *B. bruxellensis* including, e.g., differences regarding its tolerance for SO_2 (Agnolucci et al., 2010; Avramova et al., 2018; Curtin et al., 2012) and the production of volatile phenols (Agnolucci et al., 2009; Conterno et al., 2006; Crauwels et al., 2017; Di Toro et al., 2015) makes it difficult to predict its occurrence, behavior and control. In addition, most of the studies analyzing the ability of *B. bruxellensis* to tolerate SO_2 or to produce volatile phenols have used synthetic laboratory media or concentrations of phenol precursors that are not commonly found in wines, thus the results may be biased. The production of volatile phenols by *B. bruxellensis* strains in synthetic media is different from the production in the wine matrix or with wine-like conditions (Oelofse et al., 2009; Sturm et al., 2015).

The present study aimed to determine the genetic diversity of *B. bruxellensis* strains isolated from different Catalonian wine regions and compare them to the genetic diversity of isolates obtained from around the world. Also, their phenotypic ability to tolerate SO_2 and to produce volatile phenols applying wine-like conditions was investigated.

2. Materials and Methods

2.1. Yeast isolation

Samples for the isolation of *B. bruxellensis* strains were taken from wineries of different wine regions of Catalonia including AOC (controlled designation of origin, originally in French) Priorat, Montsant, Tarragona and Penedès. Samples were red wines ageing in oak barrels that had been inoculated with *S. cerevisiae* strains to do the alcoholic fermentation except in the case of Montsant wines that were fermented spontaneously. As the average time of ageing was 12 months, samples were taken at representative times including the beginning, middle and end of the ageing. The

oenologist from the Tarragona and Montsant wineries indicated that the barrels used for sampling had "Brett" character (barnyard/medicinal aroma). However, wine samples from Priorat and Penedès did not show any aromatic defects according to the enologist of those wineries. The management of the wines during the ageing was similar at the different wineries. The only difference was that wines with "Brett" character did not receive sulfitation during the ageing period. A volume of 100 μL of each sample was plated directly into modified Walerstein laboratory nutrient (WLN) medium (BD Difco, Eysins, Switzerland) with the addition of 100 mg/L of cycloheximide (Sigma Aldrich Co., St. Louis, MO, USA). WLN plates were incubated from 7 to 10 days at 28 °C. Slow-growing colonies were microscopically observed and those with typical *Dekkera/Brettanomyces* cellular morphology (long ovoid shaped cells) were streaked on WLN+cycloheximide plates and incubated again for the same period. The successfully isolated strains were grown in plates of YPD (yeast extract-peptone-dextrose) medium (2% glucose, 2% peptone, 1% yeast extract and 1.7% agar; BD Difco) for subsequently genus and species confirmation. In addition, *B. bruxellensis* strain CECT (Spanish Type Culture Collection; Valencia, Spain) 1009 isolated from Lambic beer, was grown on YPD medium and used for comparison purposes.

2.2. Yeast identification using RFLP (restriction fragment length polymorphism) analysis of 5.8S-ITS (5.8S rDNA-internal transcribed-spacer) and partial sequencing of the ITS1-ITS4 gene region

Isolated yeasts were grown in 4 mL of liquid YPD medium, at 28 °C with agitation of 120 rpm for 48 h. DNA extraction was done as previously described by Querol et al. (1992) with some modifications. Briefly, cells were spun down at 8,000 x g for 5 min (Eppendorf microcentrifuge 5424, Sigma Aldrich), washed with sterile distilled water and resuspended in buffer solution 1 (0.9 M sorbitol, 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 7.5). Then, zymolyase and lyticase

(from *Arthrobacter luteus*) enzymes (10 mg/mL each; Sigma Aldrich) were added for cell wall digestion at 37 °C for 1 h. After centrifugation, the resulting pellet was resuspended in solution 2 (50 mM Tris, 20 mM EDTA, pH 7.4). After that, a solution of 10% sodium dodecyl sulfate (SDS) and 5 M potassium acetate was added and samples were incubated on ice for 5 min. Samples were centrifuged twice (8,000 x g for 5 min) and the supernatant was transferred to a new microfuge tube for DNA precipitation using 1 volume of isopropanol. The resulting DNA was washed with 70% (v/v) absolute ethanol (Sigma Aldrich), air-dried and dissolved in 20 μL of TE solution (10 mM Tris–HCl, 1 mM EDTA, pH 8).

The DNA was used for yeast identification based on the RFLP of the PCR-amplified ITS-5.8S rDNA region as described by Esteve-Zarzoso et al. (1999). Briefly, 1 μL of DNA was used for the PCR amplification of the region between the 18S and the 26S rDNA using the primers ITS1 (5'-TCCGTACGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTATTGATATGC-3'). The amplified fragment was digested in a 20 μL reaction at 37 ºC overnight by the enzymes *Cfo*I, *Hae*III and *Hin*fI (Roche Diagnostics, Basel, Switzerland) using 1 μL of each enzyme with 2 μL of specific buffer for each enzyme, 8 μL of amplified DNA and 10 μL of water. The digestion product was visualized using electrophoresis (MSMINIDUO horizontal electrophoresis system, Sigma Aldrich) on a 3% agarose gel. The RFLP patterns obtained were compared with those of the database included at https://www.yeast-id.org/ following the method described by Esteve-Zarzoso et al. (1999) and grouped within the profiles of *B. bruxellensis* or other known yeasts species. The isolates with a *B. bruxellensis* profile were confirmed by sequencing the DNA of the corresponding amplified ITS1-ITS4 region using PCR (Macrogen Inc., Madrid, Spain). The sequences were compared with those of the type strains included in the GenBank using the BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Identification at the species level was achieved with homologies with type strains >99% for sequence lengths of 418-425 bp.

2.3. Genotyping using intron splice site PCR (ISS-PCR) and microsatellite analysis.

147

148 Identification of *B. bruxellensis* isolates at the strain level was done using a multiplex PCR of the
149 ISS using 1 µL (80-100 ng) of the extracted DNA as a template. For that, 2 primers pairs that
150 mapped to ISS, DbEI1 (5′-CTGGCTTGGTGTAAGT-3′) and La2 (5'-CGTGCAGGTGTTAGTA-
151 3') and El1 (5'-CTGGCTTGGTGTATGT-3') and La2, were used for the reaction as described by
152 Vigentini et al. (2011). The PCR products were separated using the horizontal electrophoresis
153 system on 2% agarose gels containing ethidium bromide (4%) for 8 h at 50 V. The Invitrogen
154 100bp DNA ladder (Fisher Scientific, Madrid, Spain) was included in each agarose gel as a control
155 (Fig. S1) and the images digitalized using a UV transilluminator with a digital camera (MiniBIS,
156 DNR, Neve Yamin, Israel).
157 The resulting DNA patterns were processed using GelJ v.2.0 software (Department of Mathematics
158 and Computer Science of University of La Rioja, Spain). A composite dendrogram was constructed
159 with the unweighted pair group method using arithmetic averages (UPGMA) (Guzzon et al., 2018).
160 The similarity matrix was constructed using Dice's similarity coefficient (Vigentini et al., 2011)
161 allowing a tolerance for band migration of 5.0%. Strains were clustered together when the
162 coefficient of genetic similarity was >90%. To check the reproducibility of the ISS-PCR method,
163 two biological replicates of the collection strain CECT 1009 were tested.
164 Isolate genotyping was done using microsatellite analysis as described by Avramova et al. (2018).
165 To investigate the genetic relationship between the Catalonian isolates and other isolates obtained
166 from around the world, the microsatellite data from the isolates of the present study were compared
167 to the dataset used in Avramova et al. (2018) and a dendrogram tree was constructed using Bruvo's
168 distance and Neighbour Joining clustering using the Poppr package in R (3.1.3 version,
169 https://www.r-project.org) as previously described (Avramova et al., 2018).
170 Both ISS-PCR and microsatellite clustering results were used to create resemblance matrices based
171 on Euclidean distance ('proxy' package in R) and their correlation with the strains' origin was

tested using the Spearman method ('ecodist' package in R). The level of statistical significance considered for the test was $p < 0.05$.

2.4. Resistance to sulfur dioxide in laboratory and wine-like conditions

A total of 19 *B. bruxellensis* strains and the type strain CECT 1009 were used to study their sulfite tolerance and their volatile phenols production. Two different media were used to evaluate the sulfite tolerance in *B. bruxellensis* strains. The first medium tested consisted of a yeast nitrogen base (YNB, BD Difco) (6.70 g/L) and glucose (5 g/L). The second one, the synthetic wine medium (SWM), consisted of YNB (1.70 g/L), tartaric acid (4 g/L), glycerol (5 g/L), sodium acetate (0.134 g/L) and ethanol (10% v/v). Both media were adjusted to pH 3.5 and supplemented with different volume concentrations of $K_2S_2O_5$ solution to obtain the final concentrations of 0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 mg/L of total SO_2. To calculate the total SO_2 it was assumed that $K_2S_2O_5$ contained 50% SO_2. The free SO_2 (fSO_2) concentration was also assessed on days 0, 7 and 20 from non-inoculated controls of both YNB and SWM with a commercial kit (GAB system SL, Barcelona, Spain) based on the Ripper method (Vahl & Converse, 1980). Briefly, the commercial kit is a colorimetric method based on the redox reaction in which SO_2 reacts with iodine. Additionally, the molecular SO_2 (mSO_2) was calculated for the parameters used (pH 3.5, 10% v/v ethanol and 28ºC) for each concentration of fSO_2. Thus, for a concentration of 60 mg/L fSO_2, 2,67 mg/L of mSO_2 was calculated.

For the preparation of the inoculum, one colony of each selected isolate was grown in 20 mL of YPD medium in sterile tubes at 28 °C and 120 rpm for 48 h. Two mL of the grown culture was added to 30 mL of YNB medium and incubated using the previous conditions. For the SWM medium, additional steps were added to adapt the isolates to the ethanol concentration that this medium contains (10% v/v). After incubation in YPD, two consecutive steps in YNB containing 4 and 8% (v/v) ethanol were done before the transfer to SWM. Cells were finally adapted in 100 mL

of SWM at 28 °C and 120 rpm for 96 h before the inoculation to SWM containing different concentrations of SO_2.

Each well of a microtiter plate (96 wells) containing 195 µL YNB or SWM with different concentrations of SO_2 was inoculated with 5 µL of the preculture to obtain a final concentration of $2 \cdot 10^6$ cells/mL (in the microscope, the preculture had an average concentration of $8 \cdot 10^7$ cells/mL) and incubated at 28 °C without agitation. Each isolate was tested in triplicate with the different SO_2 concentrations.

The tolerance to SO_2 was followed by growth of the selected *B. bruxellensis* isolates in the two media (YNB and SWM) containing different mSO_2 concentrations. Growth was monitored using OD measurements (600 nm) with a shaking spectrophotometer SPECTROstar (BMG Labtech Co., Cary, NC, USA) each 48 h during 7 days for YNB medium and each 4-5 days during 30 days for SWM. Before OD measurements, the 96 well plates were shaken for 1 min at 120 rpm using the spectrophotometer to avoid interferences by cells deposits.

2.5. Volatile production of phenols with wine-like conditions

The selected 19 *B. bruxellensis* isolates were also evaluated for volatile phenol production with wine-like conditions. Specifically, the production of 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) from their hydroxycinnamic acids (HCA) precursors (*p*-coumaric and ferulic acids) was analyzed. A concentration of $2 \cdot 10^6$ pre-adapted cells/mL grown in YNB with 8% (v/v) ethanol were inoculated in duplicate in 100 mL of SWM and incubated at 25 ºC in screw-tap tubes without agitation. The SWM contained the HCA precursor *p*-coumaric and trans-ferulic acids (Sigma Aldrich) at a ratio of 8:1 with a final HCA concentration of 10 mg/L. This concentration and proportion simulates the amount of HCA commonly found in red wines (Hixson et al., 2012). The growth and viability of *B. bruxellensis* isolates was followed using OD (600 nm) measurements and CFU (colony forming units) counting on solid YPD plates. For the volatile phenol analysis, 10 mL

of each culture was taken after 7 and 30 days of incubation, centrifuged (8,000 x g for 5 min) and the supernatant frozen at -20 °C for 3 wk until its analysis by the metabolomics facility of the Centre for Omic Sciences (COS, Reus, Spain). Briefly, the volatile phenols were extracted with dichloromethane as the solvent and analyzed using GC-MS (gas chromatography-mass spectrometry) with a chromatographic column CP-Sil 24 CB (30 m x 0.25 mm i.d., 0.25 μm film) (Agilent Technologies Co., Santa Clara, CA, USA). Compounds were detected using electronic impact (EI). Standards of 4-EP and 4-EG were used to prepare the calibration curves using *p*-cresol (Sigma Aldrich) as an internal standard.

3. Results and Discussion

3.1. Yeasts identification and typing of *B. bruxellensis* strains

The negative impact of *B. bruxellensis* on the wine industry due to its spoilage activity has led to numerous studies analyzing both the phenotypic and the genetic diversity of this yeast in different geographical areas (Agnolucci et al., 2009; Curtin et al., 2007; Di Toro et al., 2015). However, little attention has been previously paid to the biodiversity of *B. bruxellensis* strains isolated from Spanish wines. Furthermore, the genetic comparison between different strains from different parts of the world might allow a better understanding of the spoilage potential of the *B. bruxellensis* strains.

During the present study, 172 colonies were selected on plates of WLN+cycloheximide medium inoculated with ageing wine samples from different Catalonian wine regions. From these colonies, 65 were confirmed as *B. bruxellensis* strains both using RFLP analysis of the 5.8S-ITS gene region and partial sequencing of the ITS1-ITS4 gene region. The rest of the yeast colonies had a RFLP pattern that did not correspond to that of *B. bruxellensis*. A high proportion of the *B. bruxellensis* strains came from samples from Tarragona, which had “Brett” character at the time of sampling. However, Montsant samples also had “Brett” character and the number of isolates was even lower

than in the other two regions (Priorat and Penedès).

The typing of the *B. bruxellensis* strains was done using ISS-PCR and resulted in 9 genetic clusters (C1-C9) at the 90% level of similarity (Fig. 1). Cluster C3 included the duplicated fingerprints of the collection strain CECT 1009 resulting from different DNA extractions to show both reproducibility (Fig. S2) and correct clustering of the fingerprints (Fig. 1). The rest of the clusters include the 65 *B. bruxellensis* isolated strains.

Cluster distribution was mostly depending on the wine region from where the strains were isolated ($p=0.001$; $R=0.433$). Cluster C1 included isolates from Penedès, clusters C2 and C6 included isolates from Priorat and clusters C4 and C5 from Tarragona. Other clusters like C7 and C8 included mostly isolates from Priorat and Tarragona, respectively. However, cluster 9 was the largest one and included isolates from diverse regions (Penedès, Montsant and Tarragona). Nevertheless, wine regions closer geographically did not necessarily cluster together (e.g., C6 and C7) although some clusters (such us C1 and C5) included isolates from the same geographical area and had a high level of similarity (Fig. 1 and S3). These results might be potentially explained by the adaptation of *B. bruxellensis* to the environment of each specific wine region and the lack of spreading of this species in the area studied.

Additionally, 40 isolates from the present study were genotyped using microsatellite analysis and compared to the database used in Avramova et al. (2018). That database includes 1488 isolates of *B. bruxellensis* isolated from 29 countries and 9 different substrates. As shown in Fig. 2, most of the 40 *B. bruxellensis* isolates resulted in unique clones using the microsatellite analysis (22 different clones from this study marked as dots within the colored circles). The isolates identified by the same clone shared the same microsatellite pattern. The 22 clones fall within three of the 6 genetic clusters described by Avramova et al. (2018). All isolates within the wine/beer-triploid-SO_2 sensitive group belonged to clusters C1, C6, C5 except one isolate of C3 and C4. Isolates within the wine triploid-SO_2 tolerant group belonged to clusters C3, C4, C5, C7 and C8. The kombucha/wine-diploid-SO_2 sensitive group contained just isolates from cluster C3 (Fig. 2). Some clones were

found in different regions (e.g., clone 5 in Tarragona and Penedès or clone 17 in Tarragona and Montsant) but the great majority of clones were unique and found just in one region (Fig. 1). No clear relation was found between the three genetic groups and the geographical area or the winery of origin (p=0.3; R= 0.195)(Fig. 1).

In cross-national studies, Conterno et al. (2006) found a certain relation between clusters and site of isolation, while Vigentini et al. (2012) and Guzzon et al. (2018) described that strains of distant geographical origin appeared to be closely related at genetic level, indicating a poor correlation of clusters and the geographic location of isolation. Other study analyzing the biodiversity in various winemaking regions of Australia, highlighted the presence of three genotypes found across multiple winemaking regions (Curtin et al., 2007). At a regional level, Di Toro et al. (2015), highlighted that strains isolated from the same area could be grouped into different clusters. Recently, Avramova et al. (2018) found a significant influence of the geographical origin in the structure of the *B. bruxellensis* population for non-wine strains (54.8% of variation explained). However, when looking only for wine strains, this global association with geographic origin is reduced to 5%, indicating the low association of *B. bruxellensis* diversity with geography among the wine yeast clusters at a world scale.

3.2. Resistance to sulfur dioxide in laboratory and wine-like conditions

SO_2 is the most common antimicrobial agent used in wine industry to control *B. bruxellensis*. The molecular SO_2 (mSO_2) concentration recommended to control *Brettanomyces* spp. ranges from 0.5 to 0.8 mg/L (Oelofse et al., 2009) and from 20 to 30 mg/L of fSO_2 (Ribéreau-Gayon et al., 2006). Several studies evaluated the resistance to SO_2, with contradictory results depending on the strains and the media used (Conterno et al., 2006). Some of these studies were able to link the SO_2 resistance phenotype to specific genotypic groups (Curtin et al., 2007, Curtin et al., 2012), but other authors found no relation between SO_2 resistance and genetic groups though the range to maximum

SO_2 tolerance varied from two- to five-fold, probably due to the lack of resolution of the typing method (Conterno et al., 2006). In the present study, the collection strain CECT 1009 and two strains from each of the clusters (19 strains in total) were selected for the SO_2 tolerance test. The strains were evaluated for their ability to grow both on YNB and SWM medium in the presence of different concentrations of SO_2. At day 0, the total and free SO_2 (fSO_2) were coincident for both media at pH 3.5 (Fig. S4) and thus, the mSO_2 concentration was between 0 and 2.67 mg/L. However, the fSO_2 decreased over time for non-inoculated YNB medium though it remained constant over time for non-inoculated SWM (Fig. S4).

At the 3rd day of incubation with laboratory conditions (growth in YNB), all the strains were able to grow without SO_2 and at least with 5 mg/L of fSO_2 or 0.22 mg/L mSO_2 (Fig. 3A). Also, most of the *B. bruxellensis* strains grew with <10 mg/L of fSO_2 (0.3 mg/L mSO_2) on the third day of incubation and 47% of the strains coped with up to 20 mg/L fSO_2 (0.44 mg/L mSO_2) at the 5^{th} day of incubation. The tolerance to SO_2 from all strains increased with further incubation time even if their growth was initially affected at ≥10 mg/L (Fig. 3A). However, the strains of cluster C6 at the 7^{th} day of incubation showing high growth at 30 mg/L of total SO_2 were at ~10 mg/L of fSO_2 and thus, 0.44 mg/L of mSO_2 because it was demonstrated that the concentration of fSO_2 decreased with time in the non-inoculated control of YNB (Fig. S4). Isolates belonging to the same cluster and location usually showed similar SO_2 tolerance at the 5^{th} day, e.g, clusters C1, C2, C4, C5, C6 and C9. Nevertheless, strains of the same cluster but isolated from different location showed differences in their SO_2 tolerance, e.g., isolates from clusters C7 and C8 (Fig. 3A). When considering microsatellite genetic groups, strains from the so-called wine triploid group (Fig. 2, red group, isolates I, II, III, IV, IX, X and XIII) showed higher tolerance to SO_2 than the others from kombucha/wine (XIV) or wine/beer (V, VI, VII, VIII, XVI). Six of 7 wine triploid strains grew at the 7^{th} day at 20-30 mg/L of total SO_2 equivalent to 10 mg/L of fSO2 and 0.44 mg/L mSO_2 (all strains except II), while only 1 strain of 5 wine/beer triploid strains (VI) was able to do so. The collection strain CECT 1009, included in the cluster C3, and the strain XIV showed an average

328 tolerance to 5-10 mg/L total SO_2 (0.22-0.27 mg/L mSO_2) from the beginning to the end of the
329 incubation, being in general more sensible to SO_2 than most of the strains isolated from the wine
330 samples (Fig. 3A). Results in YNB are consistent with Agnolucci et al. (2010) as most of *B.*
331 *bruxellensis* isolates decreased in growth with the same concentration of mSO_2 (0.22 mg/L).
332 Nevertheless, no relation was found between the genetic clustering using ISS-PCR analysis and the
333 resistance to SO_2 consistent with Conterno et al. (2006). However, the 19 strains analyzed is a low
334 number that might limited the significance of this result.
335 In wine-like conditions, the growth of all the *B. bruxellensis* strains was inhibited compared to the
336 growth on YNB, probably due to the ethanol concentration (10% v/v) and low carbon in SWM (Fig.
337 3B). No significant growth was observed up to the 7^{th} day and then, the growth and SO_2 tolerance
338 moderately increased for some strains from the 20^{th} to the 30^{th} day. However, clusters C1, C2, C3
339 (including CECT) and C4, hardly grew on SWM. Different from the YNB medium, the
340 concentration of total and fSO_2 remained constant in SWM across time (Fig. S4). Thus, most of the
341 strains able to grow on SWM were highly tolerant to SO_2 (52.6% of isolates) and were growing
342 after 30 days in, at least, 30 mg/L of fSO_2 equivalent to 1.33 mg/L of mSO_2 (Fig. 3B). Strains VII
343 and VIII from cluster C5 and some strains from clusters C4, C7 and C9 (strains VI, XVI and XVIII)
344 grew even at 30-60 mg/L fSO_2 (1.33-2.67 mg/L mSO_2) being the most resistant isolates. These
345 results suggested an adaptation of the strains to SO_2 with time and it is important for the prevention
346 of their growth by the wine industry. Although the trend in SO_2 tolerance was in some cases similar
347 for isolates from the same cluster (e.g., cluster V), wine-like conditions generally increased the
348 differences in the observed SO_2 tolerance for the tested strains (Fig. 3B).
349 The isolates able to grow on SWM and tolerating higher concentration of SO_2 (strains VI, VII, VIII
350 and XVI) were genetically classified using the microsatellite analysis within the orange or
351 wine/beer-triploid-SO_2 sensitive group according to the study of Avramova et al. (2018). These
352 strains grew easily on SWM without SO_2 while the rest seemed not to be adapted to SWM showing
353 poor growth even in the absence of SO_2 (Fig. 3B). On the other hand, the isolates growing at least at

20 mg/L of fSO_2 in YNB (0.44 mg/L mSO_2) fell within the red or wine-triploid-SO_2 tolerant group. However, as commented before, the growth on YNB was not impaired. The different response of the strains in the different media may be explained by the stress conditions represented by the composition of the SWM medium. Avramova et al. (2018) used YNB medium supplemented with 5% of ethanol, which may be comparable to the YNB used with the laboratory conditions, while the cells were growing less in SWM indicating their incapability to adapt to that medium. These result indicated that, even if the strains had been isolated from wine, differences with SWM composition or multiple stresses could impair their growth. Further study is needed to learn more about the reason for this, but these results highlight the importance of using growing conditions similar to the real environment to have more reliable results.

3.3. Volatile phenols production with wine-like conditions

The accumulation of volatile phenols in wine is considered the most detrimental effect directly attributable to *Brettanomyces*. The production of 4-EP and 4-EG by the isolated strains was evaluated with wine-like conditions (SWM). Previous studies used assay conditions, which were often far from vinification conditions including rich media, no ethanol, elevated pH and/or high concentration of HCA precursors. Each strain was inoculated in SWM containing HCA precursors of 4-EP and 4-EG, respectively. The growth of the *B. bruxellensis* strains was inhibited with wine-like conditions achieving an OD (600 nm) that ranged from 0.13±0.01 to 0.44±0.06 after 30 days of incubation (Fig. 4). Isolates from the same cluster showed similar final growth value (e.g., clusters C4, C5 and C9). Various isolates showed low growth with wine-like conditions as observed in the previous experiment, e.g., clusters C1, C2, C6 and the CECT 1009 included in cluster C3 (Fig. 4). These values were significantly lower than the respective OD values resulting from the SO_2 tolerance experiment with SWM at 0 mg/L SO_2. The presence of the HCA in the medium has been shown to have an inhibitory effect on the growth of *B. bruxellensis* (Harris et al., 2010).

Besides, no agitation and lower temperature were used to simulate wine-like conditions. The lack of agitation reduces the amount of oxygen and inhibits the growth of this yeast increasing the production of phenolic volatile compounds (Curtin et al., 2013).

High proportion of the isolates (52.6%) showed lower CFU on YPD plates than the initial inoculum (10^6 cells/mL) even after 30 days of incubation, indicating that the cultivability of those strains decreased with time during incubation (Fig. 5). The remaining isolates either showed an increase on CFU at the 7^{th} day but then decreased through the 30^{th} day of incubation (clusters C6 and C7 and the isolates VIII and XIII from clusters C5 and C8, respectively) or they kept increasing until the 30^{th} day of incubation (isolates XVII and XVIII from cluster C9).

Regarding the volatile phenol production, all isolated strains, even if they showed limited growth, were able to produce 4-EP and 4-EG from their precursors and the concentration of both phenols increased with incubation time. At the 7^{th} day, final of the exponential phase, the maximum registered 4-EP and 4-EG were 2.1 ± 0.1 and 0.2 ± 0.1 mg/L, respectively, whereas after 30 days of incubation the maximum values were 4.2 ± 0.1 mg/L of 4-EP and 0.5 ± 0.1 mg/L of 4-EG (Fig. 5). The levels largely exceeded the detection threshold of these compounds in wine (230 and 47 μg/L, respectively) (Chatonnet et al., 1992). These concentrations were consistent with the previously reported results using 6 or 11 mg/L of HCA (Curtin et al., 2013; Sturm et al., 2015).

It remains unclear whether metabolic capacity to produce volatile phenols or ability to grow in wine is the key determinant of spoilage potential of *B. bruxellensis*. Previous studies indicated that the production of 4-EP and 4-EG was strain dependent (Agnolucci et al., 2009; Conterno et al., 2006; Guzzon et al., 2018). Other studies evidenced a relationship between the physiological state of *B. bruxellensis* and its ability to produce ethylphenols, being culturable populations able to synthesize large quantities (Sturm et al., 2015). Additionally, Capozzi et al. (2016) reported conclusive evidence for the assessment of viable-but-not-culturable state as a strain dependent character.

The strains with higher OD (600nm) at the final sampling point (30 days) were not the ones with higher phenol production. For example, strains IV, X and XIII had low final growth (OD ranging

406 from 0.190 to 0.203) but produced similar concentrations of phenols than others strains that showed
407 higher growth (OD ranging from 0.263-0.443) (Fig. 4). Additionally, strains with low growth like
408 IV, X and XIII produced higher phenol concentrations than the strain CECT 1009 from cluster C3
409 (Fig. 6) with similar final growth (Fig. 4). The *B. bruxelllensis* isolates that produced the lowest
410 phenol compounds were strains from cluster C1 (1.10 ± 0.01 mg/L of 4-EP and 0.17 ± 0.04 of 4-
411 EG) that also showed the lower final growth (OD 0.134 ± 0.004) and the collection strain in cluster
412 C3 (0.91 ± 0.01 mg/L of 4-EP and 0.15 ± 0.05 of 4-EG). Furthermore, strains with higher numbers
413 of CFU/mL were not necessarily those with the higher amount of volatile phenols. With the
414 exception of the strains from cluster C9, the number of CFU of the rest of the strains decreased
415 from the 7^{th} to the 30^{th} day but increased both their 4-EP and 4-EG production (Fig. 6).
416 Altogether, these results suggested that with wine-like conditions most of the strains lose
417 culturability but phenols are still increasing over time. Thus, an early detection of *B. bruxellensis* in
418 wines is recommended to avoid high populations because even if its population decreases, e.g., by
419 SO_2 addition, it could be able to spoil the wine. Therefore, molecular techniques such as qPCR or
420 RNA-FISH with specific primers and probes for *B. bruxellensis* would be useful for their
421 sensitiveness and quick results.
422

423 **4. Conclusions**

424 High intraspecific diversity of *B. bruxellensis* strains in Catalonia with wide representation within
425 the global phylogenetic tree of isolates from around the world was observed. Most of the isolates
426 clustered separately according to the wine region of isolation and were represented by unique
427 clones as shown using ISS-PCR and microsatellite analysis, respectively. The tolerance of those
428 strains to SO_2 was similar when it was evaluated with laboratory conditions with the most tolerant
429 isolates falling within the recognized as resistant to SO_2 genetic group (Avramova et al., 2018).
430 However, with wine-like conditions, strains showed limited growth and were more sensitive to the
431 presence of SO_2. The response to different SO_2 concentrations was diverse depending on the strain.

Additionally, the production of phenols was variable for the different strains and increased with incubation time even if the growth was limited or cell concentration decreased. This complexity makes it difficult to monitor and prevent the spoilage by *B. bruxellensis* strains.

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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521

522 **Figure captions**

523 **Figure 1:** Dendrogram of multiplex ISS-PCR profiles from *B. bruxellensis* strains isolated from
524 Catalonian wine regions. The different color of the isolates represents each of the 6 phylogenetic
525 groups classified in Avramova et al. (2018). Catalonian clones are included within the red (wine-
526 triploid-SO_2 resistant), the orange (wine/beer-triploid-SO_2 sensitive) and the green
527 (kombucha/wine-diploid-SO_2 sensitive) groups.

528 **Figure 2:** Phylogenetic tree based on microsatellite analysis of the 1488 *B. bruxellensis* isolates of
529 the study of Avramova et al. (2018) showing the position of the 22 different clones from this study
530 marked as dots within the colored circles.

531 **Figure 3:** Heat maps representing the growth of the selected strains as OD (600 nm) measurements
532 with increasing concentration of SO_2 (0-60 mg/L) in laboratory conditions (A) and in SWM (B).

533 **Figure 4:** Heat map representing the growth of the selected strains as OD (600 nm) measurements
534 in wine-like conditions with *p*-coumaric and ferulic acids as precursors of 4-EP and 4-EG,
535 respectively.

536 **Figure 5:** CFU counts of selected strains on YPD plates after 7 and 30 days of incubation with
537 wine-like conditions with SWM and *p*-coumaric and ferulic acids as precursors of 4-EP and 4-EG,
538 respectively.

539 **Figure 6:** Production of 4-EP (A) and 4-EG (B) by the selected strains after 7 and 30 days of
540 incubation with wine-like conditions with SWM and *p*-coumaric and ferulic acids.

541 **Figure S1:** Agarose gels of *B. bruxellensis* ISS-PCR patterns. "M" indicates the DNA ladder used
542 as reference for DNA bands size. "–C" indicates the negative control for ISS-PCR.

543 **Figure S2:** Agarose gel of the ISS-PCR pattern of triplicate DNA extraction of one of the isolated
544 strains and the reference strain CECT 1009.

545 **Figure S3:** Map of the regions in Catalonia with the regions sampled during the study marked
546 within a square.

547 **Figure S4**: Concentration over time of total, free molecular SO_2 (fSO2) and molecular SO_2 (mSO_2)

548 calculated for pH 3.5, 10% ethanol v/v and 28 ºC in YNB (A) and SWM (B) medium.

Figure 1

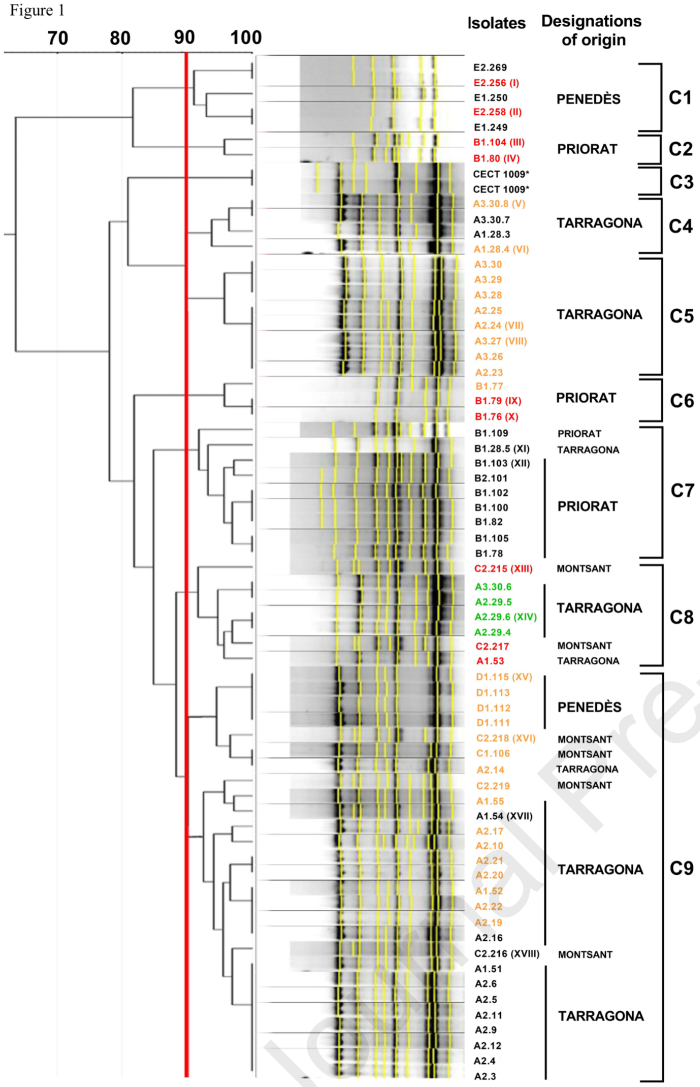


Figure 2

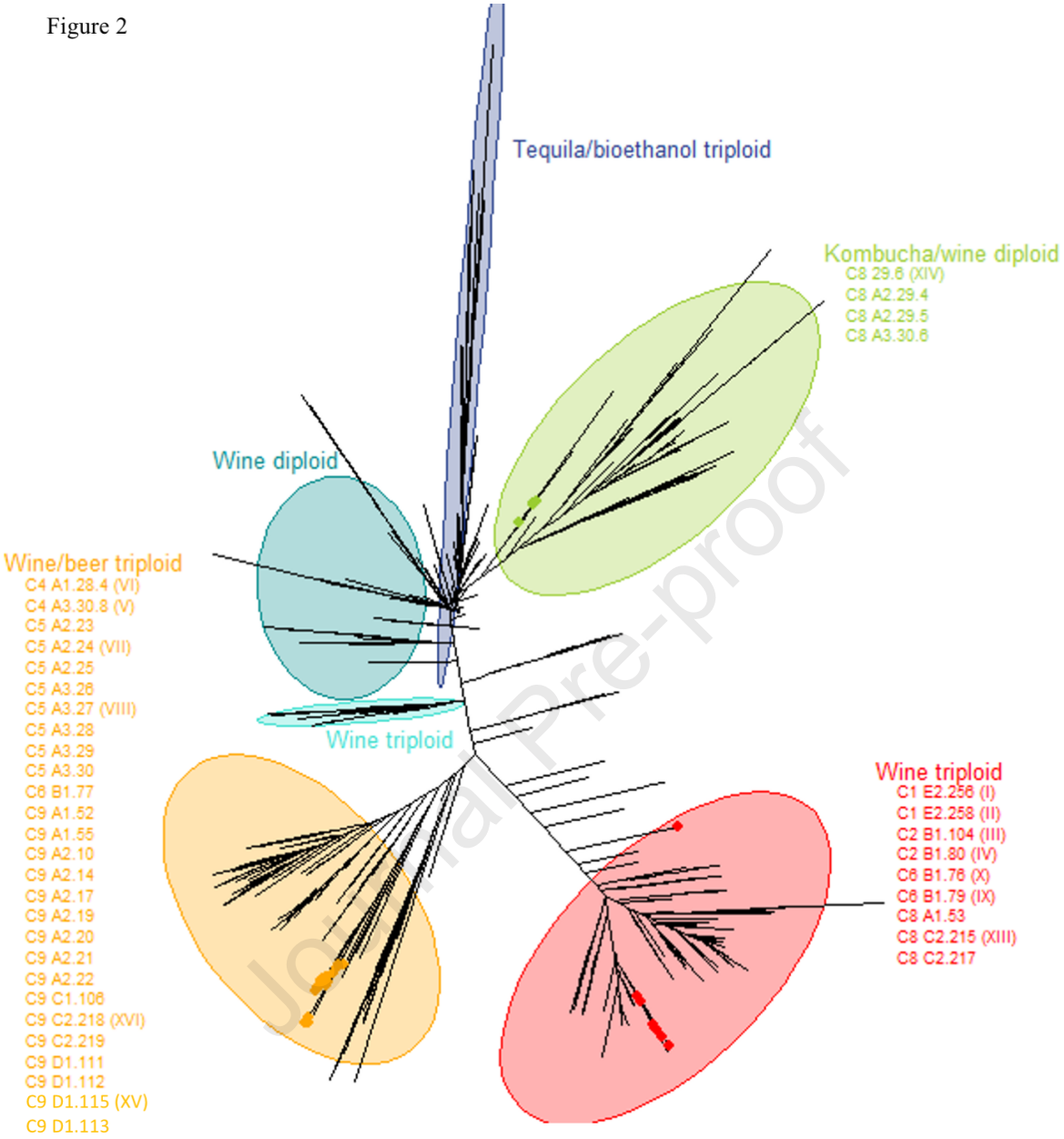


Figure 3

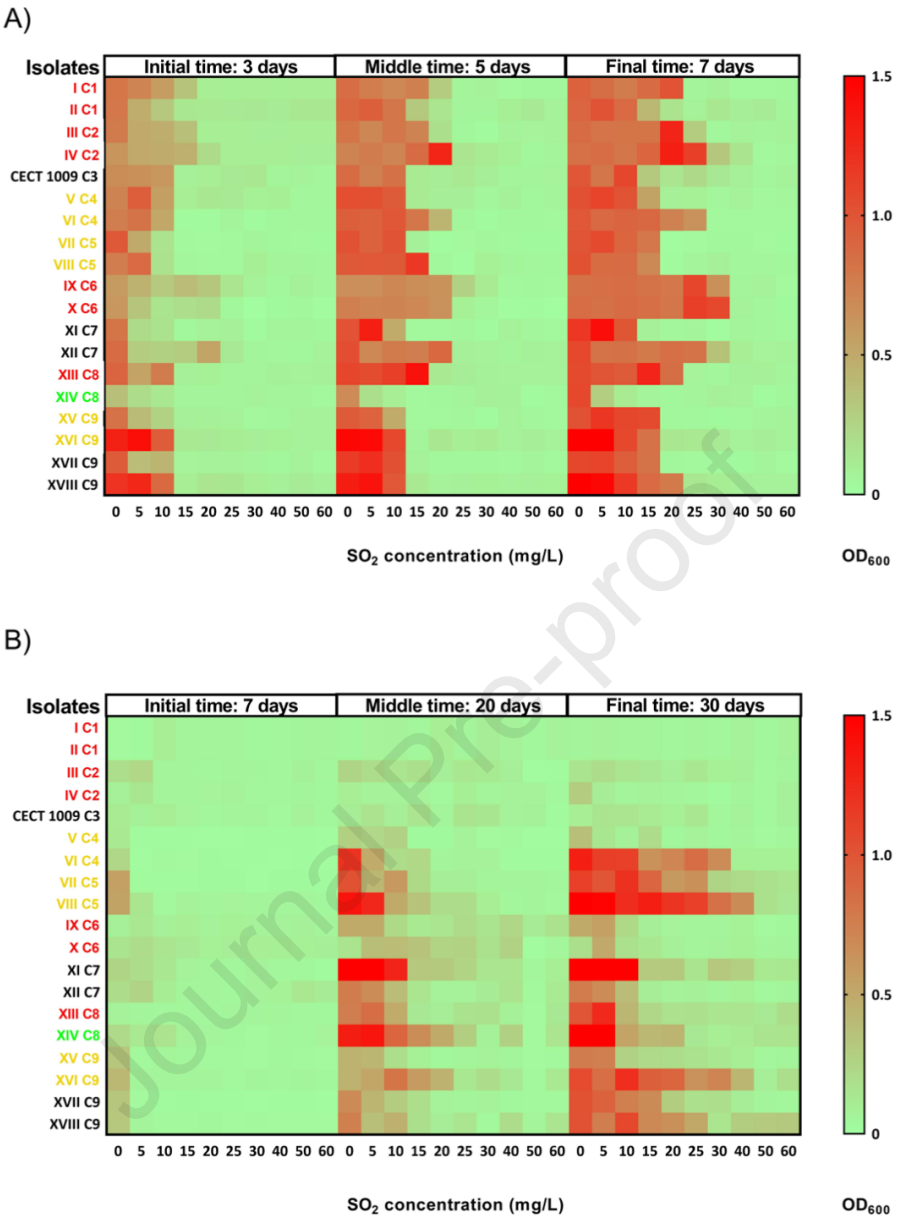


Figure 4

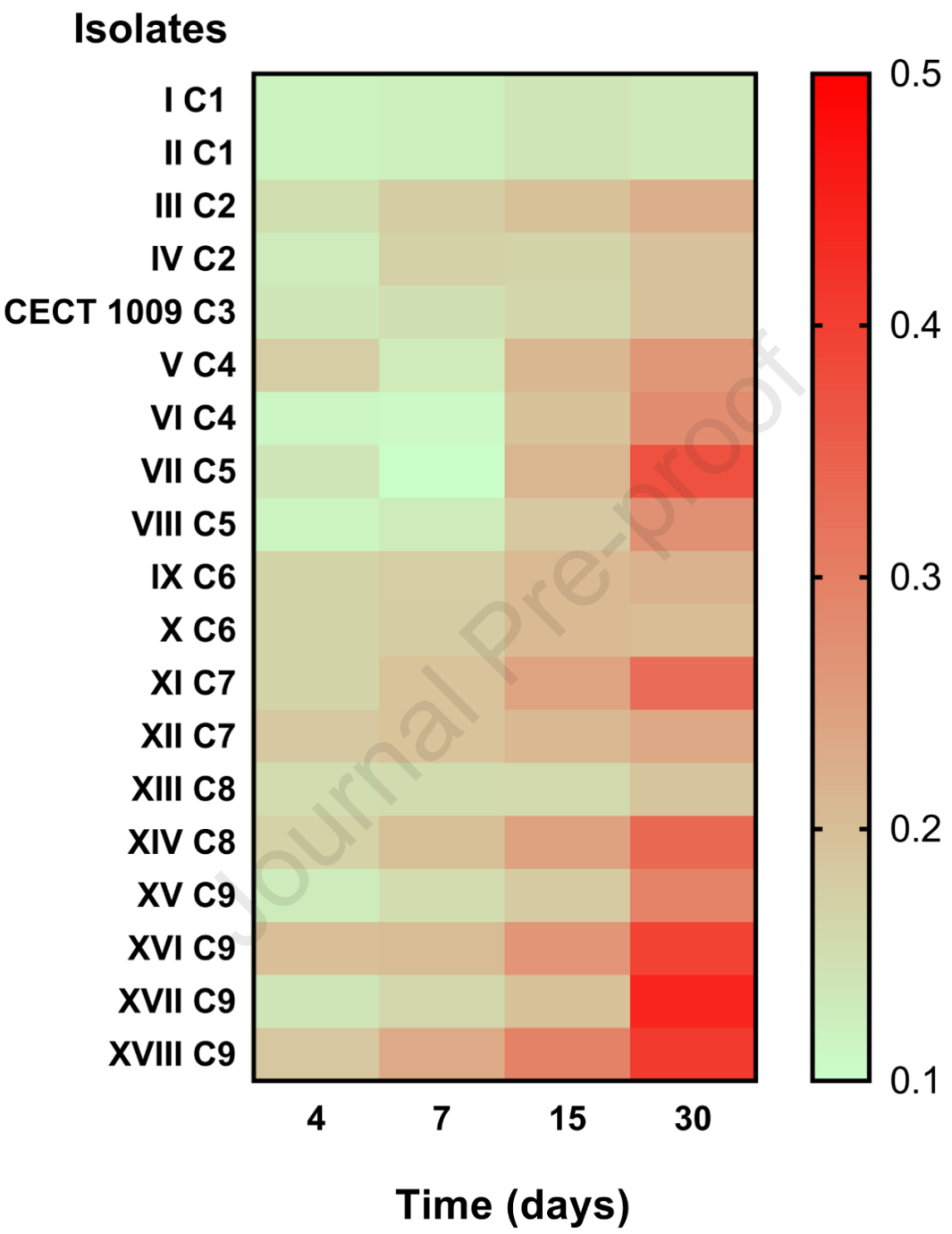


Figure 5

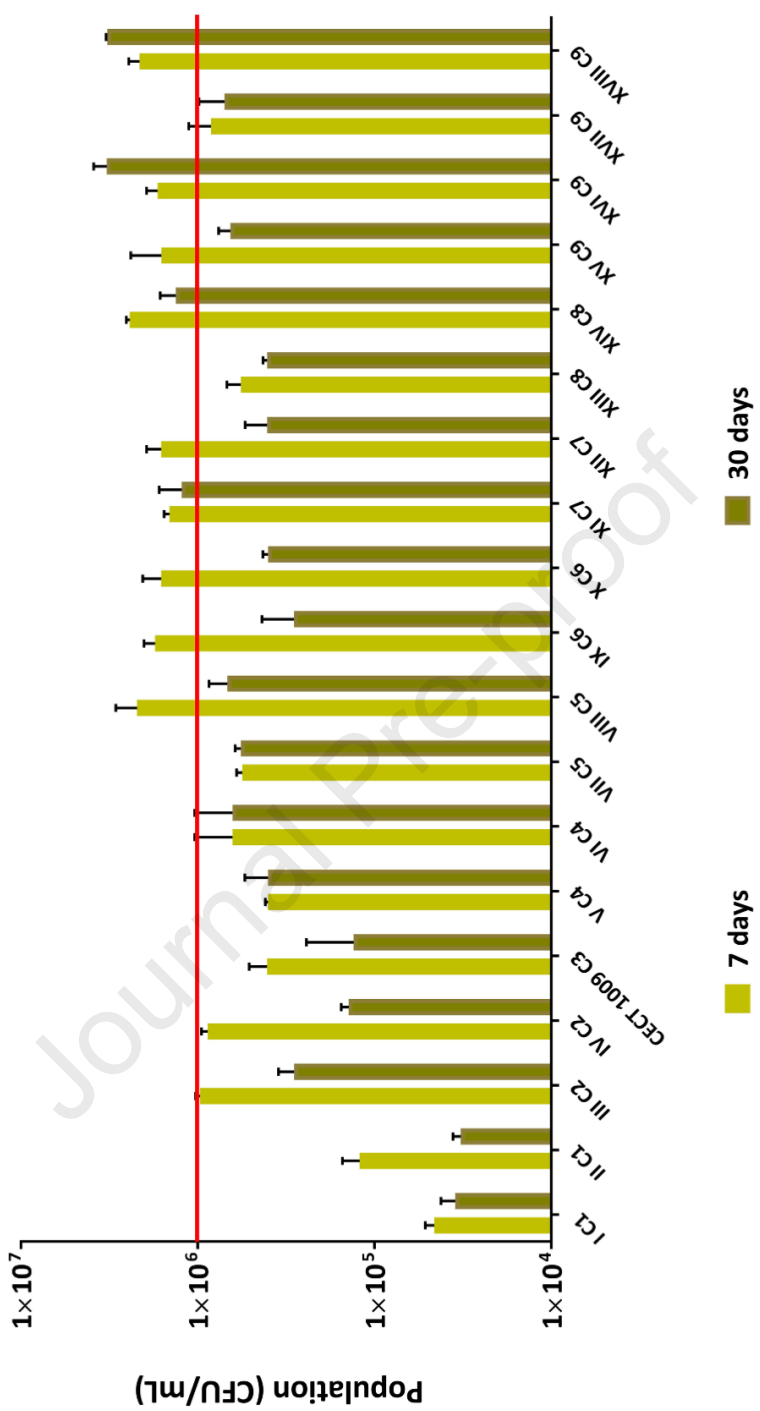
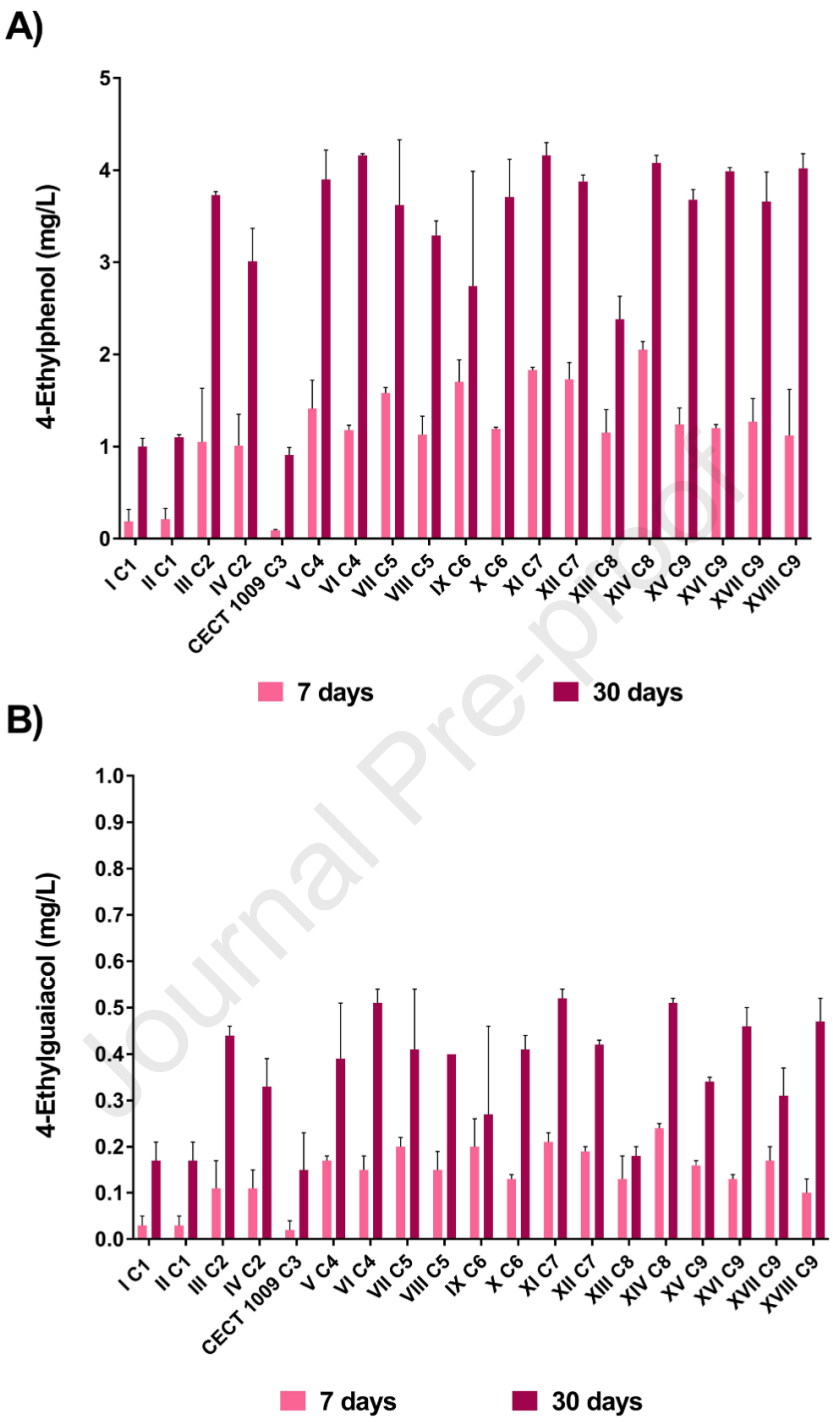


Figure 6



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: