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Viable But Not Culturable (VBNC) state of *Brettanomyces bruxellensis* in wine: New insights on molecular basis of VBNC behaviour using a transcriptomic approach

Vittorio Capozzi [a], Maria Rosaria Di Toro [a], Francesco Grieco [b], Vania Michelotti [c], Mohammad Salma [d], Antonella Lamontanara [c], Pasquale Russo [a], Luigi Orrù [c], Hervé Alexandre [d], Giuseppe Spano [a, *]

[a] Department of Agricultural, Food and Environmental Sciences, University of Foggia, via Napoli 25, 71122 Foggia, Italy
[b] Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle Ricerche, Unità Operativa di Supporto di Lecce, Lecce, Italy
[c] Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Centro di ricerca per la genomica vegetale, 29017, Fiorenzuola d'Arda, Italy
[d] UMR PAM Université de Bourgogne-AgroSup Dijon Laboratoire VALMIS Institut Universitaire de la Vigne et du Vin Jules Guyot, Université de Bourgogne, 21078 Dijon cedex, France

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ABSTRACT

The spoilage potential of *Brettanomyces bruxellensis* in wine is strongly connected with the aptitude of this yeast to enter in a Viable But Non Culturable (VBNC) state when exposed to the harsh wine conditions. In this work, we characterized the VBNC behaviour of seven strains of *B. bruxellensis* representing a regional intraspecific biodiversity, reporting conclusive evidence for the assessment of VBNC as a strain-dependent character. The VBNC behaviour was monitored by fluorescein diacetate staining/flow cytometry for eleven days after addition of 0.4, 0.6, 0.8, 1 and 1.2 mg/L of molecular SO_2 (entrance in the VBNC state) and after SO_2 removal (exit from the VBNC state). Furthermore, one representative strain was selected and RNA-seq analysis performed after exposure to 1.2 mg/L SO_2 and during the recovery phase. 30 and 1634 genes were identified as differentially expressed following VBNC entrance and 'resuscitation', respectively. The results reported strongly suggested that the entrance in the SO_2-induced VBNC state in *B. bruxellensis* is associated with both, sulfite toxicity and oxidative stress response, confirming the crucial role of genes/proteins involved in redox cell homeostasis. Among the genes induced during recovery, the expression of genes involved in carbohydrate metabolism and encoding heat shock proteins, as well as enriched categories including amino acid transport and transporter activity was observed. The evidences of a general repression of genes involved in DNA replication suggest the occurrence of a true resuscitation of cell rather than a simple regrowth.

1. Introduction

Brettanomyces bruxellensis is considered the main yeast spoilage in red wines (Oelofse et al., 2009). It is a serious problem for wine industry because it is able to convert, by enzymatic transformation, the hydroxycinnamic acids into volatile phenols, conferring the final product off-odours, described as 'spicy', 'barnyard', 'animal', 'horse sweat' and 'medicinal'. This metabolic effect leads to an unacceptable oenological productions, entailing huge economic losses computable into hundreds of thousands of dollars (Oelofse et al., 2009; Childs et al., 2015). In the oenological environment, *B. bruxellensis* could be detected in vineyards, soil, grape must, fermentation tanks and barrels (Rodrigues et al., 2001). According to Rodrigues et al. (2001), it is mainly found in oak barrels, where the wine is placed for aging with the aim to improve its sensory properties. The critical characteristic of oak barrels is their porosity and *Brettanomyces* is able to settle in the pores of the wood and survive even after washing (González-Arenzana et al., 2016). Following this way, *Brettanomyces* can also move from one wine to

* Corresponding author.
E-mail address: giuseppe.spano@unifg.it (G. Spano).

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another during the aging process (Albertin et al., 2014; Garde-Cerdán et al., 2008).

Although a large number of culture-dependent techniques are available to assess the presence of this undesired yeast during the vinification processes, in several cases *Brettanomyces* is undetectable, while the corresponding final products are affected by phenolic odors caused by the presence of the above yeast species (Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012). This phenomenon has been explained studying the aptitude of *Brettanomyces* cells to enter in a Viable But Not Culturable (VBNC) state, a physiological condition characterized by a cells inability to divide on a specific media for *B. bruxellensis* medium, even if they are still alive and maintain the metabolic activities and cell functions (Agnolucci et al., 2010; Divol and Lonvaud-Funel, 2005; Du Toit et al., 2005).

Although the entrance in the VBNC state is a serious problem in order to manage microbial stability and safety in fermented food (Millet and Lonvaud-Funel, 2000; Li et al., 2014), few studies have investigated the VBNC behaviour in yeasts (Du Toit et al., 2005; Agnolucci et al., 2010; Serpaggi et al., 2012; Zuehlke and Edwards, 2013; Salma et al., 2013). Referring to the winery environment, several studies have shown that sulphur dioxide, an antimicrobial agent used in food preservation, induces the VBNC state in *B. bruxellensis* (Du Toit et al., 2005; Agnolucci et al., 2010; Serpaggi et al., 2012; Zuehlke and Edwards, 2013; Agnolucci et al., 2013). Serpaggi et al. (2012) demonstrated that the strains may 'resuscitate', returning to a culturable state, by increasing the pH in order to remove the SO_2 from the medium (Serpaggi et al., 2012). Effectively, in microbes, the resuscitation process can often start by simply removing the stress that initially induced the VBNC response (Du Toit et al., 2005; Oliver, 2010; Li et al., 2014). Concerning intraspecific variability, Zuehlke and Edwards (2013), aiming to assess the good combination of molecular SO_2 and temperature to limit the spoilage by *Brettanomyces*, described a certain diversity in culturability among three *B. bruxellensis* strains using fluorescence microscopy. Recently, Agnolucci et al. (2013), analysed by vital staining with tripan-blue seven strains representative of 85 isolates from Sangiovese grapes in different Tuscan wineries of the Chianti area. The results reported indicated that concentrations up to 1 mg/L of molecular sulphur dioxide induced the non-culturable state to a different extent after 24 h of exposure (Agnolucci et al., 2013).

With concern of molecular mechanisms involved, it is important to underline that, when compared to the corresponding model organism *Saccharomyces cerevisiae*, laboratory method for sporulation and transformation are poorly developed (Curtin and Pretorius, 2014), leading to little insights in the biological understanding of the major feature of *B. bruxellensis* during fermentations. On the other hand, recent advances in complete genome sequencing provides gene catalogue data useful to perform transcriptomic and comparative genomic analysis (Curtin and Pretorius, 2014).

In this work, we aimed to verify if the SO_2-induced VBNC state in *Brettanomyces* cells could be a strain-dependent character. To test this hypothesis, we measured the ability of seven strains representing a regional intraspecific biodiversity (Di Toro et al., 2015) to enter in the VBNC state (after sulphite stress) and in the subsequent 'resuscitation' (after stressor removal). The VBNC behaviour was monitored by fluorescein diacetate staining/flow cytometry for eleven days after addition of 0.4, 0.6, 0.8, 1 and 1.2 mg/L of molecular SO_2 and after molecular SO_2 removal. In addition, a strain selected in reason of resistance to SO_2 stressor, was analysed using transcriptomic approach with the aim to identify genes involved in VBNC phenomena.

2. Materials and methods

2.1. Yeast strains

Seven strains (Unifg 8 = strain A; Unifg 14 = strain B; Unifg 39 = strain C; Unifg 47 = strain D; Unifg 29 = strain E; Unifg 27 = strain F; Unifg 46 = strain G) representative of intraspecific biodiversity of *Brettanomyces bruxellensis* isolated and previously characterized from Apulian wines were analysed (Di Toro et al., 2015).

2.2. Analysis of the VBNC state

B. bruxellensis strains were grown on YPD agar at 28 °C for 5 days. VBNC studies were performed in a synthetic wine medium (10% v/v ethanol, 3 g/L D-L malic acid, 0.01% acetic acid, 0.1 g/L potassium sulphate, 0.025 g/L magnesium sulphate, 1 g/L yeast extract, 1.5 g/L glucose, 1.5 g/L fructose) as described by Serpaggi et al. (2012). The pH was adjusted at pH 3.5 and the medium was sterilized by filtration through a 0.2 μm filter (Millipore, France). A YPD-grown colony was used to inoculate 10 mL of this synthetic wine, which was then incubated at 28 °C for 3 days. The biomass produced was used to inoculate one liter of synthetic wine, which was incubated until a cell density of about 10^7 CFU/mL was reached. Five aliquots consisting of hundred millilitres of this last culture were separately added with incremental amounts of 67.4% (w/v) sodium metabisulphite to respectively give a final concentration of 15.2, 23, 30, 40 and 46 mg/L of free SO_2. The above addition respectively corresponded to a final concentration of 0.4, 0.6, 0.8, 1 and 1.2 mg/L of molecular SO_2. The culture containing the middle concentration of molecular SO_2 (0.8 mg/L) was chosen as model to study the 'resuscitation' phenomenon. The pH increment, obtained by addition of NaOH until the pH of the medium reached 4.0, was sufficient to decrease the concentration of molecular sulphur dioxide to almost 0 mg/L (Usseglio-Tomasset, 1995). Cells were returned to a culturable state after the removal of sulphur dioxide from the medium. All the trials were performed in independent duplicate biological experiments.

2.3. Culturability and viability assays

In order to determinate the total viable and culturable populations, aliquots of *B. bruxellensis* cultures were removed at various times after incubation at 28 °C in synthetic wine medium. Cell culturability was assessed by a plate assay using YPD agar. The percentage of cells that were culturable was expressed in respect to total cell count. Cell viability was determined by staining with fluorescein diacetate (FDA). Cells were stained for 15 min with FDA, at a final concentration of 15 mM, in 0.5 M sodium phosphate buffer at pH 7. The suspension of FDA-stained cells was analysed by flow cytometry with the EasyCyte Plus SSC4C cytometer (Guava Technologies Inc., USA). Green fluorescence was read using the PMT3 detector with a 525 nm ± 30 nm bandpass filter. Measurements were processed with GuavaCytosoft software and the results expressed as the number of viable yeast per millilitre of the original sample.

2.4. Library preparation and sequencing

Transcriptomic analysis was performed on the strain Unifg 14 = strain B. RNA samples were collected from three biological replicates for each condition and used for total RNA extraction using the Mammalian RNA Extraction Kit (Sigma Aldrich, Italy). The RNA integrity was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Libraries were prepared from

1 μg of total RNA as template, using the Illumina Truseq RNA library prep kit according to the manufacturer's instructions. Libraries quality was evaluated with the Agilent 2100 Bioanalyzer. Library concentration was determined by Real Time PCR. Sequencing was performed using an Illumina GA IIx platform generating 75 bp single end reads. Fastq file generation was performed by CASAVA v1.8.2.

2.5. RNA-seq analysis

Raw reads generated from sequencing were cleaned using PRINSEQ v0.20.4 software (Schmieder and Edwards, 2011) in order to remove low quality reads. The quality-filtered reads were mapped to the *Brettanomyces bruxellensis* AWRI1499 reference genome and counted using the CLC Genomics Workbench software. The RPKM (Reads per Kilobase per Million) gene expression values were calculated using the CLC Genomics Workbench software. Differential expressed genes (DEG) were determined using the R package DESeq (Anders and Huber, 2010). Gene Ontology (GO) terms were retrieved using the functionality of InterPro annotations in Blast2GO software (Conesa et al., 2005). Gene Ontology enrichment analysis was performed using the GOseq package (Young et al., 2010).

The project was submitted to NCBI BioProject with BioProject ID: PRJNA318157. The Illumina sequencing data were deposited in NCBI SRA database with the accession numbers SRX1692984, SRX1692985, SRX1693001, SRX1701770, SRX1701771, SRX1701772, SRX1701773, SRX1701778, SRX1701806.

3. Results

3.1. SO_2-induced VBNC behaviours in Brettanomyces bruxellensis

In order to investigate the correlation between *B. bruxellensis* strain diversity and its VBNC state in wine, we analysed the VBNC behaviours of seven strains representative of intraspecific biodiversity of *B. bruxellensis* isolated from Apulian (Southern Italy) wines (Di Toro et al., 2015). To trigger the VBNC physiological state in *B. bruxellensis*, SO_2 was used as 'inducing' factor under wine-like stress conditions (Divol and Lonvaud-Funel, 2005; Du Toit et al., 2005; Agnolucci et al., 2010; Serpaggi et al., 2012). Total and viable cells were observed by flow cytometry and viable and culturable cells were monitored by colonies counting on YPD-agar medium. All seven strains analysed by flow cytometry remained viable for the entire experiment and no statistically significant difference among the strains were detected (data not shown). Indeed, the losses in culturability recorded in the different tests provided a measure of VBNC phenomena (Figs. 1–2).

When cultured in a wine-like medium in absence of SO_2, a slight decrease in culturability is observed and all the strains showed a slight entrance in VBNC state, probably due to ethanol content and starvation condition (Figs. 1–2; blue line). However, it appeared clear the role of SO_2 as 'VBNC inducer'. In fact, the increase of SO_2 concentration in the medium caused the falling of the number of non-culturable cells in a strain-dependent manner (Figs. 1–2). About the 'resuscitation' character of these strains, they were differently able to exit from the VBNC state. The ability of cells to exit from this state was investigated choosing as model the 0.8 mg/L concentration of molecular SO_2, which was reduced to 0 by increasing the pH from 3.5 to 4.0 (Salma et al., 2013; Serpaggi et al., 2012). One day after removal of the stress, all the strains showed a concentration of culturable cells ranging from 10^3 to 10^4 CFU/mL, which was lower than that observed before the entry in the VBNC state. We found that the strain A was culturable at a concentration of molecular SO_2 equal to 0.4 mg/L until 10 days (Fig. 1). At 0.6 mg/L of molecular concentration of SO_2, culturability was lowered to $2.60 \cdot 10^2$ CFU/mL after only 2 days and decreased at eighth day, until reached a concentration of 10 CFU/mL. Complete loss of culturability was observed after three days at either 0.8 or 1.2 mg/L SO_2 concentrations. However, the removal of SO_2 allowed an immediate resuscitation, with a recover of culturability that reached up to $3 \cdot 10^2$ CFU/mL (Fig. 1). Strains B and C remained culturable at a concentration of molecular SO_2 equal to 0.4 mg/L and 0.6 mg/L until 10 days. After stress was removed, there is a recovery phase in which the strain B reached a high level of culturability ($5.97 \cdot 10^5$ CFU/mL) (Fig. 2). Strain C immediately lost the culturability at a concentration of 1.2 mg/L of molecular SO_2, already after two days, after three days at 1 mg/L of molecular SO_2 and after 10 days at a concentration of 0.8 mg/L, then it returned culturable after the SO_2 (0.8 mg/L concentration of molecular SO_2) was removed (Fig. 1). In contrast, strain D was cultivable only in presence of 0.4 mg/L of SO_2 ($2 \cdot 10^4$ CFU/mL). Concentrations of molecular SO_2 equal to 0.6, 0.8, 1 and 1.2 mg/L, made the strain non-culturable after only two days. However, even in this case, removing the stress allowed the strain to return in a culturable state ($7.51 \cdot 10^4$ CFU/mL) (Fig. 1). The strains E and F were culturable, until 10 days, in presence of molecular SO_2 equal to 0.4 or 0.6 mg/L. Culturability was already lost after three days at a concentration of 0.8 mg/L, 1 mg/L and 1.2 mg/L of SO_2. A resumption of culturability up to a value of $1.02 \cdot 10^4$ CFU/mL and $1.10 \cdot 10^4$ for strains E and F, respectively, was observed after the SO_2 stress (0.8 mg/L concentration of molecular SO_2) was removed (Fig. 1). Finally, strain G remained culturable in presence of molecular SO_2 equal to 0.4 mg/L and 0.6 mg/L up to $2.65 \cdot 10^2$ CFU/mL and $2.87 \cdot 10^1$ CFU/mL respectively after 10 days and it lost culturability after 9 days at the concentration of 0.8 mg/L and after 8 days at the concentration of molecular SO_2 equal to 1 mg/L and 1.2 mg/L. The strain returned in a culturable form (up to $1.30 \cdot 10^4$ CFU/mL) after the stressing condition (0.8 mg/L concentration of molecular SO_2) was eliminated (Fig. 1).

3.2. Transcriptome profile of B. bruxellensis strain B after exposure to 1.2 mg/L SO_2 and after SO_2 removal

The RNA-seq approach was used in order to investigate the global transcriptional change in *B. bruxellensis* strain B following exposure to 1.2 mg/L SO_2 (after 20 min) and during the first recovery phase (twenty minutes after SO_2 removal). Transcriptome analysis was performed in cells grown without treatment (C), cell exposure to 1.2 mg/L SO_2 treatment (E) and cells recovered after the removal of sulphur dioxide from the medium (R). Three mRNA samples were sequenced for each condition. The reads obtained from the sequencing were cleaned, mapped on the reference genome and counted using the CLC software. A total of 4956 genes were found expressed in the control (C), 4961 genes in the treatment (E) and 4960 genes in the recovery phase (R). Genes were considered expressed if they showed an expression level greater than 0.1 RPKM (Reads per Kilobase per Million). Differentially expressed genes (DEG) were identified using the R package DESeq. Genes were considered differentially expressed if they possessed an absolute value of $\log_2$-fold change ≥ 1 and an adjusted P-value $\leq$ 0.01. The analysis with DESeq generated two set of DEG corresponding to two comparisons: 1) cells growth without treatment vs cells in VBNC state (C vs E) and 2) cells in VBNC state vs cells in the recovery phase (E vs R). The number of differential expressed genes found in the two comparisons is reported in Table 1 and Table S1. As shown, we found 30 genes differentially expressed following VBNC state induced condition (C vs E) while 1634 genes were found differentially expressed in the E vs R comparisons (Table 1 and Table S1). The GO functional categories enrichment analysis were

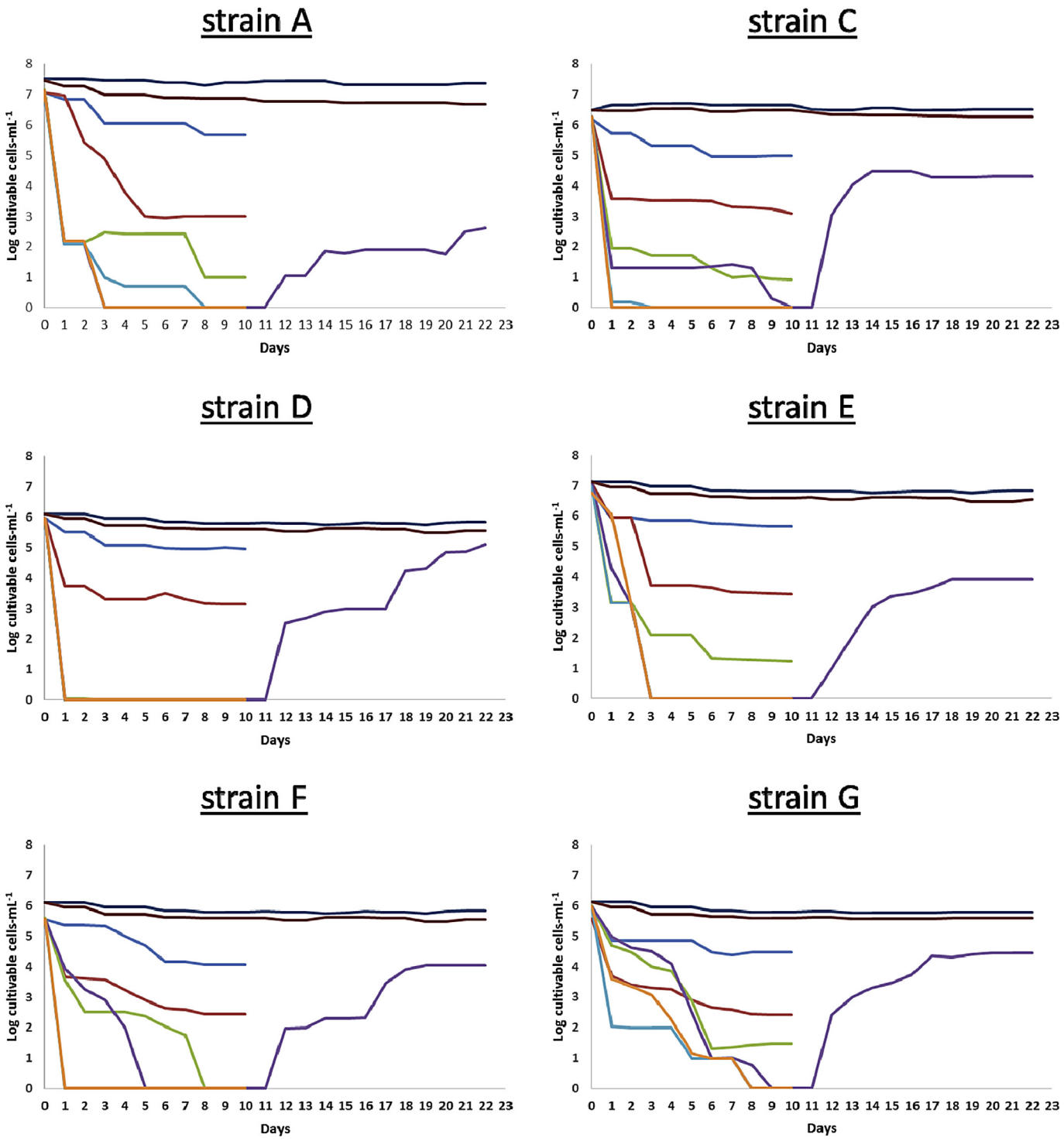

Figure 1. Total, viable and culturable cells of strain A, C, D, E, F, G. Total, viable and culturable cells of samples A, C, D, E, F, G monitored by flow cytometry and colonies count on YPD-agar medium, at different concentrations of molecular SO_2 added to 10^7 cells/mL in stationary phase. The eleventh day indicates the point in which the SO_2 was removed by pH increase to 4.0 in all concentration of SO_2. The values reported are the means of two independent experiments. The differents lines indicated the different growth curves of *B. bruxellensis* in absence of SO_2 (blue line) and in presence of diverse concentrations of molecular SO_2 (red line: 0.4 mg/L of molecular SO_2 concentration; green line: 0.6 mg/L; violet line: 0.8 mg/L; light blue line: 1.0 mg/L; orange line: 1.2 mg/L). Dark blue line indicated total cells and magenta line indicated viable cells.

performed among the differentially expressed genes in order to identify the key molecular processes involved in VBNC state induction and in the recovery of culturability. The GO enrichment tests were performed separately for genes up and down regulated in each pairwise comparison. Among the down-regulated genes, we observed five genes involved in the oxidative stress response, suggesting that the regulation of this class of genes may be involved in the induction of the VBNC state (Table 2). With this exception, no GO categories were found to be significantly over-represented among the 30 genes found differentially expressed when

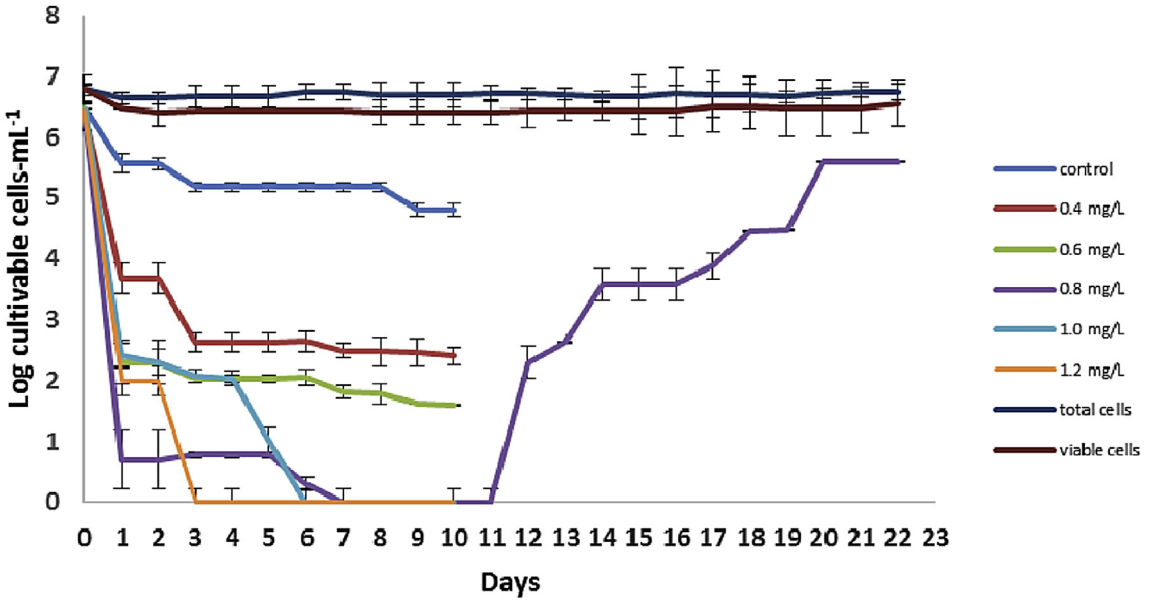

Figure 2. Total, viable and culturable cells of strain B. Total, viable and culturable cells of samples B monitored by flow cytometry and colonies count on YPD-agar medium, at different concentrations of molecular SO_2 added to 10^7 cells/mL in stationary phase. The eleventh day indicates the point in which the SO2 was removed by pH increase to 4.0 in all concentration of SO_2. The values reported are the means of two independent experiments. The differents lines indicated the different growth curves of *B. bruxellensis* in absence of SO_2 (blue line) and in presence of diverse concentrations of molecular SO_2 (red line: 0.4 mg/L of molecular SO_2 concentration; green line: 0.6 mg/L; violet line: 0.8 mg/L; light blue line: 1.0 mg/L; orange line: 1.2 mg/L). Dark blue line indicated total cells and magenta line indicated viable cells.

inducing the VBNC state. The possible implications of oxidative stress response genes among the principal determinants of VBNC state in *B. bruxellensis* was supported by the up-regulation detected for these five genes when E vs R were compared (Table 2). The genes up-regulated during recovery of culturability, included genes codify for amino acid transport (GO:0006865), nucleobase transmembrane transporter (GO:0015205), transporter activity (GO:0005215), oxidoreductase (GO:0016491) and cytochrome-c oxidase (GO:0004129). Overall, our results suggest an active transport of amino acids, sugar and nucleobases across membrane during the recovery phase as well an active energetic metabolism (Fig. 3 and Table S2). Among the down regulated genes the enriched GO terms involved genes categories related to the DNA replication and the DNA repair (Fig. 4 and Table S2). Although not found among the enriched GO terms, several genes involved in carbohydrate metabolism were observed to be up-regulated during the recovery phase (Table 3) as well as several heat shock proteins (Table 4).

4. Discussion

The aptitude of *B. bruxellensis* to survive when exposed to wine-like stress conditions by entering in a viable but not culturable state (VBNC) has already been described by several authors (Du Toit et al., 2005; Agnolucci et al., 2010; Serpaggi et al., 2012). For instance, Vigentini and collaborators (2013), analysing 108 strains of *B. bruxellensis*, highlighted strain-dependent sensitivity to the SO_2 concentration. In accordance, we observed that the strains analysed have different VBNC behaviour as a function of molecular SO_2 concentration, corroborating the evidence of a strain-dependent sulphite sensitivity character within the *B. bruxellensis* species. With this concern, it is interesting to notice that in the case of strains A and B, 1.0 mg/L of SO_2 leads to lower levels of culturability loss than 0.8 mg/L. It is possible to speculate that this behaviour is related to a different intensity of the stress response induced by diverse extents of the 'sulphite stressor'. The 'resuscitation' character has been widely debated (Nyström, 2003; Ramamurthy et al., 2014). According to these authors, the recovery of culturability is due to the presence and sudden growth of a few residual cells with a normal metabolism in a population predominantly non-culturable. Recently, Salma et al. (2013) demonstrated that the removal of environmental stresses was sufficient to induce the exit from the VBNC state. Indeed, the recovery of the culturable state was shown as a true 'resuscitation' character and not a simple growth of a few residual cells with a normal metabolism (Salma et al., 2013).

We found that the ability of the analysed *B. bruxellensis* strains to enter in a VBNC state and even to 'resuscitate' is greatly flexible. Our findings are in accordance with other studies on the variability of strains sensitivity to SO_2 (Agnolucci et al., 2010; Du Toit et al., 2005; Serpaggi et al., 2012). In addition, our results strongly confirm

Table 1
Number of differentially expressed genes found in the two comparisons in *B. bruxellensis* strain Unifg 14 (strain B of this study).

	UP	Down	Total
C2_vs_E1	16	14	30
E1_vs_R2	971	663	1634

Table 2
Genes coding for proteins involved in the oxidative stress response found significantly differentially expressed (Log2 fold change) i) in the comparison between cells growth without treatment and cells in VBNC state (C vs E) and ii) comparing cells in VBNC state vs cells in the recovery phase (E vs R)..

Gene	Product	C vs E	E vs R
AWRI1499_2182	manganese-superoxide dismutase	−1.34	3.19
AWRI1499_2246	peroxiredoxin *tsa1*	−1.66	4.35
AWRI1499_2588	thioredoxin reductase	−1.57	3.88
AWRI1499_2856	superoxide dismutase	−1.13	1.57
AWRI1499_3810	glutathione peroxidase	−1.58	4.89

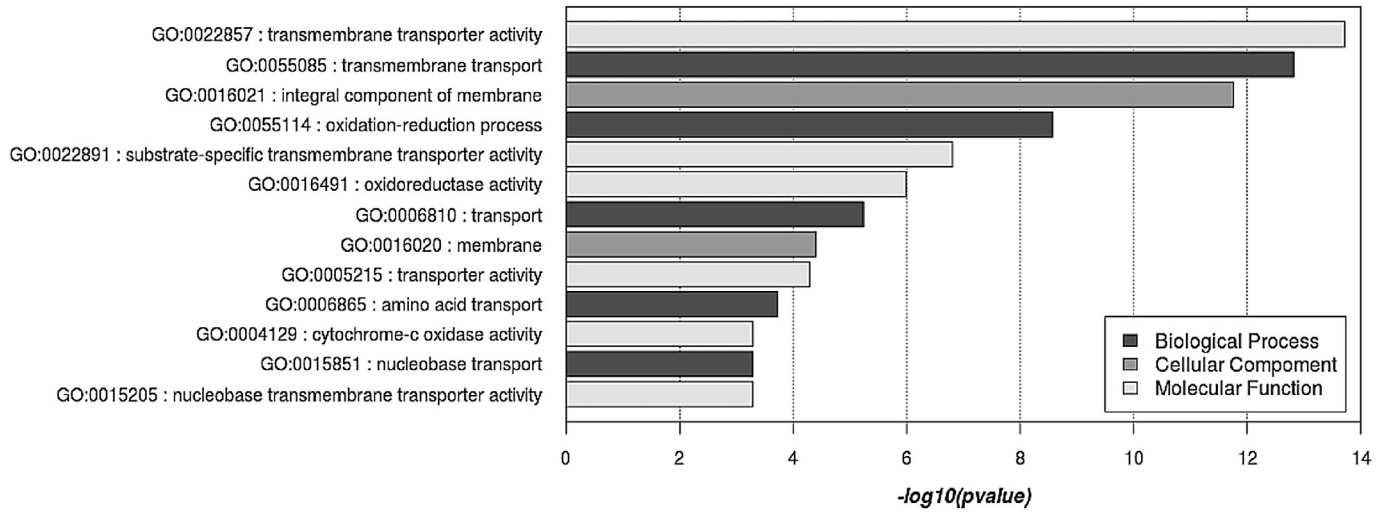

Figure 3. Enrichment among the up-regulated E vs R. Gene ontology (GO) terms enriched among the up-regulated genes in E vs R comparison in *B. bruxellensis* strain Unifg 14 (strain B of this study).

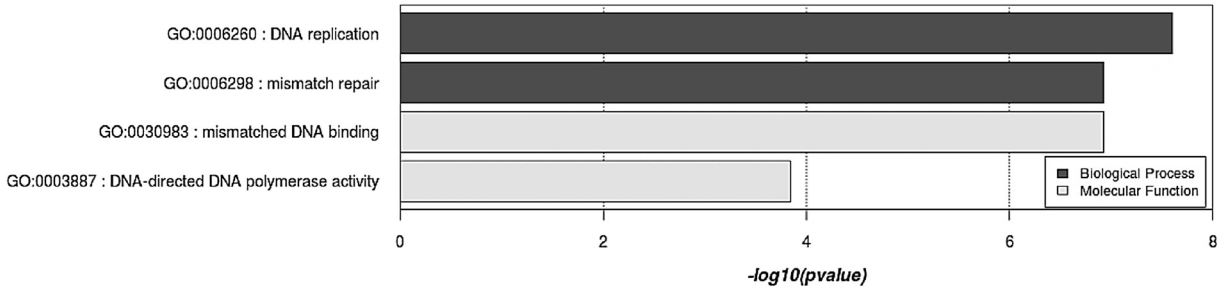

Figure 4. Enrichment among the down-regulated E vs R. Gene ontology (GO) terms enriched among the down-regulated genes in E vs R comparison in *B. bruxellensis* strain Unifg 14 (strain B of this study).

evidences already reported by Zuehlke and Edwards (2013) and by Agnolucci et al. (2013) on the strain-dependent variability of VBNC state. Moreover, we evidenced that the VBNC behaviour in *B. bruxellensis* varies in a strain-dependent manner as a function of molecular SO_2 concentration. To the best of our knowledge, it is the first demonstration of VBNC state as a strain-dependent character

Table 3
Genes coding for proteins involved in carbohydrate metabolism found significantly up-regulated in the comparison between cells in VBNC state and cells in the recovery phase (E vs R).

Gene	Product	Log2 fold change
AWRI1499_0020	hexokinase	3.95
AWRI1499_0477	ribulose-phosphate 3-epimerase	1.72
AWRI1499_0109	hxk2p	3.24
AWRI1499_1190	non-oxidative pentose phosphate pathway	3.58
AWRI1499_1904	emi2p	1.22
AWRI1499_1928	glucosamine-6-phosphate deaminase	1.20
AWRI1499_1937	sucrose-6-phosphate hydrolase	3.47
AWRI1499_1425	malate mitochondrial precursor	4.14
AWRI1499_2554	malate nad-dependent	2.32
AWRI1499_2675	glucosidase ii catalytic subunit	1.84
AWRI1499_3210	phosphoglucomutase	5.66
AWRI1499_3615	sol3p	2.13
AWRI1499_3896	1,4-alpha-glucan branching enzyme	3.04
AWRI1499_3904	fructose- -bisphosphatase	1.96
AWRI1499_4351	isomaltase	2.13
AWRI1499_4603	udp-glucose:sterol glucosyltransferase	1.58
AWRI1499_4728	gal10 bifunctional protein	5.44
AWRI1499_4351	isomaltase	2.13
AWRI1499_4731	maltase	4.98
AWRI1499_4884	maltase	1.38
AWRI1499_4733	beta-glucosidase	1.54
AWRI1499_4832	putative carbohydrate kinase	1.23

Table 4
Genes coding for heat shock proteins found significantly up-regulated in the comparison between cells in VBNC state and cells in the recovery phase (E vs R).

Gene	Product	Log2 fold change
AWRI1499_0167	heat shock protein 60	2.08
AWRI1499_0363	10 kda heat shock mitochondrial	2.82
AWRI1499_0727	heat shock protein *hsp20*	5.61
AWRI1499_0839	heat shock protein 70	4.98
AWRI1499_2042	heat shock protein *hsp88*	4.02
AWRI1499_2449	heat shock protein 104	5.69
AWRI1499_2450	heat shock protein *hsp98*	6.29
AWRI1499_2918	heat shock protein *ssa2*	1.63
AWRI1499_3200	heat shock protein 90	3.38
AWRI1499_4466	heat shock protein *sti1*	4.16
AWRI1499_4467	heat shock protein *sti1*	4.47
AWRI1499_4656	heat shock protein *hsp20*	5.55

in yeast and this variability concerns several phases of VBNC state: entrance in VBNC state, rapidity of the entrance, influence of molecular SO_2 concentration, percentage of 'resuscitation'. Our observations may also explain an apparent contradiction between previous investigations on this phenomenon. For example, Divol and Lonvaud-Funel (2005) found that SO_2 removing was not sufficient to exit from the VBNC, while Serpaggi et al. (2012) reported a complete ability of *B. bruxellensis* strains to recover culturability after stressor was removed. In fact, the strain-dependent character of 'resuscitation' percentage shed new light on these phenomena. Our results confirm the high level of phenotypic polymorphism existing in *B. bruxellensis* species, already evidenced at the level of sulphite sensitivity/tolerance (Curtin et al., 2012; Vigentini et al., 2013).

After the study of VBNC behaviour, we selected one strain to perform a molecular analysis at the transcriptomic level. The strain B has been chosen in reason of the intermediate VBNC entrance after sulphite stress and the good performances in the recovery phase. Concerning transcriptomic outcomes, we highlight a first relevant difference in the number of genes regulated after 1.2 mg/ml of SO_2 exposure and after sulphite stressor removal. Even though RNA-seq analysis have been already applied to investigate dormant/VBNC bacteria (Carvalhais et al., 2014; Meng et al., 2015), to the best of our knowledge, this is the first transcriptomic analysis concerning VBNC state in eukaryotic microorganisms. Considering that we select an identical time (twenty minutes both after stressor exposure and stressor removal), it appears clear that the transcriptomic changes related with the two biological phenomena were radically different. However, only future studies will assess how representative are these two 'snapshot' of the evolution of mRNA populations associated with SO_2-associated entrance and exit from the VBNC state in *B. bruxellensis* strain Unifg 14 (strain B of this study). Our evidences report additional proofs of the crucial role, in *B. bruxellensis* VBNC SO_2-induced state, of genes/proteins involved in the redox cell homeostasis and in reactive oxygen species detoxification. These findings are in accordance with the existence, in association with VBNC entrance, of a response to cope with changes in redox potential reported by Serpaggi et al. (2012) adopting a proteomic approach. Other than the genes reported in Table 2, belonging to the general class of oxidative stress response, we have clear indications of a general machinery involved in the redox balance. In particular, with different extents, we found the major biological mechanisms involved in sulphite detoxification reported in the model organism *S. cerevisiae* (Park and Hwang, 2008). Sulfite reductase has a direct role in sulphite consumption (Park and Hwang, 2008), with an important side effect on wine sensory quality, being responsible for the production of hydrogen sulfide (associated with the characteristic rotten egg off-flavor) (Swiegers and Pretorius, 2007). Inorganic phosphate transporter might be relevant in phenomena of sulphite efflux, while the other transporters (e.g. plasma membrane ATP-binding cassette multidrug transporter) could be related to other detoxification processes (Jungwirth and Kuchler, 2006). Alcohol dehydrogenases and lactate dehydrogenases, other than involved in regeneration of NAD^+ regeneration from NADH, might also be important for the formation of non-toxic adducts with acetaldehyde (Park and Hwang, 2008). Finally, remaining in the field of redox-homeostasis, it is interesting to underline how, in other eukaryotic microorganisms, the negative transcriptional regulator NmrA has been found to discriminate between the oxidized and reduced forms of $NAD(P)^+$/NAD(P)H, with a general possible role in redox sensing (Lamb et al., 2003). All these results report consistent proofs that the entrance in the SO_2-induced VBNC state in *B. bruxellensis* is associated with both, sulfite toxicity and the consequent oxidative stress response.

This first transcriptomic study on VBNC in eukaryotic microorganisms suggests the presence of common biological mechanisms shared with prokaryotes. In fact, several studies highlighted the entrance in VBNC state of bacteria after an oxidative stress (e.g. Cuny et al., 2005; Atack and Kelly, 2009; Reuter et al., 2010). On the other hand, in some cases the involvement of the oxidative stress response in the induction of VBNC state has been suggested in prokaryotes (e.g. Desnues et al., 2003; Wang et al., 2013; Postnikova et al., 2015). In addition, VBNC bacterial cells have also been shown to be tolerant, together with a wide range of environmental stressors, to oxidative challenges (Nowakowska and Oliver, 2013). More broadly, in prokaryotes, a variety of stresses induces VBNC state suggesting this persistence form has evolved as a general environmental stress response that allows a subpopulation of cells to survive environmental stress (Ayrapetyan et al., 2015). With this regards, considering the specific nature of our study, it is difficult to speculate on the possible general role of VBNC state in yeasts with respect of bacteria.

Concerning the exit from the VBNC, an intriguingly feature deals with the class of 'DNA replication-related genes'. In fact, the evidences of a general repression of genes involved in DNA replication add a further piece to the puzzle suggesting that true resuscitation of cell rather than a simple regrowth occurs (Oliver, 2010).

Overall, with a low number of regulated genes, our results supported the hypothesis that *B. bruxellensis* acclimation mechanisms are mainly a sort of adaptation to exogenous stresses (Nardi et al., 2010), a cellular response clearly different when compared to the more articulate remodelling metabolic changes of the model eukaryote microorganism *S. cerevisiae* (Nardi et al., 2010). The number of genes associated with removal of SO_2 stressor, and with the consequently exit from the VBNC state, clearly described a general reactivation of the cell metabolism after a dormancy condition. From this point of view, the involvement of genes coding for heat shock proteins (HSP) was not surprising considering their relevance in protein homeostasis (Hartl et al., 2011). It is well known the basal or housekeeping role (of some HSPs during the normal growth of microorganisms (Haslbeck et al., 2004; Fiocco et al., 2010; Capozzi et al., 2011), in peculiar physiological state probably connected with the folding need of a cell with massive synthesis of new proteins (as suggested by the great numbers of induced genes).

Concluding, the major outcomes reported in the present study indicate VBNC behaviour as a new key standard in the characterization of spoilage potential of *B. bruxellensis* strains, suggesting possible molecular basis. From an industrial point of view, the results are important to design physicochemical and biotechnological strategies for *B. bruxellensis* control in regional wines.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.06.007.

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