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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

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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

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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,

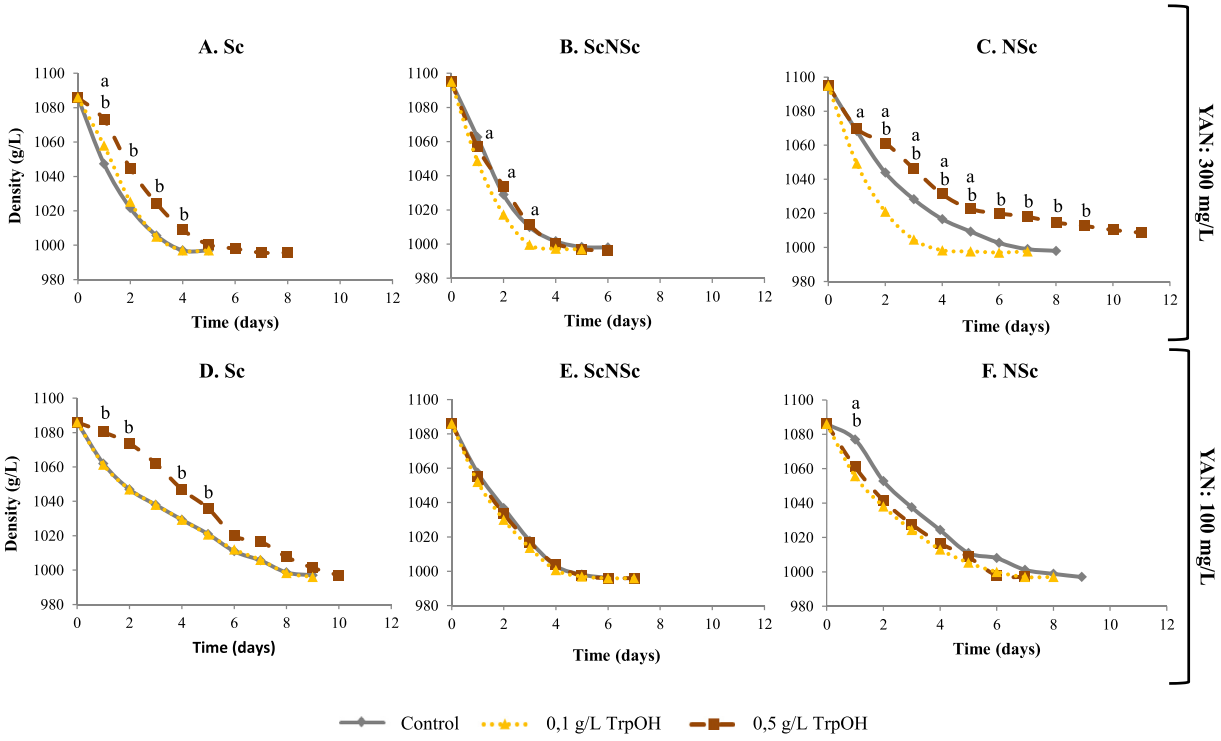

Fig. 1. Fermentation kinetics with added tryptophol (TrpOH) and control in synthetic must containing 300 mg/L (A, B, C) and 100 mg/L (D, E, F) of yeast assimilable nitrogen. Fermentations were inoculated with (i) *S. cerevisiae* (A, D), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B, E) and (iii) a mixture of four non-*Saccharomyces* species (C, F). Values are means of triplicate fermentations. Letters represent statistically significant differences.

although the effects were dependent on nitrogen conditions and the presence of different yeasts in the medium (Fig. 1). When only *S. cerevisiae* was present in the medium (Sc), the highest concentration tested (0.5 g/L) produced a delay in fermentation that was significant throughout the entire process. The same effect was observed for the two nitrogen concentrations tested (Fig 1A and D). This effect clearly disappeared when *Saccharomyces* was inoculated together with other non-*Saccharomyces* strains (ScNSc), as shown in Fig 1B and E. Thus, alcoholic fermentations presented similar kinetics with one exception: when 0.1 g of TrpOH/L was added in the presence of 300 mg/L of YAN. In this case, fermentation proceeded faster than under the other two conditions. This effect was more noticeable when only non-*Saccharomyces* yeasts (NSc) were present in the medium (Fig 1C and F). In this case, at 300 mg/L of YAN, fermentations containing 0.1 g TrpOH/L were also significantly faster than the control, whereas a higher concentration of TrpOH (0.5 g/L) slowed down fermentation and even failed to consume all sugars. This effect disappeared in media with a low nitrogen concentration (100 mg/L of YAN).

The population dynamics of fermentation were analysed to determine the relationships between the yeast population and fermentation kinetics. Regarding the overall population, no significant variations were observed (Supplementary Table 1). As expected, non-*Saccharomyces* populations recovered on LYS plates were lower than the total population (counted on YPD medium) under ScNSc conditions. The results obtained with WL medium allowed us to discriminate between five species based on colony morphology, and *Saccharomyces* was the only species detected when it was inoculated individually (results not shown). In the mixed fermentation (NSc and ScNSc), there were several notable features (Fig. 2): *M. pulcherrima* was hardly recovered on any plate, and, when it was recovered, it occurred practically only at the beginning of fermentation, while *H. uvarum* was only found at the beginning and mid-fermentation. When present, *S. cerevisiae* was predominant at mid and at the end of fermentation. Additionally, when *S. cerevisiae* was not present, *T. delbrueckii* normally was predominant, although *S. bacillaris* also had a significant presence (Fig. 2B and D). However, there were certain deviations from this general pattern due to the presence of TrpOH. At low nitrogen concentrations in ScNSc, although *S. cerevisiae* imposed at the end of the process, *H. uvarum* was the main species detected in the middle of the process, when the highest concentration of TrpOH was in the medium. On the other hand, at high concentrations of nitrogen in the presence of 0.1 g/L of TrpOH, *T. delbrueckii* was the main species detected at the end of the process, resulting, as explained in fermentation kinetics, in a faster fermentation (Fig. 1B and 2A). Similarly, in NSc fermentations in the same conditions (high nitrogen and 0.1 g/L of TrpOH), also the imposition of *T. delbrueckii* resulted in faster fermentations than the control, whereas the imposition of *S. bacillaris* at 0.5 g/L TrpOH resulted in stuck fermentations (Fig. 1C and 2B).

3.2. Effects of melatonin addition on fermentation kinetics

The addition of MEL exerted more limited effects than TrpOH in terms of fermentation kinetics. Although some significant differences were observed, those were normally restricted to single points during fermentations (Fig. 3). With the NSc inoculum most of the fermentations were significantly faster than the control when the highest concentration of MEL was used. In general, in the absence of *S. cerevisiae* the presence of MEL appeared to help alcoholic fermentation because the control fermentation was always slightly delayed. This also happened in the presence of *S. cerevisiae* but only at high nitrogen concentrations. No significant differences were observed in the yeast population with the exception of specific time points, and there was no correlation with fermentation kinetics (Supplementary Table 1). When the population was analysed at the species level, similar observations to those obtained with TrpOH were detected (Fig. 4). Remarkably, non-*Saccharomyces* species persisted at the end of the fermentation, in some cases comprising up to 50% of the total population, when MEL was available in ScNSc fermentations with low nitrogen concentrations. Indeed, under low nitrogen concentrations, the proportion of *S. bacillaris* increased at the end of fermentation with MEL supplementation.

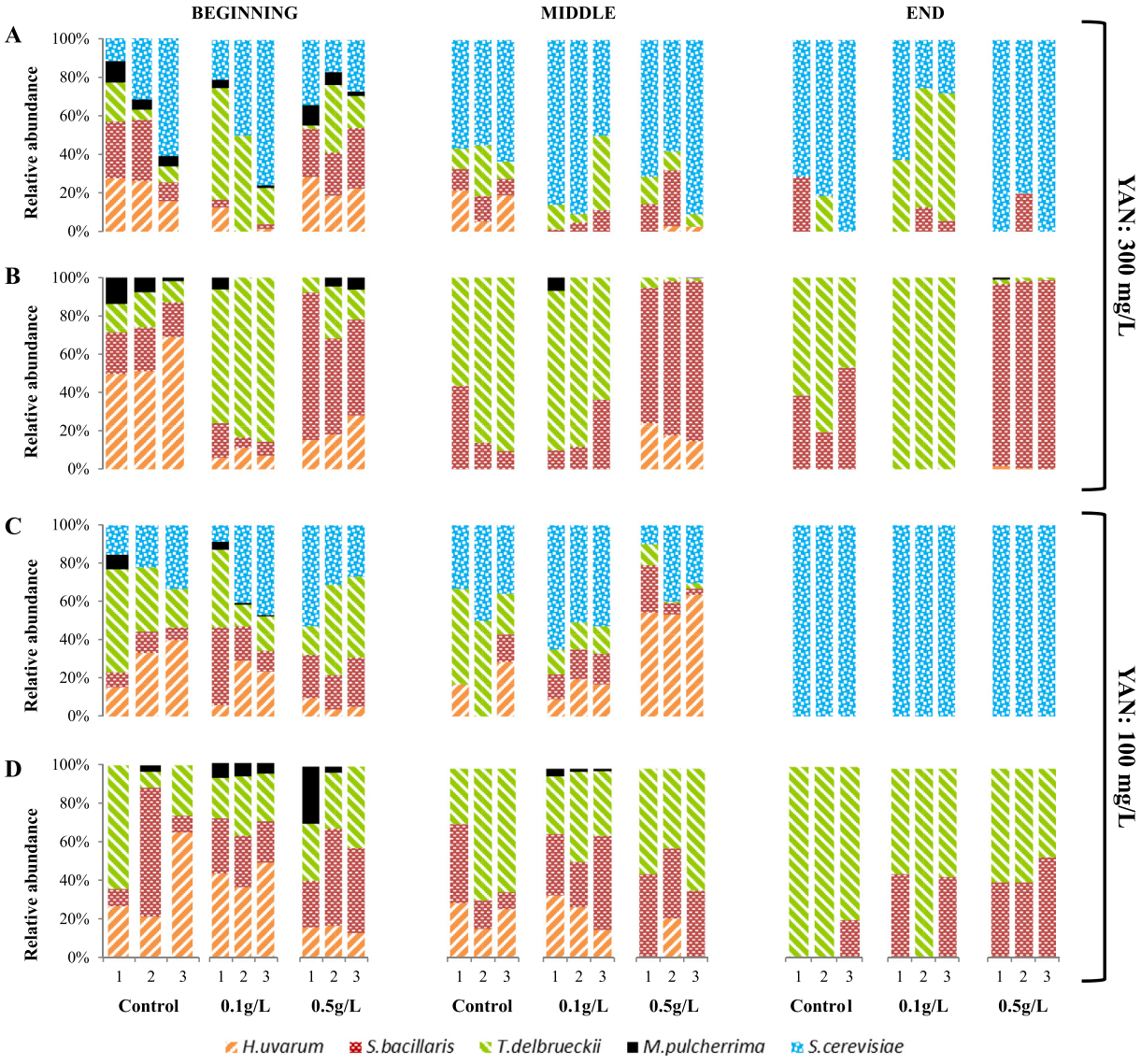

Fig. 2. Distribution of yeast species at the beginning (24 h after inoculation), middle and end of the process in control fermentations and fermentations with tryptophol (TrpOH) supplementation (0.1 g/L and 0.5 g/L). A) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 300 mg/L of yeast assimilable nitrogen (YAN) in the must; B) mixture of non-*Saccharomyces* in musts containing 300 mg/L of YAN C) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN; D) non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN.

3.3. Tryptophol and melatonin production during fermentation

Extracellular samples obtained from control fermentations for the three different inocula and two nitrogen concentrations were analysed for TrpOH and MEL production at three different time points: the beginning, middle and end of fermentation.

In all the cases, higher amount of TrpOH was synthesized in the musts containing high concentration of nitrogen (Fig. 5). At 100 mg/L of YAN, the TrpOH concentration was around 20 ppm, while it was between 50 and 70 ppm at 300 mg/L. The Sc inoculum produced the highest concentration of TrpOH (Fig. 5A), while the presence of non-*Saccharomyces* in the medium resulted in a lower amount of TrpOH, regardless the presence of *S. cerevisiae* (Fig. 5B and C).

As in the case of TrpOH, the amount of MEL in the medium was always lower in must containing 100 mg/L of YAN than in must containing 300 mg/L (Fig. 6). However, the amounts of MEL were much lower than those of TrpOH throughout the fermentation. Moreover, the proportion between the production of MEL in a medium containing 100 and 300 mg/L of YAN was not maintained as it was in TrpOH. Depending on the inocula, MEL presented the highest concentrations at different time points during fermentation regardless of the nitrogen concentration in the initial must. Sc-inoculated fermentations presented the maximum MEL concentration at the beginning of fermentation, 24 h after inoculation (Fig. 6A). Then, MEL levels decreased progressively until the end of the fermentation. However, when inoculation was performed using the ScNSc mixture (Fig. 6B), the MEL concentration increased during fermentation. Finally, fermentations performed with the NSc inoculum (Fig. 6C) presented maximum MEL concentrations in the middle of fermentation, while MEL levels decreased at the end. Moreover, in these fermentations, the MEL contents were the lowest; and thus, the presence of *Saccharomyces* seems to promote a higher presence of MEL in the medium.

4. Discussion

Alcoholic fermentation is the result of a complex microbial succession involving many interactions between different yeast species. The initial non-*Saccharomyces* yeast species, which are more abundant in

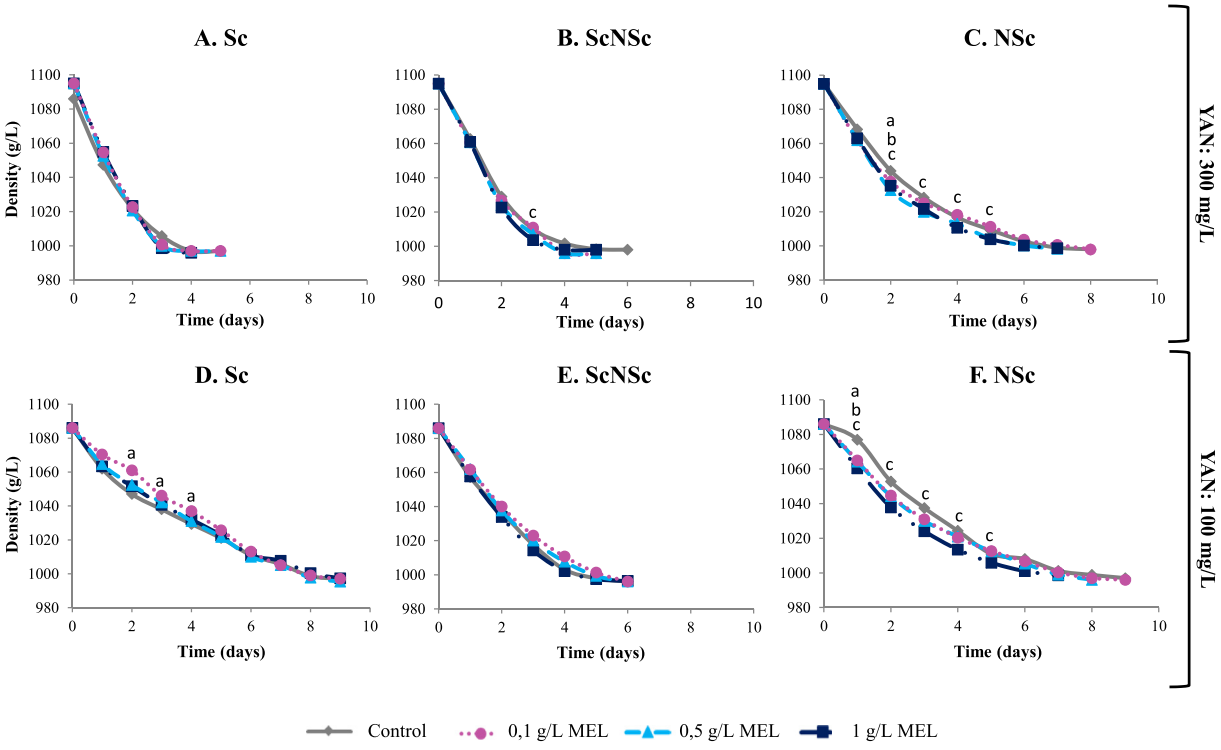

Fig. 3. Fermentation kinetics with added melatonin (MEL) and control in synthetic must containing 300 mg/L (A, B, C) and 100 mg/L (D, E, F) of yeast assimilable nitrogen (YAN). Fermentations were inoculated with (i) *S. cerevisiae* (A, D), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B, E) and (iii) a mixture of four non-*Saccharomyces* species (C, F). Values are means of triplicate fermentations. Letters represent statistically significant differences.

grapes and grape must, have been considered undesirable for many years. To avoid their effects, fermentation control was favoured by the inoculation of selected strains of *S. cerevisiae*. However, in the recent years, good properties and contribution to the wine of non-*Saccharomyces* species has been described, turning the use of mixed cultures of *Saccharomyces* and non-*Saccharomyces* species as a good alternative for wine production (Ciani et al., 2010, Fleet, 2008, Jolly et al., 2014, Mas et al., 2016). The presence of *S. cerevisiae* ensures complete sugar consumption, while conversely, non-*Saccharomyces* species contribute to higher complexity, increasing the aromatic profile of wines (Comitini et al., 2011, Fleet, 2003). The growth of several species sharing the same environment causes competition for nutrients, but other interactions between yeast species remain unknown. In addition, additive effects are caused by the production of metabolites such as ethanol, killer toxins or fatty acids to induce the death of other sensitive yeasts (Ciani and Comitini, 2015; Pérez-Nevado et al., 2006; Wang et al., 2015). There are other additional mechanisms that contribute to this complex scenario, such as quorum sensing-like responses regulated by yeast metabolites, which some authors have identified as a possible mechanism of yeast interaction and not just morphological changes (Ciani and Comitini, 2015; González et al., 2018b).

Regarding competition for nutrients, the reduced availability of nitrogen in must causes slow or sluggish fermentation and limited biomass formation (Varela et al., 2004). Indeed, nitrogen concentration below 140 mg/L has been reported to limit the growth and fermentation rate of *S. cerevisiae* (Bell and Henschke, 2005, Martínez-Moreno et al., 2012, Tesnière et al., 2015). Nitrogen was not a limiting substrate in the present study, because all fermentations with 100 mg/L of N finished successfully and the only fermentation that was halted contained a high nitrogen concentration. Nitrogen needs were likely covered by previous growth in YPD during inoculum preparation, permitting internal nitrogen accumulation (Lleixà et al., 2016). In general, non-*Saccharomyces* yeasts are considered high nitrogen consumers (Andorrà et al., 2010, 2012). However, nitrogen requirements are species-dependent. Thus, in the present work, the initial nitrogen concentration determined different fermentation kinetics, likely due to the differential sensitivities of yeast species to the lack of nitrogen. Padilla et al. (2016) reported that the *H. uvarum* strain used in our study was detected at the beginning and middle points of spontaneous fermentation and exhibited high population levels. However, Lleixà et al. (2016) showed that this *H. uvarum* strain was sensitive to low nitrogen conditions in fermentations performed with mixed yeast inocula. According to our results, *H. uvarum* was more persistent in time under low nitrogen conditions, although at the beginning of fermentations, it seems to be more competitive at high nitrogen concentrations. When fermentations were carried out with NSc inoculum and high nitrogen, *S. bacillaris* was able to survive until the end of the fermentation, which has been observed by other authors working with the same strain (Lleixà et al., 2016; Padilla et al., 2016). *S. cerevisiae* was the only species detected at the end of fermentation under low nitrogen conditions. However, at the midpoint of fermentation, the proportion of this species was reduced compared with the proportion observed under high nitrogen conditions. This is explained by the high proportion of *T. delbrueckii* found under low nitrogen conditions. Indeed, *T. delbrueckii* was able to complete fermentation in most processes performed only with non-*Saccharomyces* yeasts, even under low nitrogen conditions. Recent studies have reported that killer strains of *Torulaspora delbrueckii* can complete wine fermentations in single inoculation, although these fermentations were pretty slower than the ones performed with *S. cerevisiae* (Ramírez et al., 2016; Velázquez et al., 2015). Moreover, *T. delbrueckii* was recovered at the end of fermentation in the presence of *S. cerevisiae*, likely because *T. delbrueckii* strains have been characterized by their good fermentation capacity as well as their ethanol tolerance (Bely et al., 2008; Lleixà et al., 2016). A previous study reported that *T. delbrueckii* and *S. bacillaris* were able to maintain their culturability longer than *H. uvarum* upon inoculation with *S. cerevisiae* (Wang et al., 2016). Conversely, *M. pulcherrima* was unable to survive until the end of fermentation. This species is known to have a low tolerance to

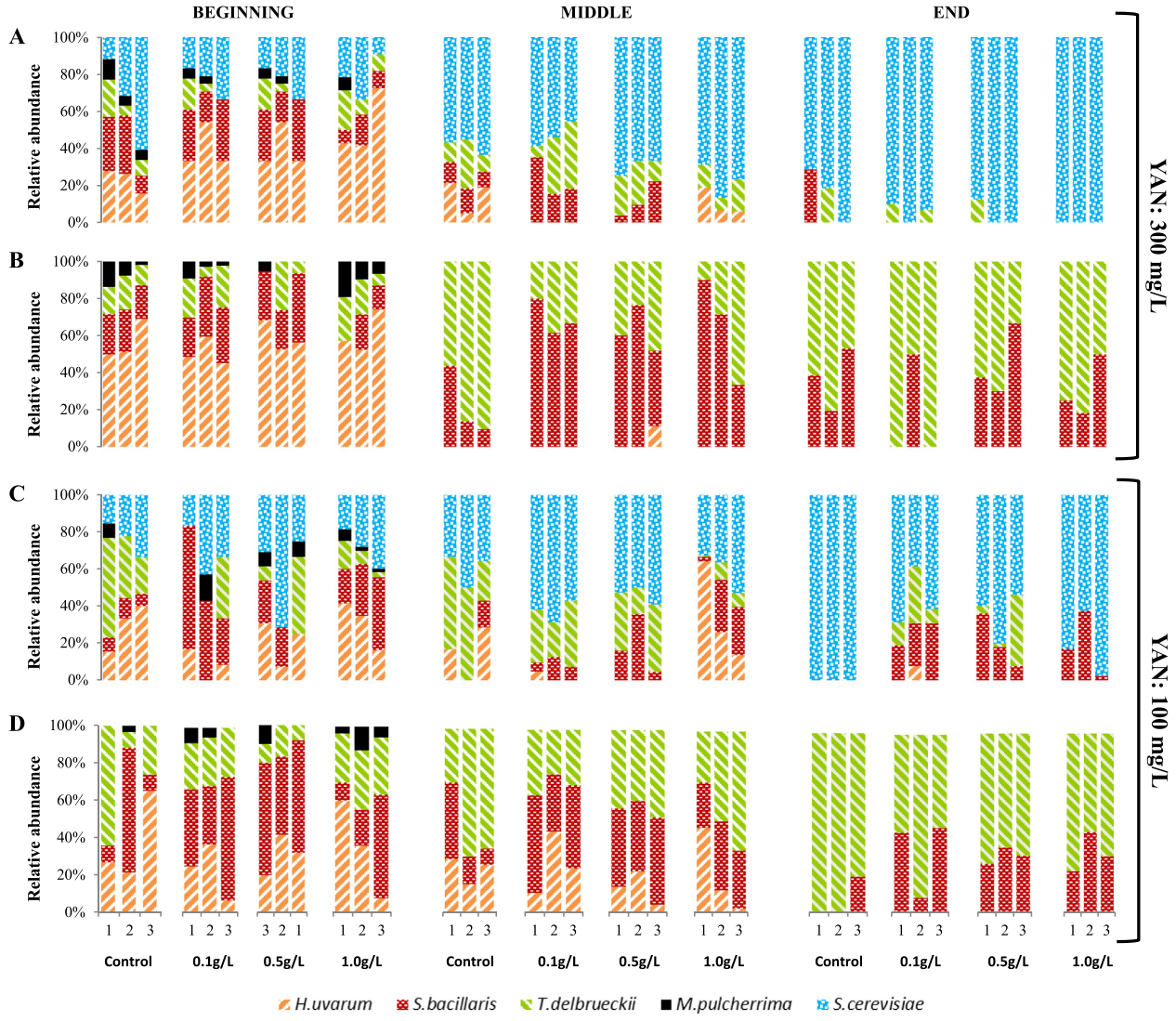

Fig. 4. Distribution of yeast species at the beginning (24 h after inoculation), middle and end of the process in control fermentations and fermentations with melatonin (MEL) supplementation (0.1 g/L, 0.5 g/L, 1 g/L). A) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 300 mg/L of yeast assimilable nitrogen (YAN); B) mixture of non-*Saccharomyces* in musts containing 300 mg/L of YAN; C) *Saccharomyces* and non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN; D) non-*Saccharomyces* inoculum in musts containing 100 mg/L of YAN.

alcohol; some authors have reported that it is unable to survive in concentrations of 2–3% (v/v) of ethanol (Kunkee and Amerine, 1970). Our results agree with those of González-Royo et al. (2015), with a very low recuperation of this strain after inoculation.

The addition of TrpOH resulted in different effects on species distribution and fermentation kinetics depending on the added concentration. This compound has been highlighted as a quorum sensing molecule (Zupan et al., 2013). Quorum sensing-like responses in yeast have been mainly investigated to explain morphological changes, however, the involvement of this phenomenon in some yeast interactions cannot rule out (Ciani and Comitini, 2015). Our results confirmed that *S. cerevisiae* growth, similar to that of many other non-*Saccharomyces* species, is affected by the presence of TrpOH in the medium, as previously reported in single fermentations (González et al., 2018a). A high TrpOH concentration limited the fermentation performance of *S. cerevisiae*, although this effect was reversed by the presence of non-*Saccharomyces* yeast. On the other hand, low TrpOH concentration improved the fermentation performance in ScNSc conditions in high-nitrogen musts, although the major species at the end of these fermentations was *T. delbrueckii* instead of *S. cerevisiae*. Similarly, with NSc inoculum, low TrpOH supplementation also resulted in faster fermentations, being again particularly evident in samples obtained from high-nitrogen where *T. delbrueckii* was the only species detected at the end. On the other hand, high TrpOH concentrations under high nitrogen conditions favoured the growth of *S. bacillaris*, but the fermentation kinetics was slower. Control fermentations showed an intermediate situation, with the presence of both species and an intermediate kinetics. Thus, *T. delbrueckii* as the major species accelerated fermentation kinetics compared with the control, while high populations of *S. bacillaris* slowed down fermentation.

MEL, another tryptophan derivative, presents characteristics that point to a putative role as a signalling molecule during fermentation. In previous studies, MEL was detected in extracellular medium during wine fermentations conducted by *S. cerevisiae*. This MEL was pulsatilely released in the first hours of wine fermentation, but it also disappeared rapidly from the extracellular medium (Rodríguez-Naranjo et al., 2012). Moreover, in this study, the synthesis of MEL has been related to the yeast growth phase and the concentration of reducing sugars, suggesting a role of MEL as a growth signal. The effect of MEL on fermentation kinetics was less evident than that observed for TrpOH, although the presence of high concentrations of MEL (0.5 and 1 g/L), reduced fermentation time in mixed fermentations, especially in NSc

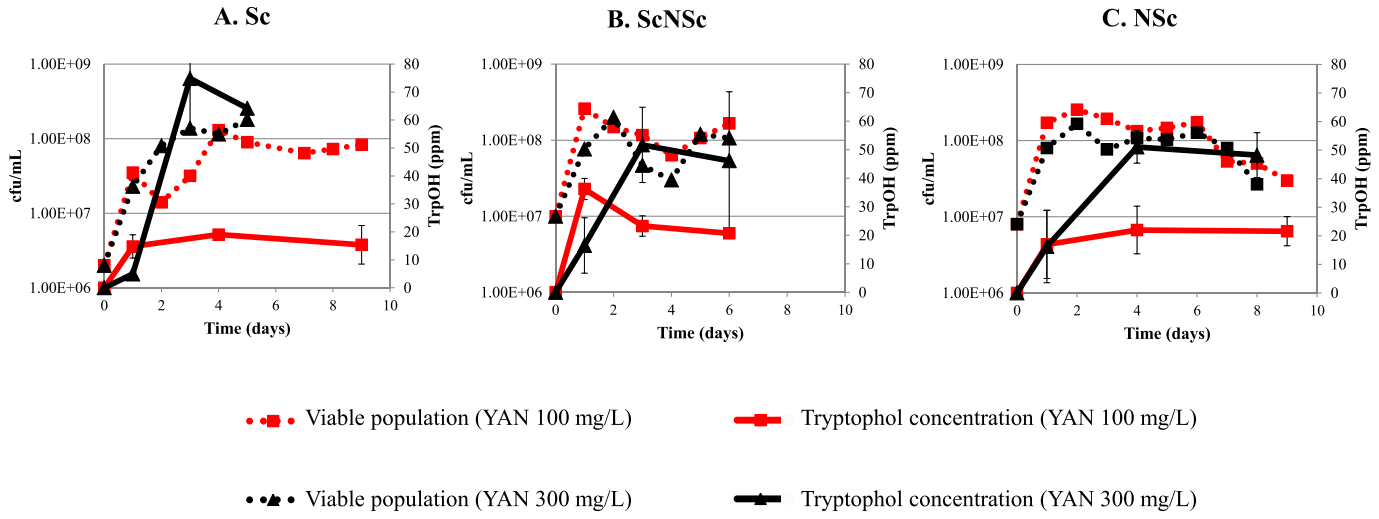

Fig. 5. Yeast populations recovered on YPD medium during fermentation and tryptophol (TrpOH) concentration produced at the beginning, middle and end of fermentation in control fermentations (unsupplemented with TrpOH) containing 300 mg/L or 100 mg/L of yeast assimilable nitrogen (YAN). Fermentations were inoculated with (i) *S. cerevisiae* (A), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B) and (iii) a mixture of four non-*Saccharomyces* species (C). Values are means of triplicate fermentations. Bars represent standard deviation.

conditions. Additionally, non-*Saccharomyces* viability was increased by the addition of MEL in the first hours after inoculation when nitrogen concentration was high. Instead, under low nitrogen conditions, MEL addition extended the survival of the non-*Saccharomyces* yeasts in presence of *S. cerevisiae* until the end of the fermentation and of *S. bacillaris* in NSc fermentations.

TrpOH is produced by the Ehrlich pathway and thus, its synthesis is a way for cells to eliminate tryptophan and use nitrogen (Mas et al., 2014). This agree with the profile of synthesis obtained in this study, since its synthesis occurred during the first stages of the fermentation, when nitrogen is consumed, reaching the highest concentration in the beginning and middle of fermentation, under low and high nitrogen concentrations, respectively. Moreover, the higher is the nitrogen concentration, the higher is the TrpOH synthesis. However, in the case of MEL, the concentration was rather low in comparison to that of TrpOH and the profile of synthesis was very different according to yeast populations. Furthermore, the lack of relation with the concentration of nitrogen present in the medium might point towards different role of this compound in yeasts.

In conclusion, TrpOH and MEL addition caused changes in fermentation kinetics, viability and species distribution during fermentation. Additionally, their presence contributed in different ways to each yeast species studied. Few studies have been performed to describe yeast interactions during wine fermentation. TrpOH and MEL are tryptophan derivatives that have been previously identified or proposed to be growth regulators and associated with cell signalling. The present study focused on this putative role of these compounds as signalling molecules during wine fermentation. The conditions to perform the experiments were selected according to the limited information available about the impact of these molecules in the fermentation process.

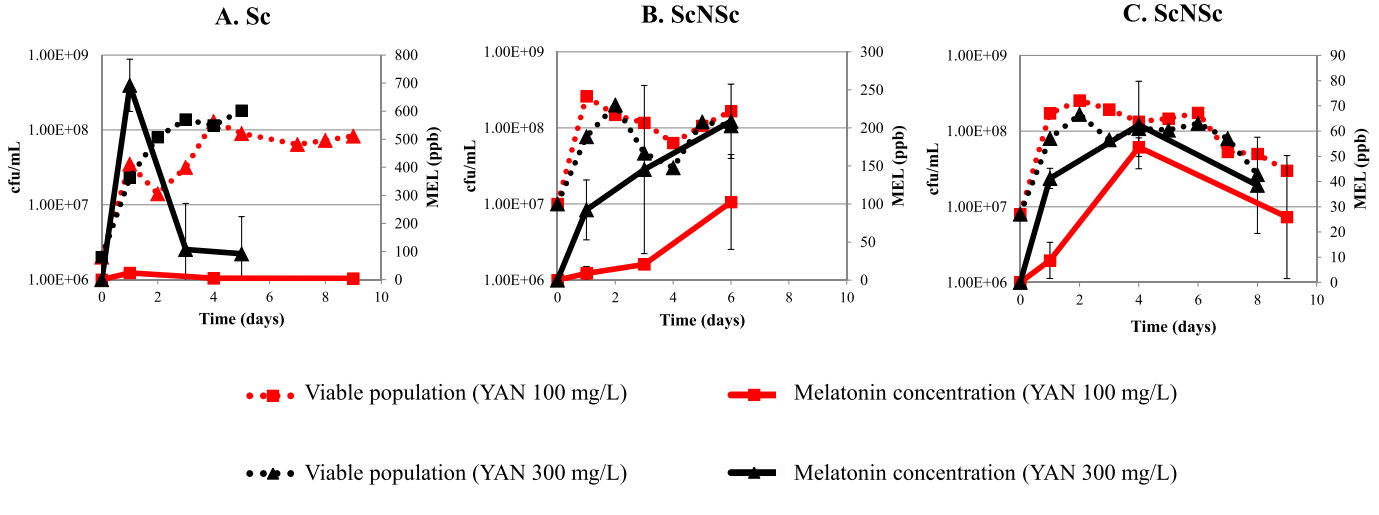

Fig. 6. Yeast populations recovered on YPD medium during fermentation and melatonin (MEL) concentration produced at the beginning, middle and end of fermentation in control fermentations (unsupplemented with MEL) containing 300 mg/L or 100 mg/L of yeast assimilable nitrogen (YAN). Fermentations were inoculated with (i) *S. cerevisiae* (A), (ii) *S. cerevisiae* and a mixture of four non-*Saccharomyces* species (B) and (iii) a mixture of four non-*Saccharomyces* species (C). Values are means of triplicate fermentations. Bars represent standard deviation.

This is a first attempt to study the relation between tryptophan derivatives and interaction between yeast from oenological environments. Further studies must be carried out to elucidate the mechanisms in which they are specifically involved and how to apply these findings in winemaking industry.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.09.013.

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