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Use of propidium monoazide for the enumeration of viable *Brettanomyces bruxellensis* in wine and beer by quantitative PCR

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ABSTRACT

Brettanomyces bruxellensis is a current problem in winemaking all over the world, and the question if *B. bruxellensis* has a positive or negative impact on wine is one of the most controversial discussions in the world. The presence of live *B. bruxellensis* cells represents the risk of growth and an increase in cell numbers, which is related to the potential production of volatile phenols. In this work, the optimisation of a PMA-quantitative PCR (qPCR) method to enumerate only viable cells was carried out using the standard strain *B. bruxellensis* DSMZ 70726. The obtained detection limits were 0.83 log CFU/mL in red wine, 0.63 log CFU/mL in white wine and 0.23 log CFU/mL in beer.

Moreover, the quantification was also performed by Reverse Transcription quantitative PCR (RT-qPCR), and the results showed a higher detection limit for all of the trials.

1. Introduction

Alteration of wine and beer by microorganisms needs to be managed during production because microorganisms can affect the flavours and aromas of the final products. Yeasts, such as *Saccharomyces cerevisiae*, are used to drive alcoholic fermentation, but others species, such as *Brettanomyces bruxellensis*, may be responsible for the development of unpleasant flavours (Willenburg and Divol, 2012).

The widespread use of oak barrels to age red wines has contributed to the expansion of *B. bruxellensis*, and thus *B. bruxellensis* has become one of the main subjects in the field of microbial wine and beer spoilage.

B. bruxellensis can be present on grapes, cellar equipment and barrels and can flourish during wine ageing and bottling (Deak and Beuchat, 1996; Gerbaux et al., 2000; Renouf and Lonvaud-Funel, 2007; Coulon et al., 2010). *B. bruxellensis* has been shown to be the main organism responsible for the formation of volatile compounds, such as 4-ethylphenol and 4-ethylguaiacol, that are often described as “Brett” taint when perceived in red wine (Chatonnet et al., 1995). The threshold for the sensorial detection of ethyl phenols is low (Suarez et al., 2007), and even small amounts can considerably reduce the olfactory quality of wine. Descriptors such as “phenolic,” “animal,” “horse sweat,” and “stable” have been used to describe the presence of these compounds (Chatonnet et al., 1992, 1995; Rodrigues et al., 2001).

In addition to the production of ethyl phenols, which is the most important indicator of the activity of *B. bruxellensis* in red wine, the presence of this species can have detrimental effects on the visual and organoleptic quality of wines by causing film formation, cloudiness, loss of colour, production of volatile acidity, and production of “mousy” off-flavours (tetrahydropyridines) (Fugelsang et al., 1993; Fugelsang, 1997, 1998; Mansfield et al., 2002; Aguilar-Uscanga et al., 2003; Heresztyn, 1986; Snowdon et al., 2006; Romano et al., 2008). *Brettanomyces* species are less frequently found in white wines (Licker et al., 1998; Dias et al., 2003). The loss of viability and the consequent absence of ethylphenol levels in white wines are largely attributed to the efficiency of sulphur dioxide (SO_2) at lower pH conditions (Loureiro and Malfeito-Ferreira, 2006).

B. bruxellensis has also been isolated from beer samples, and its presence in this product can result in an organoleptic alteration (Manzano et al., 2012); however, this microorganism is often considered responsible for the development of the aromatic profile of some particular types of beer (e.g., Belgian Lambic) (Piškur et al., 2012).

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Due to the reasons mentioned above, it is necessary to be able to detect *B. bruxellensis* contamination and to have a rapid and accurate technique to quantify the viable, metabolically active cells in wine and beer. The best strategy to correct *B. bruxellensis* contamination remains monitoring winery and brewery equipment, barrels and raw materials, but the conventional identification methods for *Brettanomyces* are inadequate because its low cell density and metabolic characteristics results in slower growth in yeast media than other yeasts (Chatonnet et al., 1992; Suarez et al., 2007). This is problematic for winemakers and beer producers because they have to delay the corrective treatments required to alleviate the presence of high numbers of *Brettanomyces*; these corrective treatments include the addition of sulphur dioxide, filtration and thermal inactivation (Couto et al., 2005).

Many different methods have been used to detect the presence of *B. bruxellensis* in wine and beer. These methods include molecular biology techniques such as mitochondrial DNA restriction analysis, restriction fragment length polymorphism analysis (RFLP) PCR, random amplified polymorphic DNA (RAPD) PCR, PCR and nested PCR (Martorell et al., 2006; Dias et al., 2003; Esteve-Zarzoso et al., 1999; Agnolucci et al., 2009; Mitrakul et al., 1999; Cocolin et al., 2004; Campolongo et al., 2010; Ibeas et al., 1996). However, these culture-dependent techniques require the use of an enrichment step to extract the DNA and determinate the cell concentration, and this enrichment step lacks precision (Willenburg and Divol, 2012). Thus, culture independent techniques, such as qPCR, have been developed to rapidly detect and quantify yeast and bacteria from wine and beverages without enrichment (Hierro et al., 2006; Rawsthorne and Phister, 2006; Rawsthorne et al., 2009; Jara et al., 2008; Andorrà et al., 2010). In particular, the identification and enumeration of *B. bruxellensis* in wine using qPCR has been the main goal of many recent papers, but none of the developed methods have a detection limit lower than 10 CFU/mL or can discriminate between dead or alive cells (Delaherche et al., 2004; Phister and Mills, 2003; Agnolucci et al., 2007; Tessonniere et al., 2009; Campolongo et al., 2010).

Recently, the DNA-intercalating agent propidium monoazide (PMA) was used in conjunction with qPCR to selectively detect live cells of pathogenic and spoilage microorganisms (Mamlouk et al., 2012; Elizaquìvel et al., 2012; Yokomachi and Yaquchi, 2012; Vendrame et al., 2013). Therefore, we report here the development of the first PMA-qPCR assay for *B. bruxellensis*. This assay can enumerate live cell concentrations lower than 10 CFU/mL and thus allows winemakers and beer producers to choose an efficient corrective measure to remove or prevent the growth of this spoiling microorganism.

2. Materials and methods

2.1. Brettanomyces bruxellensis strain and cell suspension preparation

The strain *B. bruxellensis* DSMZ 70726 was chosen for the optimisation of the PMA-qPCR technique. It was cultured to reach cell concentrations of 10^7 colony-forming units (CFU)/mL (equivalent to an optical density at 600 nm [OD600] of 1) for approximately 7 days at 30 °C in tubes containing 20 mL of Malt Extract (ME) broth medium (Oxoid, Milan, Italy).

Ten-fold dilutions of each culture were prepared in red wine (Tavernello, Faenza, Italy, alcohol content 11.5% v/v), white wine (Tavernello, Faenza, Italy, alcohol content 11% v/v), and beer (Peroni, Rome, Italy, alcohol content 4.7% v/v) to obtain suspensions of *B. bruxellensis* for standard curve construction. The final concentration of the cells in red wine, white wine and beer was between 10^7 and 1 CFU/mL. The 72 h culture of *B. bruxellensis* used to contaminate the wine and beer samples was enumerated on ME agar medium (Oxoid, Milan, Italy) to determine the exact CFU/mL spiked into the samples. Plates were incubated under aerobic conditions at 30 °C for 9 days, the colonies were counted, and the numbers of viable *B. bruxellensis* were determined from those counts.

Next, samples of red wine (Tavernello, Faenza, Italy, alcohol content 11.5% v/v), white wine (Tavernello, Faenza, Italy, alcohol content 11% v/v) and beer (Peroni, Rome, Italy, alcohol content 4.7% v/v) were inoculated with a mixture of live and dead *B. bruxellensis* cells to assess the efficiency of PMA to differentiate between live and dead cells.

To obtain a suspension of 10^7 CFU/mL dead cells, tubes containing 30 mL of *B. bruxellensis* culture ($OD_{600} = 1$) were pasteurised. Different conditions of time and temperature (60 °C for 20 min, 80 °C for 10 min, and 80 °C for 20 min) were tested to determine the best method to obtain a suspension consisting solely of dead cells.

The evaluation of the presence and the enumeration of survived *B. bruxellensis* cells, similarly to other authors (Andorrà et al., 2010), was carried out by plate count on ME agar. Moreover, to evaluate the presence of stressed viable-not culturable cells (VBNC), 10 mL of the pasteurised suspension were added to 90 mL of LOM medium and incubated at 30 °C for 7 days and then streaked in triplicate on ME agar plates.

One millilitre of the suspension constituted only by dead cells was mixed with live cell suspensions to obtain a final concentration of the cells in the different wine and beer samples between 10^8 and <1 CFU/mL of live cells mixed with 10^9 CFU/mL dead cells. The inoculum was evaluated on ME agar medium (Oxoid, Milan, Italy) at 30 °C for 9 days to determine the exact CFU/mL of viable cells spiked in the samples.

All inoculations were carried out in triplicate. A non-inoculated negative control was included in each experiment. Prior to the inoculation trials, the wines and beer used were analysed both by culture methods (ME agar medium at 30 °C for 9 days) and qPCR to assure the absence of natural *B. bruxellensis* contamination.

2.2. Treatment of suspensions with PMA

A 20 mM solution of PMA (Biotium, Inc., Hayward, CA, USA) in 20% (v/v) dimethyl sulfoxide (Sigma–Aldrich, Oakville, Ontario, Canada) was prepared and stored in the dark at 4 °C. Then, 1 mL of each cells suspension previously prepared in red wine, white wine and beer was centrifuged, and the cellular pellets were resuspended in 300 μL 0.1% (w/v) peptone water and mixed with 1.5 μL of the PMA solution. Each tube was incubated on ice in the dark for 5 min. After incubation, the tubes were placed in the PhAST Blue instrument (GenIUL, Barcelona, Spain) for a 15-min photo-activation process.

2.3. DNA extraction

To extract the DNA, 1 mL of each suspension without PMA and 300 μL of the suspensions treated with PMA were centrifuged at 10,000 rpm for 7 min to obtain a cellular pellet. The DNA was then extracted from the pellets using the phenol–chloroform method (Manzano et al., 2004). After resuspension of DNA in 50 μL of milliQ sterile water, 1 μL of DNase-free RNase (Roche diagnostics, Milan, Italy) was added to each sample to digest RNA with incubation at 37 °C for 1 h (Iacumin et al., 2009). The DNA concentration was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Rodano, Italy) and standardised to 40 ng/μL by dilution with sterile DNA-free Milli-Q water.