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Effects of melatonin and tryptophol addition on fermentations carried out by *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast species under different nitrogen conditions

María José Valera, M. Ángeles Morcillo-Parra, Izabela Zagórska, Albert Mas, Gemma Beltran, María Jesús Torija*

Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Grup Biotecnologia Enológica, C. Marcel·lí Domingo 1, Tarragona, Spain

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ABSTRACT

During wine fermentation, yeasts produce metabolites that are known growth regulators. The relationship between certain higher alcohols derived from aromatic amino acid metabolism and yeast signalling has previously been reported. In the present work, tryptophol (TrpOH) or melatonin (MEL), which are putative growth regulators, were added to alcoholic fermentations. Fermentations were performed with three different inocula, combining *Saccharomyces cerevisiae* and four non-*Saccharomyces* yeast species, under two nitrogen conditions. The combinations tested were: (i) only *S. cerevisiae*; (ii) the mixture of four non-*Saccharomyces* species; and (iii) the combination of all five species together. The results revealed that the TrpOH and MEL addition caused changes in fermentation kinetics, viability and species distribution during fermentation, but it was dependent on the nitrogen present in the media and the composition of the inocula.

Low nitrogen condition seemed to favour the presence of non-*Saccharomyces* species until mid-fermentation, although at the end of fermentation the imposition of *Saccharomyces* was higher in this condition. The presence of high concentrations of TrpOH resulted in limited growth and a delay in fermentation, noticeably significant in fermentations performed with *S. cerevisiae* inocula. These effects were reversed by the presence of non-*Saccharomyces* yeast in the medium. Low TrpOH concentration allowed faster fermentation with mixed non-*Saccharomyces* and *Saccharomyces* inocula. Moreover, in the absence of *S. cerevisiae*, a low concentration of TrpOH increased the presence of *Torulaspora delbrueckii* during fermentation with high nitrogen availability but not under low nitrogen conditions, when the population of *S. bacillaris* was higher than that in the control. The effects of MEL were particularly evident at the beginning and end of the process, primarily favouring the growth of non-*Saccharomyces* strains, especially the first hours after inoculation.

1. Introduction

Wine fermentation is a complex microbial process carried out by yeasts. These microorganisms produce metabolites that are growth regulators and modulate the quorum sensing response in yeast (Albuquerque and Casadevall, 2012, Zupan et al., 2013). Yeast catabolism results in the production of fusel alcohols, which are derived from amino acids through the well-known Ehrlich pathway (Eden et al., 2001). Yeasts convert amino acids through three enzymatic steps: transamination to form α-keto acid, decarboxylation to an aldehyde, and reduction to a fusel alcohol (Dickinson et al., 2003; Hazelwood et al., 2008). In the case of tryptophol (TrpOH), a fusel alcohol derived from tryptophan, biosynthesis starts with the amino group of tryptophan, which is transaminated into 3-indole pyruvate and subsequently decarboxylated to 3-indole acetaldehyde before undergoing final reduction to TrpOH depending on the redox state of the cell (Mas et al., 2014). Fusel alcohols such as TrpOH have been described as modulators of the quorum sensing response in yeast, particularly under low nitrogen conditions (Zupan et al., 2013). On the other hand, other metabolites derived from aromatic amino acids are considered valuable molecules as bioactive compounds (Mas et al., 2014). For example, melatonin (*N*-acetyl-5-methoxytryptamine; MEL) is also a tryptophan derivative. The biosynthesis of MEL in yeast seems to be similar to that described in vertebrates. Tryptophan is hydroxylated into 5-hydroxytryptophan and decarboxylated to serotonin prior to its acetylation to *N*-acetylserotonin. Then, MEL is finally synthesized by transmethylation

* Corresponding author.
E-mail address: mjesus.torija@urv.cat (M.J. Torija).

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(Mas et al., 2014; Sprenger et al., 1999). MEL presents antioxidant activity in some organisms and, in humans, has been described as a hormone regulating circadian rhythms and reproductive functions (López et al., 2009; Serrano et al., 2010). MEL is synthesized by yeast during alcoholic fermentation, although its role remains unknown in these microorganisms (Gómez et al., 2012; Rodríguez-Naranjo et al., 2011).

Yeast metabolism presents variations based on the genetic characteristics of these microorganisms and environmental conditions. In complex environments such as wine fermentation, the interactions between different yeast species or even strains modulate their behaviour (Ciani and Comitini, 2015; Sadoudi et al., 2012). Indeed, different yeast strains in mixed cultures have either synergistic or antagonistic interactions, and this differential performance modifies the aromatic profiles of wines (Ciani and Comitini, 2015; Pérez-Nevado et al., 2006). A complex array of biological communication determines the interactions between microorganisms: killer toxins and antimicrobial compounds (Albergaria and Arneborg, 2016); nutrient limitation (Wang et al., 2016), which might result from rapid nutrient uptake; or the release of other compounds such as fatty acids or acetic acid (Sadoudi et al., 2012). The investigation of interactions between *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation is noteworthy, and understanding the modulation mechanisms performed by secondary metabolites derived from yeast activity is important to control this process (Ciani and Comitini, 2015).

Compounds such as TrpOH or MEL, which have well-known intercellular communication activities in yeasts and other organisms, may play critical roles in the interactions between different species of yeast during alcoholic fermentation. To test this hypothesis, we performed a comparative analysis of mixed cultures of *Saccharomyces* and non-*Saccharomyces* strains during wine fermentation in the presence of secondary metabolites derived from tryptophan, TrpOH and MEL. We analysed the induced changes in microbial succession in the synthetic must environment.

2. Materials and methods

2.1. Yeast strains

The following yeast species were used in this work: three commercial strains for wine production (*Saccharomyces cerevisiae* strain QA23®, *Torulaspora delbrueckii* strain Biodiva™ and *Metschnikowia pulcherrima* strain Flavia® (Lallemand Inc., Montreal, Canada)) and two strains isolated from the spontaneous fermentation of Priorat grape juice (Padilla et al., 2016) (*Hanseniaspora uvarum* strain CECT 13130 and *Starmerella bacillaris* strain CECT 13129). Overnight cultures were prepared in liquid YPD medium (2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract), grown at 28 °C and stirred at 120 rpm to be used as inocula.

2.2. Wine fermentations

To carry out fermentations, synthetic grape must was prepared as described by Riou et al. (1997) with some modifications. Two nitrogen concentrations, in terms of Yeast Available Nitrogen (YAN), were applied: 300 mg/L (150 mg/L derived from amino acids and 150 mg/L derived from NH_4Cl) and 100 mg/L (50 mg/L from amino acids and 50 mg/L from NH_4Cl). The sugar concentration in the synthetic must was 200 g/L, with the same proportion of glucose and fructose. The pH was adjusted to 3.3.

Three different inocula were used to start the fermentations: i) *S. cerevisiae* QA23 (Sc); ii) a mixed culture of four non-*Saccharomyces* strains (*T. delbrueckii*, *M. pulcherrima*, *H. uvarum* and *S. bacillaris* (NSc)); and iii) a mixed population of these four non-*Saccharomyces* strains together with *S. cerevisiae* QA23 (ScNSc). The fermentations were inoculated with 2×10^6 cells/mL of each yeast species used.

For each fermentation, 200 mL of must were dispensed in a 250-mL opaque bottle. The effects of either TrpOH or MEL were analysed for each inoculum and nitrogen concentration. Based on a previous study (González et al., 2018a), two different concentrations of TrpOH (Roche, Germany) (0.5 g/L and 0.1 g/L) and three different concentrations of MEL (Roche, Germany) (1 g/L, 0.5 g/L and 0.1 g/L) were tested by adding them to freshly prepared must. Controls without any specific metabolite supplementation were included for each nitrogen condition and inoculum.

The fermentations were carried out in triplicate at room temperature on an orbital shaker with a stirring rate of 120 rpm.

2.3. Wine sampling and yeast growth analysis during fermentation

Samples were taken every 24 h. Due to the different lengths of the fermentations, three stages were defined to compare them: beginning of fermentation (24 h after inoculation); end of fermentation, when the wines contained less than 2 g/L sugar; and the middle point of fermentation, which was considered the day that represented the median of the process. Yeast growth was determined by plate counting. Three media were used: YPD solid medium (YPD medium plus 1.7% (w/v) agar), a rich medium that was used for total yeast counts; lysine agar medium (Oxoid; USA), which is selective for non-*Saccharomyces* species; and Wallerstein Laboratory Nutrient Agar (WL) medium (Difco; USA), a differential medium that was used for the rapid identification of yeast species based on different colony morphologies (Fig. S1). Additionally, when the morphology of a colony was not clear, amplification and subsequent restriction analysis of 5.8S ITS rDNA were performed directly from colony as described by Esteve-Zarzoso et al. (1999).

To calculate the dilution required for plating, samples were counted using a Neubauer chamber (0.0025 mm^2 and 0.100 mm deep). All plates were incubated at 28 °C for 24–72 h before counting. All colonies that grew were counted, and the numbers of colonies per plate ranged from 20 to 200. However, in WL medium, the numbers of colonies counted were not greater than 50 due to difficulties distinguishing different morphologies when the colonies were too small.

2.4. Chemical analysis of wines

The fermentation process was monitored daily based on density using a digital densitometer (Mettler Toledo, Portable Lab) as an indirect value of the sugar concentration. When density remained stable for at least 2 days, the fermentations were considered finished or stuck. The final wines were analysed to evaluate residual sugars, glucose and fructose using a specific enzymatic kit (Roche, Boehringer Mannheim, Germany) according to the manufacturer's instructions.

The MEL and TrpOH concentration was analysed by performing liquid chromatography-mass spectrometry following the method described by Rodríguez-Naranjo et al. (2011) and González et al. (2018b), respectively. The system was based on a high performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Agilent G6490; Agilent Technologies, Palo Alto, USA).

2.5. Statistical analysis

The variances of the results were statistically analysed by performing one-way ANOVA and Student's *t*-test with a level of significance of 5% using IBM SPSS Statistics software.

3. Results

3.1. Effects of tryptophol addition on alcoholic fermentation and population dynamics

Alcoholic fermentation was strongly affected by TrpOH addition,