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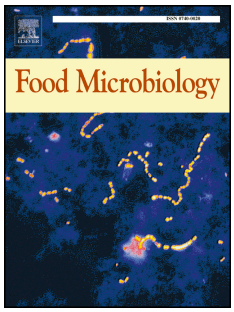

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PII: S0740-0020(19)30471-X

DOI: https://doi.org/10.1016/j.fm.2019.103265

Article Number: 103265

Reference: YFMIC 103265

To appear in: *Food Microbiology*

Received Date: 8 May 2019

Revised Date: 8 July 2019

Accepted Date: 8 July 2019

Please cite this article as: Fracassetti, D., Francesco Lo Faro, A.F., Moiola, S., Orioli, M., Tirelli, A., Iriti, M., Vigentini, I., Foschino, R., Production of melatonin and other tryptophan derivatives by *Oenococcus oeni* under winery and laboratory scale, *Food Microbiology* (2019), doi: https://doi.org/10.1016/j.fm.2019.103265.

Production of melatonin and other tryptophan derivatives by *Oenococcus oeni* under winery and laboratory scale

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Abstract

Malolactic fermentation (MLF) in Valtellina Superiore DOCG red wine was monitored in 4 cellars and the final products were analysed to determine the content of melatonin (MEL) and other tryptophan (TRP) derivatives, including tryptophan ethyl ester (TEE) and MEL isomers (MISs), and to isolate predominant *O. oeni* strains. MEL and TEE significantly increased in wines after MLF from two cellars out of four. Six strains were isolated during the MLF of red wines and under laboratory scale, in rich and synthetic wine cultural media, together with other four *O. oeni* strains able to trigger the MLF. Results showed that the presence of stressful growth factors, like ethanol and acid pH, has a pivotal role in triggering the release of TEE by oenococci. Indeed, all the strains became capable to produce also MEL and MISs, together with TEE. under harsh growth conditions, as in a synthetic wine medium. The production of these compounds was strain-dependent and a maximum amount of 0.0078±0.0023 ng_T/mL (UMB472) and 619.85±196.16 ng_T/mL (UMB436) of MEL and TEE was obtained, respectively. In particular, different MISs were detected under oenological and laboratory scale suggesting that other factors (i.e. technological and/or physico-chemical) could affect the synthesis of TRP derivatives.

Keywords: *Oenococcus oeni*, Melatonin; Tryptophan ethyl ester; Malolactic fermentation; Wine.

1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine; MEL) is a ubiquitous compound exerting several benefits on human health including the regulation of circadian and circannual physiological functions (Bonnefont-Rousselot and Collin, 2010). It is a strong antioxidant since it is able to directly scavenge free radical species (both reactive oxygen and nitrogen species) and stimulate the activity of antioxidant enzymes (Reiter et al., 2009). Several authors reported the presence of MEL in edible plants (Dubbels et al., 1995; Hattori et al., 1995; Paredes et al., 2009), where it acts as a phytohormone protecting against oxidative stress and regulating growth (Manchester et al., 2000; Tan et al., 2012a). The precursor of MEL is L-tryptophan (TRP): in animal, the MEL pathway is via 5-hydroxytryptophan (5-OH-TRP), serotonin, and N-acetyl serotonin intermediates using four serial enzymes (Sprenger et al., 1999; Tan et al., 2007). In plants, MEL is also synthetized via tryptamine instead of 5-OH-TRP (Kang et al., 2007; Lee and Back, 2016). MEL has been found in foods (Manchester et al., 2000; Sturtz et al., 2011; Garcia-Moreno et al., 2012), including grape (Iriti et al., 2006; Vitalini et al., 2011b) and wine (Mercolini et al., 2008; Iriti et al., 2010; Stege et al., 2010; Vitalini et al., 2011a), where MEL isomers (MISs) were also detected (Rodriguez-Naranjo et al., 2011a; Gomez et al., 2012; Vitalini et al., 2013). MISs differ for the position of one of both side chain that can be potentially relocated to any one of the seven positions in the indole ring (Tan et al., 2012b). MIS biosynthesis is still not clear and their activities are related to antioxidant and cytoprotective effects depending on the position of two side chains in the indole ring (Spadoni et al., 2006).

Several factors affect the MEL and MISs in grape and wine, including the environmental and agronomical conditions as well as the winemaking process (Murch et al., 2010; Boccalandro et al., 2011; Vitalini et al., 2011a). Among MISs, one of them was identified as tryptophan-ethyl

ester (TEE) (Gardana et al., 2014; Iriti and Vigentini, 2015) which resulted in higher concentration in comparison to MEL (Vigentini et al., 2015; Fernández-Cruz et al., 2017; Fracassetti et al., 2019). However, the origin and the putative nutritional role(s) of these molecules have not been clarified yet. The alcoholic fermentation (AF) step was shown to increase the content of MEL in wine as well as in other fermented beverages indicating yeasts, both *Saccharomyces* and non-*Saccharomyces*, play a role in its biosynthesis (Rodriguez-Naranjo et al., 2011b; Garcia-Moreno et al., 2012; Mena et al., 2012; Fernandez-Pachon et al., 2014). A relevant influence of grape cultivar and of the time of fermentation was demonstrated (Fernández-Cruz et al., 2018). The metabolic pathway of MEL in yeast has not been completely elucidated. Recently, Muñiz-Calvo and co-authors (2017) suggested that the TRP derivatives in yeast are released by means of synthetic route and enzymes similar to those described in vertebrates. Fernández-Cruz et al. (2019) proved the synthesis of MEL and other indolic compounds derive from TRP metabolism by yeast. However, definitive biochemical and molecular evidence is still missing. The understanding of the complete metabolic pathway by searching homolog genes to those described in vertebrates represents a key point in order to improve the synthesis of these molecules during the fermentation process.

The yeast is not the only relevant microorganism of wine; indeed, several quality wines required the intervention of lactic acid bacteria (LAB) capable to trigger the malolactic fermentation (MLF). In particular, those belonging to *Oenococcus oeni* specie are generally desired. Recently, Meng and co-authors (2017) suggested the LAB can synthesize MEL in wine during the MLF. This is in accordance to Rodriguez-Naranjo et al. (2011b) that found an increase of MEL in Tempranillo wine after the inoculation with *O. oeni*. To the best of our knowledge, this is the only publication hinting the biosynthesis of MEL due to growth and metabolism of *Oenococcus*

genus in wine. Moreover, the microbial population was not determined and the possible presence of yeasts, LAB other than the *O. oeni* strains inoculated and/or acetic bacteria cannot be excluded (Rodriguez-Naranjo et al., 2011b). Thus, the role on MEL, MISs and TEE occurrence needs to be elucidated for LAB.

This study evaluated the release of MEL and other TRP derivatives after the MLF, in order to clarify the contribution of *O. oeni* species on the content of these potential bioactive compounds in wine. For this purpose, red wines were sampled before and after the MLF and the concentrations of MEL, MISs and TEE were determined. In the same samples, the microbial populations responsive for the MLF were monitored, identified and characterized for both their genetic background (molecular characterization) and capability to release TRP derivatives (chemical characterization).

2. Materials and Methods

2.1. Vinification experiments and wine sampling

Spontaneous MLFs were monitored during vinifications of Nebbiolo grapes in 4 different cellars producing Valtellina Superiore DOCG wines in vintage 2016. All winemaking plants and practices (i.e. tanks, work volumes and cellar temperatures, sulfite addiction, wine re-filling, etc.) followed the procedures usually adopted in the wineries and by the involved winemakers. Vinifications were carried out in duplicate in two separated batches (A and B) using two identical aliquots of the wine at the end of the AF. Table 1 summarises the type of tanks, work volumes and cellar temperatures.

Wine samples were collected after racking at the end of AF, during the MLF and after the end of the latter. The sampling was carried out in sterilized flasks and conserved at 4°C maximum for 12 hours before chemical and microbiological analyses.

2.2. Chemical analyses

Quantification of glucose, fructose, citric acid, L-malic acid and L-lactic acid was performed using enzymatic kits (Scil Diagnostics GmbH, Germany). Titrable acidity (expressed as g/L of tartaric acid, endpoint pH 7.0) and pH determinations were carried out by automatic titrator Titromatic 2S (Crison, Spain). Free and combined sulfur dioxide concentrations were measured by an automatic iodometric titration (I_2 0.05 mol/L; Carlo Erba, Italy) with Ripper method (Titromatic 2S, Crison). Volatile acidity was determined by electronic oenochemical distillatory apparatus (DEE) equipped with VADE steam generator (Gibertini, Italy) and subsequent acid-base titration. Results are expressed as mean value of duplicated determinations. The MLF yield (%) was calculated as molar ratio between the malic acid consumed and the lactic acid produced. MEL, MISs, TEE and TRP in wine and laboratory samples were determined by HUPLC-ESI-MS/MS as described by Fracassetti et al. (2019). The purification was carried out by solid phase extraction (SPE) technique using Strata X-Polymeric Reversed Phase 200 mg/3 mL cartridge (Phenomenex, Torrance, CA, USA). Briefly, 5 mL of sample were loaded and the elution was carried out with methanol 100% after washing with 5 mL formic acid 0.1% (v/v) and then with 5 ml of methanol 40% (v/v). Methanol was evaporated under vacuum (Rotavapor R 110, Büchi) and the sample was re-suspended in 250 µL of methanol 10% acidified with formic acid 0.1% (v/v) corresponding to a concentration folds of 20. The chromatographic and detection

126 conditions as well as identification and quantification were as reported by Fracassetti et al.
127 (2019).
128 The concentration of free amino acids (AAs) was determined by derivatization with o-ptaldehyde
129 (OPA). The derivatization was carried out as follows: 750 µL of borate buffer 0.4 M at pH 10.5,
130 300 µL of wine samples and 150 µL of OPA (25 g/L dissolved with ethanol/borate buffer 0.4 M
131 at pH 10.5 15/85 and added with 20 g/L 3-mercaptoethanol); the reaction mix was mixed for 5
132 minutes prior the injection. Amino acids were separated in a UPLC system (HClass, Waters)
133 coupled with a diode array detector (model 2696, Waters). Chromatographic separations were
134 performed in a Kinetex Phenyl-Hexyl column (150 x 4.6 mm, with 2.6 µm particle size,
135 Phenomenex, Torrance, CA, USA) maintained at 50°C and the injection volume was 20 µL. The
136 separation was carried out by a binary gradient using citrate buffer 10 mM at pH 7.5 (A) and
137 methanol/acetonitrile/citrate buffer 10 mM at pH 7.5 70/20/10 (B) and the flow rate was 1
138 mL/min. The gradient program was 3 min, 95% A; 3-6.5 min 85% A; 6.5-9 min, 80% A; 9-12
139 min, 70% A; 12-14.5, 60% A; 14.5-23 min, 20% A. Aspartic acid, glutammic acid, asparagine,
140 serine, glutammine, istidine, threonine, arginine, alanine, tyrosine, valine, methionine,
141 phenylalanine, isoleucine, leucine, ornithine and lysine were detected at 338 nm. AAs were
142 quantified by the external standard method (linearity range: 1-100 mg/L). Chromatographic data
143 acquisition and processing were performed by Empower 2 software (Waters). The average limit
144 of detection was 0.28 mg/L and the average repeatability of the method, expressed as relative
145 standard deviation (RDS%), 95.7%.
146 The content of tryptamine was determined by derivatization with dinitrobenzoyl-chloride
147 (DNBZ-Cl) (Kirshbaum et al., 2000). Briefly, 8.1 mL and wine samples were diluted up to 10
148 mL with methanol (19%) in a volumetric flask and 2 mL were purified through a lab-made SPE

cartridge packed with 300 mg polyvinylpyrrolidone (DalCin, Sesto San Giovanni, Milan, Italy). Elution was carried out with 2 mL of 20% methanol solution, and the loaded and eluted sample was recovered in the same tube. The derivatization was carried out as follows: 80 µL of purified sample, 50 µL of NaOH 2 M, 70 µL of 2-propanoland and 210 µL of DNBZ-Cl 50 mM (dissolved in acetonitrile) were mixed and, after 10 minutes, the reaction was stopped by the addition of 100 µL of HCl 1 M. The mixture was filtered with a PVDF 0.22 µm filter (Millipore, Billerica, MA, USA) and analysed by an Acquity HClass UPLC (Waters, Milford, MA, USA) system equipped with photodiode array detector 2996 (Waters) injecting 10 µL. The column used was Kinetex C18 100 x 3 mm, 2.6 µm particle size, 100 Å pore size (Phenomenex, Torrence, CA, USA) and it was maintained at 30°C. The separation was carried out by a ternary gradient using sodium citrate 30 mM at pH 7.0 (A), sodium acetate 20 mM at pH 4.3 (B), and acetonitrile (C) (Table S1) and the flow rate was 0.6 mL/min. Tryptamine was detected at 260 nm and quantified by the external standard method (linearity range: 1-100 mg/L). Chromatographic data acquisition and processing was performed by Empower 2 software (Waters). The limit of detection was 0.33 mg/L and the repeatability of the method, expressed as relative standard deviation (RDS%), was 98.7%.

2.3. Bacterial isolation, identification and molecular characterization

Suitable decimal dilutions of each wine sample were prepared in peptone water (1% w/v meat peptone, pH 7.2) and, then, 100 µL of cell suspensions were spread on Petri dishes containing MRS (BD Difco, Thermo-Fisher Scientific, Waltham, MA, USA) solid medium (1.5% w/v agar agar) at pH 6.2, supplemented with 20% (v/v) apple juice and 0.01% (w/v) cycloheximide (MRSAJ). Cells were grown in anaerobic conditions at 30°C for 7-10 days. Bacteria isolation

was performed from Petri dishes when the initial concentration of L-malic acid in wines was halved. In detail, a number of isolates corresponding to the square root of number of colonies present on a plate (between 30 and 100 colonies) was randomly isolated for species identification and strain typing; this activity was repeated for each colony type if a different morphology or cell shape was observed. Bacteria collection was maintained in MRSAJ medium added with glycerol 20% (v/v) at -80˚C.

To assess the presence of yeasts during the MLF, 1 mL of wine sample was centrifuged at 16900 *x g* (Rotina2424, Hettich, Tuttlingen, Germany) for 3 min and the pellet was resuspended in a final volume of about 100 µL of sterile distilled water. Then, the cell suspension was spread on WL medium (Sigma-Aldrich, Darmstadt, Germany) added with 0.1 g/L chloramphenicol and the plate was incubated at 25°C for 7-10 days.

Genomic DNA was obtained from cellular cultures after a lysozyme (50 mg/mL)/proteinase K (20 mg/mL) treatment and phenol:chloroform:isoamilic alcohol (25:24:1) purification and precipitation steps (Vigentini et al., 2009; Vigentini et al., 2016). Amplification of 16S/23S rDNA spacer region was performed according to Guerrini et al. (2003). Partial sequence of 16S rDNA gene was obtained as previously described (Lane, 1991). DNA separation was obtained on 1% (w/v) agarose gels stained with ethidium bromide (0.5 µg/mL), photographed under UV light, and analysed by a GelDoc XR System (BioRad, Hercules, CA, USA).

The molecular characterization of *O. oeni* isolates collected from wines was obtained by Pulse Field Gel Electrophoresis – Restriction Fragment Length Polymorphism (PFGE-RFLP) analysis according to Vigentini et al. (2009). In order to determine the lowest value of genetic similarity at which clones potentially belonging to a same strain, genomic DNAs obtained from two

biological replicates of the isolates UMB471, 473, 475 and 497 were loaded on different gels and compared.

2.4. Assessment of the production of TRP derivatives by *O. oeni* under laboratory scale: culturing media and growth conditions

Ten *O. oeni* strains were characterised for the capability of producing TRP-derivatives: 6 strains were isolated in the present work, 3 strains derived from a previous study (Vigentini et al., 2009) and the *O. oeni* type strain DSM20252^T was included as a species reference (Dicks et al., 1995) (Table 2). Two different cultural liquid media were used: *i*) a modified rich medium, called "mMRSAJ", prepared with MRSAJ supplemented with 100 mg/L TRP, 5% (v/v) ethanol at pH 4.5, and *ii*) a chemically defined liquid medium similar in composition to a wine, called "synthetic wine", prepared according to the OIV protocol (Resolution OIV-Oeno 370-2012) with few modifications: 1.7 g/L YNB (Sigma-Aldrich, Germany), 2.5 g/L glucose, 2.5 g/L fructose, 10% (v/v) ethanol, 5 g/L glycerol, 5 g/L tartaric acid, 0.5 g/L L-malic acid, 0.2 g/L citric acid, 4 g/L L-lactic acid, 5 mg/L oleic acid, 0.5 mL/L Tween 80, 15 mg/L ergosterol, 20 mg/L uracil, pool of amino acids as indicated by Contreras and collaborators (2018), adjusted at pH 3.5 with 2MNaOH. In synthetic wine the final concentration of TRP was 500 mg/L. An aliquot of each media was incubated at the same described conditions of cell cultures to verify the possible degradation of TRP in the experimental conditions adopted.

All strains were preliminary inoculated in 5 mL of MRSAJ liquid medium adjusted at pH 6.2, in anaerobic condition at 25°C for 3 days. Cells in early stationary phase (approximately 2 $OD_{600\ nm}$) were centrifuged at 6000 *x g* (Rotina 380R, Hettich) for 10 min and washed in sterile dH_2O. Pellets were re-suspended in 5 mL of physiological solution (0.9 g/L NaCl) and the inocula were

done in the useful medium at 0.20±0.05 OD_{600nm} using the following correlation CFU/mL-OD_{600nm}: UFC/mL = $2*10^9x - 4*10^8$, $R^2 = 0.9491$ (obtained using the strain UMB473) where x corresponds to OD_{600nm}.

As far the experiments in mMRSAJ, the cellular cultures were carried out in 6-well sterile plates with 10 mL of growth medium, in anaerobic and static conditions and in a dark environment, at 25°C. The monitoring for microbial parameters was performed by collecting 1 mL of the microbial cultures at the time of the inoculum and every 48h until the cells reached the stationary phase. The sample was centrifuged at 6000 *x g* (Rotina 380R, Hettich) for 10 min and was used for the determination of the bacterial cell count. At the stationary phase, the microbial cultures were collected and centrifuged at 6000 *x g* (Rotina 380R, Hettich) for 10 min and the supernatant was kept at -20°C until HUPLC-ESI-MS/MS analysis for the detection of the analytes of interest, including MEL, MISs and TEE. The survey in synthetic wine was performed in sterile glass vials in anaerobic and static conditions and in a dark environment, at 25°C. The inoculum was obtained as described for the mMRSAJ medium. The cellular growth was monitored by OD_{600nm} and cell count determination (UFC/mL). The supernatants were maintained at -20°C and those corresponding to the maximum biomass level were submitted to the analysis of MEL and TRP derivatives. In order to compare the productions of the compounds of interest by the different *O. oeni* strains, a normalization of the volumetric productions was applied. In detail, the volumetric production of MEL, MISs and TEE (ng/mL) of each strain was divided for the corresponding cell biomass (UFC/mL) obtaining a value in ng/cell. Taking into account that during the MLF of real wines a level of about 10^7-10^8 cell/mL can be reached (i.e. Figure 1 and Table 4; González-Arenzana et al., 2012; Vigentini et al., 2016), the production per cell was multiplied for 10^8 UFC/mL obtaining the "theoretical volumetric production" (ng_T/mL).

240

241 2.5. Statistical analysis

242 Statistical analysis was performed with SPSS Win 12.0 program (SPSS Inc., Chicago, IL). The
243 equations of the calibration curves were assessed by the linear regression analysis. The values
244 were presented as means±standard deviation. Differences were evaluated by the T-test, and the
245 significances were set at a value of $p < 0.05$. The correlation coefficient between MEL release
246 and ethanol concentration was computed through the Pearson correlation.

247

248 3. Results and Discussion

249 3.1. Monitoring of the spontaneous MLFs and detection of TRP derivatives under winery
250 scale

251 The development of the MLF was followed through the detection of chemical parameters and
252 LAB plate counts (Figure 1 and Table 3). No yeast colonies were isolated before and at the end
253 of the MLF (Table 3). Wines were analysed every week until L-malic acid and L-lactic acid
254 concentrations were stable. Variations in the amounts of carbon sources (glucose, fructose and
255 citric acid), pH, volatile and total acidity were also determined (Table 3). The increase of L-lactic
256 acid was observed indicating the MLF took place in all the wines (Figure 1). MLF duration was
257 different among cellars and it corresponded to 37, 62, 56 and 158 days, respectively for cellar #1,
258 #2, #3 and #4. These differences could be due to the fermentation temperature that were 18°C for
259 winery #1, 14-19°C for wineries #2 and #3, and 11-22°C for winery #4. Even if the temperature
260 was not under control, except for cellar #1, the lowest temperature found in cellar #4 could have
261 prolonged the MLF duration. MLF yield slightly differed among cellars (90-96%) except for the
262 wine produced in cellar #1 (67%). In the MLFs at cellar #4 a major reduction of carbon sources

(glucose, fructose and citric acid) was found. As expected, all the wines showed the decrease of total acidity and an increase of pH (Table 3). Higher volatile acidity was revealed in all the wines at the end of MLF, especially the one produced in cellar #3 (+ 0.60 g/L).

The total concentration of free AAs greatly differed among the wines analysed from 411.23±30.17 mg/L for wines produced in cellar #1 to 48.48±3.59 mg/L for those produced in cellar #2 at the end of MLF (Table S2). In each vinification a specific trend and AA profile were found. Tryptophan was revealed in all the wine samples (3.44±0.34 mg/L, 0.83±0.08 mg/L, 0.64±0.06 mg/L and 1.27±0.12 mg/L for wines of cellars #1, #2, #3 and #4, respectively at the end of MLF) indicating the presence of the MEL precursor and the possible release of this bioactive compound in wine. The tryptamine content showed the highest variation (+ 7.26±0.08 mg/L) for the wine produced in cellar #3 reaching a final concentration of 13.84±0.00 mg/L. For the other wines, the increase of tryptamine was negligible or about 1 mg/L and its amounts at the end of MLF were 8.49±0.22 mg/L, 5.93±0.52 mg/L and 6.17±0.17 mg/L for wines produced in cellar #1, cellar #2 and cellar #4, respectively (Table S3). The content of tryptamine revealed in the wines was higher in comparison to data recently reported for Monastrell wine (Di Lorenzo et al., 2017). Even if LAB are usually responsible for the presence of biogenic amines in wine, *S. cerevisiae* strains able to produce melatonin showed also the ability in releasing tryptamine (Fernández-Cruz et al., 2019). The presence of biogenic amines is affected by several factors including nitrogen content, winemaking process, time and storage conditions, raw material quality, yeast and LAB strains and possible microbial contamination during winery operations. We could hypothesize the winemaking process adopted could lead to a high content of tryptamine. Further investigations will be carried out in order to confirm this hypothesis.

The wine samples of the four wineries, collected at the beginning and at the end of the MLF, were purified following the SPE purification protocol developed by Fracassetti and collaborators (2019) and the analysis was performed in HUPLC-ESI-MS/MS to identify and quantify MEL, MISs and TEE. In all the samples both MEL and TEE were detected (Table 4). In general, the production of MEL was lower than that of TEE of about 1000 folds as an average. In particular, a significant increase in MEL at the end of MLF was measured for the samples of cellars #1 (0.112±0.006 ng/mL) and #4 (0.114±0.06 ng/mL), while for TEE the increase was significant for the samples of the cellar #1 (97.44±4.35 ng/mL), #2 (22.85±1.02 ng/mL) and #4 (39.60±1.77 ng/mL). Five MISs were detected and the *O. oeni* contribution was significant for MIS 4 in wines produced in cellars #2 and #3 (Table 4). The increase of MEL for wines from cellars #1 and #4 could be of biological interest since it resulted 10 folds higher than the plasmatic level of MEL in humans during the day (< 10 pg /mL) (Bonnefont-Rousselot and Collin, 2010); however, bioavailability studies are necessary to confirm this hypothesis.

The molecular characterization showed that two *O. oeni* strains were isolated from the cellar #1; a single strain was collected in cellar #2; two strains drove the MLF in cellar #3; one strain was recognized in cellar #4. Except in cellar #1, all strains were found to be dominant in both batches of vinification used as biological replicates (Table 1). These 6 strains (represented by UMB471, 472, 474, 475, and 477 isolates) together with those listed in Table 2, were further characterised for the production of TRP derivatives in laboratory and oenological conditions.

3.2. Production of TRP derivatives by *O. oeni* under laboratory scale in mMRSAJ medium

In general, the precursor TRP was found to be definitively stable under all the experimental conditions used in this study. Aliquots of media not inoculated were stored at the same

conditions as the inoculated ones and no significant difference of TRP concentration was found (105.5±9.6 mg/L before inoculum and 98.6±9.0 mg/L after incubation, as an average). To evaluate the possible release of TRP derivatives from the strains of the collection (Table 2), preliminary experiments were performed in MRSAJ broth supplemented with 100 mg/L TRP at pH 6.2. Results showed that neither MEL nor TRP derivatives were produced during the bacterial growth in the rich medium and under the set experimental conditions. Nevertheless, antioxidant capacity (Reiter et al., 2009) and protection against stress conditions (Tan et al., 2012a; Bisquert et al., 2018) of MEL are well-known. Thus, to verify whether a production of TRP-derived compounds by the investigated *O. oeni* strains could be favored by more stressful environmental conditions, but with less impact than a wine, the bacteria were grown in mMRSAJ broth [5% (v/v) ethanol, pH 4.5]. Data revealed that under stress conditions (acidic pH and ethanol presence) all the *O. oeni* strains were able to release TEE (Table 2). Since TEE was not detected in the medium not inoculated, the metabolic activities of LAB led to the release of TEE.

3.3. Production of TRP derivatives by *O. oeni* under laboratory scale in synthetic wine

Due to the encouraging results obtained from the set of experiments performed under stressful growth conditions (mMRSAJ medium), all *O. oeni* strains considered in this study were inoculated in a synthetic wine with the aim of assessing the possible production of TRP derivatives other than TEE. Oenococci reached a maximum biomass level of about $3.6\pm0.7 \times 10^8$ UFC/mL and the production of MEL was always detected in synthetic wine (Table 2). The strains were classified into three categories: low (MEL $\leq$ 0.0015 ng_T/mL), medium (0.0016 < MEL < 0.0030 ng_T/mL) and high (MEL $\geq$ 0.0030 ng_T/mL) producers. One strain belonged to the first category of low producers, UMB473. Six bacteria were classified as medium producers,

including UMB436, 438, 468, 471, 474, and 475, and four strains (UMB434, 462, 472 and 477) were enclosed in the high producer category. In particular, the UMB472 strain was the highest producer being able to release about 0.0078 ± 0.0023 ng_T/mL of MEL under our experimental conditions. Following the data obtained in synthetic wine tests, it was possible to observe that, in the monitored vinifications, the MLF was driven by: medium-high producer strains in cellar #1, one medium MEL producer in cellar #2, low-medium producing strains in cellar #3, and high producer LAB in winery #4. Moreover, the concentrations produced in synthetic wine medium could further increase in a real wine as reported data in Table 4.

Considering ethanol as a stress factor for microorganisms, a positive correlation (0.83) between alcohol content, in natural wines, and MEL production could be observed; in fact, the wines produced by cellar #4 were the ones with the highest ethanol content, followed by the cellar #1, from which medium producer strains were isolated. In the wines from winery #3, characterized by an even lower ethanol content, the medium-low producer strains were collected and, finally, the cellar #2, including only a low producer strain, produced the wines with the lowest ethanol percentage. Beside ethanol, other stress chemical parameters could affect the production of MEL. Wine from cellar #3 showed the highest level of total sulfur dioxide and the highest increase of both volatile acidity (+ 0.60 g/L) and tryptamine (+ 7.26±0.08 mg/L). Even if the latter compound has been reported as intermediate of the MEL pathway in plants (Kang et al., 2007), the mechanisms and the enzymes involved in microorganisms have not been described, yet. We could hypothesize only the pathway via 5-OH TRP takes place, like in animals (Sprenger et al., 1999; Tan et al., 2007), and the possible accumulation of tryptamine limits the conversion of TRP into 5-OH TRP and, as a consequence, into MEL. Regarding the UMB434, 436 and 438 strains, isolated from a previous research (Vigentini et al., 2009), they were here all

354 classified among the medium-high producers of MEL. In particular, it has to be noted that they
355 are capable of growing under extreme environmental stress conditions of ethanol [up to 12%
356 ethanol (v/v)] and of temperature (5°C). With regard to this last feature, the UMB434 strain
357 which was also the highest producer of MEL was able to develop in just 14 days from the
358 inoculation compared to the other two (UMB436 and UMB438) that could grow after about 35
359 days from inoculation. Comparing the amounts of MEL produced by the investigated *O. oeni*
360 strains with those released by yeasts (Vigentini et al., 2015), MEL levels resulted lower of about
361 500-folds. This indicates the yeast is a more efficient producer of MEL. However, the amount
362 produced by the high-producer strains could have a biological significance due to the potential
363 increase of the plasmatic concentration of MEL in humans at day time (Bonnefont-Rousselot and
364 Collin, 2010), thanks also to the combined consumption of fermented foods.
365 Similarly to MEL, all the investigated strains produced TEE. Based on the amount of TEE
366 released, three categories were identified: low (TEE $\leq$ 10 ng_T/mL), medium (10 < TEE < 30
367 ng_T/mL) and high (TEE $\geq$ 30 ng_T/mL) producers. The group of low producers was the most
368 numerous and included the UMB434, 438, 462, 475, 477 strains. The group of medium
369 producers grouped the strains UMB469, 472, 473, 474 and, finally, the two strains UMB436 and
370 471 were found to be the major producers where the former produced about 619.85±196.19
371 ng_T/mL of TEE. In the case of TEE, in wines of cellar #1 two *O. oeni* strains conducted the MLF
372 that resulted one medium and one high producers; in winery #2 wines only one strain was
373 dominant and it belonged to medium producers; MLF was dominated by medium-low producer
374 strains in cellar #3; a low producer was isolated from wines produced in cellar #4. A relation
375 between the release of MEL and TEE could be hypothesized: considering the ratio between TEE
376 and MEL produced, UMB462, 472, and 477, grouped among the low-medium TEE producers

and high MEL producers, showed the lowest ratio. On the other hand, the opposite effect could not be detected whereas UMB436 and 471, identified as high TEE producers, were capable of release only a medium amount of MEL ($0.0015 < MEL < 0.0030$ ng_T/mL). Contrarily to MEL, TEE produced by LAB was comparable, at the same order of magnitude, to that produced by yeasts (Vigentini et al., 2015). Concerning the MISs, only three strains of *O. oeni* released MIS 5 in concentration comparable or higher than MEL (Table 2). In any case, TEE represented the major TRP derivative produced in our experimental conditions. Amino acid esters rapidly cross the cell membrane where they can generate the native amino acids through hydrolysis (Jonas and Butler, 1989). The accumulation of TEE could represent a possible cell strategy for preserving TRP and metabolize it in case of nitrogen deficiency, starvation or for the synthesis of TRP derivatives (*i.e.* MEL) or other amino acids.

4. Conclusions

In this study, the capability of *O. oeni* species in producing MEL and other TRP derivatives was proved. The increase of TRP derivatives was found in red wines after the MLF, especially where the ethanol concentration was higher. Other factors, such as the volatile acidity and the production of tryptamine, in particular, seemed to affect the release of TRP derivatives. Under laboratory scale, the LAB synthesized these compounds when the environmental conditions were stressful due to the presence of ethanol and the acidic pH. The released amounts were different among the investigated strains indicating that the production of TRP derivatives is a strain-dependent feature. In all the cases, TEE was the most abundant TRP-derived compound. Our results showed the production of different MISs under winery and laboratory scale suggesting that other factors (i.e. winery practices, content of phenols, microbial growth

400 inhibitors/enhancers, etc.) could affect the synthesis of TRP derivatives. Further studies focusing
401 on the TRP metabolism will be necessary to better understand the pathway(s) behind the
402 synthesis of TRP derivatives. Attention will be given to the winemaking and the wine
403 composition in order to improve the production of these healthy compounds.

404

405 **Acknowledgements**

406 We are grateful to the cellars for making the wine and donating the samples.

407

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573

574 **Figure captions**

575 **Figure 1**

576 Malolactic fermentation trends observed in Valtellina Superiore DOCG red wines produced in 4
577 wineries.

578

579 **Figure S1**

580 Dendrogram of genetic similarity among the *O. oeni* strains isolated from Valtellina Superiore
581 DOCG red wines on the basis of the Pulse Field Gel Electrophoresis – Restriction Fragment
582 Length Polymorphism (PFGE-RFLP) profiles.

583

584 **Table 1**

585 Winemaking conditions used during malolactic fermentations (MLFs) and *O. oeni* isolates in this study.
586 Underlined numbers represent the *O. oeni* strains selected for the determination of MEL and TRP-
587 derivative production in MRSAJ medium supplemented with 100 mg/L TRP [medium *i*)] and in synthetic
588 wine [medium *ii*)]. Pulsotype numbers derive from the molecular characterization of all 34 isolates of *O.*
589 *oeni* collected from the vinification experiments. A and B letters identify the two batches used for the
590 vinification replicates.

591

Cellar (#)	Batch type	Batch capacity (hL)	MLF temperature (˚C)	Isolate designation (UMB#)/Pulsotype (PT)/ Replicate (A or B)
1	Wood barrels	33	Controlled, 18	**472/6/B**, **471/2/B**, 487/2/B, 489/2/B, 490/2/B, 505/6/B, 506/6/A, 507/6/B
2	Steel tanks	1	Not controlled, 14-19	**473/1/A**, 478/1/A, 479/1/A, 480/1/A, 481/1/B, 482/1/B, 483/1/B, 485/1/B, 486/1/B
3	Wood barrels	11	Not controlled, 11-15	**474/4/A**, **475/3/B**, 476/3/A, 491/3/B, 493/3/B, 494/3/A, 495/3/A, 496/3/B, 497/4/A, 498/4/B, 499/4/A
4	Steel tanks	20	Not controlled, 11-22	470/5/B, **477/5/B**, 501/5/A, 502/5/A, 503/5/B, 504/5/A

Table 2

Bacterial growth (CFU/mL) and theoretical volumetric production of tryptophan derivatives (ng_T/mL) in MRSAJ broth [5% ethanol (v/v), pH 4.5] and in synthetic wine medium (n=3). MEL: melatonin; TEE: tryptophan ethyl ester; MIS: melatonin isomer. MEL and MISs were not detected in MRSAJ broth.

Strain	Origin	Isolation	MRSAJ broth		Synthetic wine medium							
			CFU/mL	TEE	CFU/mL	MEL	TEE	MIS 1	MIS 2	MIS 3	MIS 4	MIS 5
O. oeni UMB434	Wine	Vigentini et al., 2009	$2.2\pm0.2x10^9$	12.30±0.83	$3.1\pm0.6x10^8$	0.0036±0.0007	7.06±3.16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
O. oeni UMB436	Wine	Vigentini et al., 2009	$3.2\pm0.3x10^9$	16.67±1.91	$2.8\pm0.6x10^8$	0.0022±0.0008	619.85±196.16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
O. oeni UMB438	Wine	Vigentini et al., 2009	$3.6\pm0.4x10^9$	2.27±0.11	$2.7\pm0.6x10^8$	0.0027±0.0004	4.71±0.41	< LOQ	< LOQ	< LOQ	< LOQ	0.0116±0.0024
O. oeni UMB462 (DMS20252^T)	Wine	Dicks et al., 1995	$1.6\pm0.2x10^9$	2.96±0.14	$2.0\pm0.5x10^8$	0.0049±0.0010	7.66±0.97	< LOQ	< LOQ	< LOQ	< LOQ	0.0103±0.0066
O. oeni UMB471	Wine	This study	$3.2\pm0.3x10^9$	15.42±0.63	$2.2\pm0.5x10^8$	0.0026±0.0009	609.71±17.23	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
O. oeni UMB472	Wine	This study	$3.4\pm0.3x10^9$	1.38±0.13	$2.3\pm0.6x10^8$	0.0078±0.0023	10.03±2.26	< LOQ	< LOQ	< LOQ	< LOQ	0.0061±0.0035
O. oeni UMB473	Wine	This study	$3.6\pm0.4x10^9$	2.54±0.15	$3.3\pm0.6x10^8$	0.0013±0.0002	15.97±6.41	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
O. oeni UMB474	Wine	This study	$3.7\pm0.4x10^9$	10.39±0.97	$2.5\pm0.6x10^8$	0.0021±0.0005	15.64±2.52	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
O. oeni UMB475	Wine	This study	$3.3\pm0.3x10^9$	3.32±0.48	$3.6\pm0.7x10^8$	0.0025±0.0013	4.04±1.26	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
O. oeni UMB477	Wine	This study	$4.0\pm0.4x10^9$	15.67±1.67	$3.3\pm0.6\pm x10^8$	0.0049±0.0007	6.46±0.71	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

596 **Table 3**

597 Chemical composition and microbial counts of Valtellina Superiore DOCG red wines determined at the end of AF and MLF. n.d.: not

598 detected.

Parameter/cellar	#1		#2		#3		#4	
	End AF	End MLF	End AF	End MLF	End AF	End MLF	End AF	End MLF
Glucose (g/L)	0.80±0.00	0.19±0.01	0.13±0.01	0.13±0.03	0.18±0.00	0.11±0.00	0.21±0.12	0.21±0.00
Fructose (g/L)	2.90±0.73	0.82±0.22	0.12±0.02	0.12±0.01	0.15±0.01	0.15±0.00	2.07±0.00	1.68±0.12
L-malic acid (g/L)	2.52±0.07	0.04±0.01	2.93±0.02	0.02±0.01	3.46±0.00	0.14±0.06	3.90±0.01	0.10±0.06
L-lactic acid (g/L)	0.05±0.00	1.15±0.00	0.13±0.00	2.02±0.01	0.38±0.01	2.56±0.10	0.07±0.00	2.43±0.13
Citric acid (g/L)	0.41±0.00	0.27±0.00	0.33±0.01	0.23±0.02	0.32±0.01	0.12±0.01	0.38±0.09	0.26±0.03
Ethanol (% v/v)	13.04±0.00	13.19±0.09	11.12±0.03	11.66±0.00	11.79±0.00	11.90±0.00	15.51±0.06	15.86±0.00
pH	3.56±0.03	3.95±0.01	3.22±0.01	3.28±0.02	3.47±0.00	3.65±0.01	3.72±0.00	3.80±0.00
Total acidity (g/L)	6.85±0.24	5.66±0.25	8.51±0.01	7.21±0.01	7.27±0.02	5.35±0.07	8.48±0.03	5.96±0.00
Volatile acidity (g/L)	0.39±0.00	0.57±0.00	0.30±0.00	0.41±0.02	n.d.	0.60±0.00	0.39±0.00	0.53±0.06
Free SO_2 (mg/L)	10.50±0.71	10.00±1.41	5.00±0.00	5.00±0.00	10.50±0.71	9.00±0.00	7.00±0.00	8.00±1.41
Total SO_2 (mg/L)	40.50±4.95	34.00±5.66	19±1.14	17±0.00	49.00±0.00	43.50±0.71	21.50±0.71	23.00±1.41
Cell count (Log CFU/mL)	4.18±1.49	7.21±0.12	5.03±0.05	7.24±0.17	6.63±0.00	5.54±0.34	4.29±0.41	6.18±0.04
Yeasts (CFU/mL)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

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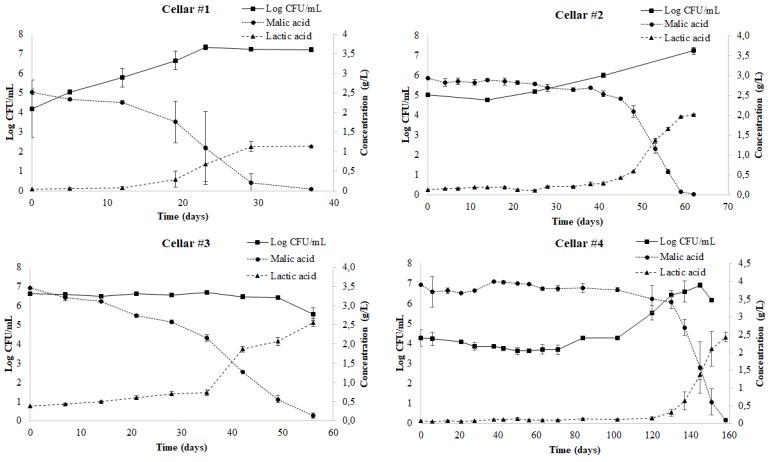
Table 4

Quantification of TRP derivatives (ng/mL) determined in Valtellina Superiore DOCG red wines at the end of AF and MLF. MEL: melatonin; TEE: tryptophan ethyl ester; MIS: melatonin isomer. The symbol * indicates significant difference ($p < 0.05$) between the end of AF and the end of MLF.

TRP derivative/ Cellar	#1		#2		#3		#4	
	End AF	End MLF	End AF	End MLF	End AF	End MLF	End AF	End MLF
MEL	0.009±0.000	0.121±0.007*	0.014±0.002	0.022±0.002	0.077±0.004	0.068±0.004	0.098±0.005	0.212±0.011*
TEE	4.89±0.22	102.33±4.57*	43.19±1.93	66.03±2.95*	40.93±1.83	41.24±1.24	20.75±0.93	60.34±2.69*
MIS1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
MIS 2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
MIS 3	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
MIS 4	0.072±0.004	0.085±0.005	0.051±0.003	0.082±0.004*	0.004±0.000	0.015±0.001	0.146±0.008	0.156±0.008
MIS 5	0.125±0.007	0.111±0.006	< LOQ	< LOQ	< LOQ	< LOQ	0.134±0.007	0.093±0.005



Production of melatonin and other tryptophan derivatives by *Oenococcus oeni* under laboratory and oenological conditions

Highlights

- Tryptophan derivatives were detected during the malolactic fermentation
- Release of tryptophan derivatives by *Oenococcus oeni* strains was investigated
- Stress factors (ethanol and acid pH) trigger the production of tryptophan derivatives by *O. oeni*
- *O. oeni* produced melatonin and tryptophan derivatives in synthetic wine medium
- Oenococci-mediated release of tryptophan derivatives resulted strain-dependent

Production of melatonin and other tryptophan derivatives by *Oenococcus oeni* under winery and laboratory scale

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Declarations of interest: none.