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Aromatic amino acid-derived compounds induce morphological changes and modulate the cell growth of wine yeast species

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Author contribution statement

BG design and perform experiments, analyze and discuss results, and writing the manuscript. JV perform experiments, analyze and discuss results. PC design experiments, discuss results and manuscript. AM, MJT and GB design experiments, analyze and discuss results, writing the manuscript and rise funding.

Keywords

Aromatic alcohols, Serotonin, tryptamine, Quorum Sensing, Pseudohyphal growth, Non-Saccharomyces, Invasive growth

In review

Abstract

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Yeasts secrete a large diversity of compounds during alcoholic fermentation, which affect growth rates and developmental processes, like filamentous growth. Several compounds are produced during aromatic amino acid metabolism, including aromatic alcohols, serotonin, melatonin and tryptamine. We evaluated the effects of these compounds on growth parameters in 16 different wine yeasts, including non-Saccharomyces wine strains, for which the effects of these compounds have not been well-defined. Serotonin, tryptamine and tryptophol negatively influenced yeast growth, whereas phenylethanol and tyrosol specifically affected non-Saccharomyces strains. The effects of the aromatic alcohols were observed at concentrations commonly found in wines, suggesting a possible role in microbial interaction during wine fermentation. Additionally, we demonstrated that aromatic alcohols and ethanol are able to affect invasive and pseudohyphal growth in a manner dependent on nutrient availability. Some of these compounds showed strain-specific effects. These findings add to the understanding of the fermentation process and illustrate the diversity of metabolic communication that may occur among related species during metabolic processes.

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9

10 **Keywords: Aromatic alcohols, serotonin, tryptamine, quorum sensing, pseudohyphal growth,**
11 **non-Saccharomyces, invasive growth**

In review

12 **Abstract**

13 Yeasts secrete a large diversity of compounds during alcoholic fermentation, which affect growth rates
14 and developmental processes, like filamentous growth. Several compounds are produced during
15 aromatic amino acid metabolism, including aromatic alcohols, serotonin, melatonin and tryptamine.
16 We evaluated the effects of these compounds on growth parameters in 16 different wine yeasts,
17 including non-*Saccharomyces* wine strains, for which the effects of these compounds have not been
18 well-defined. Serotonin, tryptamine and tryptophol negatively influenced yeast growth, whereas
19 phenylethanol and tyrosol specifically affected non-*Saccharomyces* strains. The effects of the aromatic
20 alcohols were observed at concentrations commonly found in wines, suggesting a possible role in
21 microbial interaction during wine fermentation. Additionally, we demonstrated that aromatic alcohols
22 and ethanol are able to affect invasive and pseudohyphal growth in a manner dependent on nutrient
23 availability. Some of these compounds showed strain-specific effects. These findings add to the
24 understanding of the fermentation process and illustrate the diversity of metabolic communication that
25 may occur among related species during metabolic processes.

26 1. Introduction

27 Wine is produced by alcoholic fermentation, in which grape sugars are metabolized into ethanol by
28 yeast. During grape ripening, the surfaces of berries are primarily colonized by non-*Saccharomyces*
29 yeast, such as *Hanseniaspora*, *Starmerella* (sym *Candida*), *Hansenula* or *Metschnikowia*.
30 Microorganisms belonging to the *Saccharomyces* genus are present in low abundance and are difficult
31 to detect in initial must (Ribéreau-Gayon et al., 2006). For this reason, during spontaneous
32 fermentation, non-*Saccharomyces* yeasts are responsible for initiating alcoholic fermentation and are
33 then out-competed by *S. cerevisiae* throughout fermentation (Fleet, 2003; Heard and Fleet, 1988;
34 Ribéreau-Gayon et al., 2006). Traditionally, the low ethanol tolerance and competitiveness of non-

35 *Saccharomyces* yeasts compared to *Saccharomyces* species (Ribéreau-Gayon et al., 2006) has resulted
36 in a lack of interest in these yeast species for many years. However, recently, the importance of non-
37 *Saccharomyces* strains in alcoholic fermentation has become appreciated, particularly in terms of their
38 contribution to wine aroma, during the early steps of fermentation. Indeed, these species have been
39 reported to impact, sometimes positively, winemaking via the production of high amounts of aromatic
40 compounds, such as aromatic alcohols, ethyl esters and acetate esters (Belda et al., 2017; García et al.
41 2010; Jolly et al., 2014; Romano et al., 2003). Furthermore, these strains appear to be present
42 throughout much of the fermentation process, although this finding has been neglected because such
43 strains are difficult to culture (Millet and Lonvaud-Funel, 2000; Wang et al., 2015a, 2016).

44 *Saccharomyces cerevisiae* is a unicellular fungi that reproduce asexually by budding and is able to
45 undergo filamentous growth to scavenge for nutrients (Cullen and Sprague, 2012; Verstrepen and Klis,
46 2006; Wendland and Philippsen, 2001). Filamentous growth includes morphological changes that
47 involve the global reorganization of cellular processes to produce a new cell type. Cells alter their
48 budding pattern, becoming more elongated and remaining attached to each other through the formation
49 of pseudohyphae. Moreover, under certain conditions, yeast cells penetrate surfaces through a process
50 known as invasive growth (Roberts and Fink, 1994). Although much of the genetic characterization of
51 this response has been performed in *S. cerevisiae* strains on the ∑1278b background (Cullen and
52 Sprague, 2000; Gimeno et al., 1992), the response has also been studied in many strains and genera
53 (Gimeno and Fink, 1994; Lo and Dranginis, 1998; San-blas et al., 1997). For example, the human
54 pathogen *Candida albicans* (Biswas et al., 2007; Chen et al., 2004; Hornby et al., 2001; Kruppa, 2009)
55 undergoes pseudohyphal and hyphal growth (pathogenic form), which confers the ability to infect
56 human tissues (Leberer et al., 2001; Lo et al., 1997; Rocha et al., 2001). Filamentous growth in yeasts
57 has been reported to occur in response to cell density and several molecules, such as aromatic alcohols
58 and ethanol, have been identified as stimuli that induce these morphological changes (Dickinson, 1996;
59 Gimeno et al., 1992; González et al., 2017; Lorenz et al., 2000). Indeed, aromatic alcohols, tyrosol
60 (TyrOH), tryptophol (TrpOH) and phenylethanol (PheOH), which are derived from the amino acids
61 tyrosine, tryptophan and phenylalanine, respectively, have been suggested to act as quorum sensing
62 molecules (QSMs) in yeasts, regulating cell density and evoking morphogenetic transitions (Chen et
63 al., 2004; Chen and Fink, 2006). Moreover, nitrogen limitation results in the increased production of
64 aromatic alcohols, leading to elevated filamentous growth in *S. cerevisiae*. In this species, PheOH and
65 TrpOH act as inducers of morphogenesis, while TyrOH has no detectable effects (Chen and Fink,
66 2006). However, in *C. albicans*, these alcohols exhibit the opposite behaviour: TyrOH promotes
67 pseudohyphal growth, whereas PheOH and TrpOH inhibit it. The finding that different aromatic
68 alcohols exert different responses on morphogenesis depending on the yeast species implicates these
69 molecules as inducers of species-specific effects (Chen and Fink, 2006). In a recent study, González et
70 al. (2017) showed that ethanol specifically induced filamentous growth under nitrogen-limiting
71 conditions, whereas aromatic alcohols did not. Thus, environmental conditions impact the efficacy of
72 these compounds. Non-*Saccharomyces* yeasts, such as *Hanseniaspora uvarum*, *Pichia kudriavzevii*
73 and *Pichia fabianii*, undergo filamentous growth under nutrient-limited conditions (nitrogen or carbon)
74 or in the presence of other stress factors (Pu et al., 2014; van Rijswijck et al., 2015), but the roles of
75 these alcohols have not been extensively explored.

76 During alcoholic fermentation, yeast synthesizes compounds that, depending on the concentration, can
77 be inhibitory to their own growth or the growth of other yeast species. A primary example is ethanol,
78 which is a potent inhibitory compound for growth. Other metabolites, such as short-to-medium-chain
79 fatty acids (e.g., acetic, hexanoic, octanoic and decanoic acids) and yeast killer toxins, also inhibit
80 growth and even induce the death of certain yeast species, including strains of *S. cerevisiae* (Pérez et
81 al., 2001). Recently, interactions between species were shown to be impacted by the secretion of

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82 compounds by yeast during alcoholic fermentation (Albergaria and Arneborg, 2016; Ciani and
83 Comitini, 2015; Wang et al., 2015b). To our knowledge, there have been no studies investigating the
84 effects of aromatic alcohols or other QSMs synthesized during alcoholic fermentation on the growth
85 and vitality of wine yeasts. Moreover, the effects of aromatic alcohols on the filamentous growth of
86 non-*Saccharomyces* wine yeast species have not been explored. The investigation of these areas might
87 help to unravel the possible roles of QSMs in the interactions between yeasts during alcoholic
88 fermentation. Moreover, direct microbial interactions (i.e., through physical contact) are reportedly
89 involved in the growth inhibition of non-*Saccharomyces* yeast, although such mechanisms are
90 dependent on cell density, when cultures are competing for space (Nissen et al., 2003, 2004; Pérez-
91 Nevado et al., 2006; Renault et al., 2013).

92 Additionally, through tryptophan metabolism, yeasts also produce other metabolites that are related to
93 indoles, such as serotonin, melatonin or tryptamine. Serotonin and melatonin are of special relevance
94 for their bioactivity in higher organisms, including humans. Rodriguez-Naranjo et al. (2012)
95 demonstrated that melatonin is produced during alcoholic fermentation by yeast, and different strains
96 and species synthesize this compound at different concentrations. The role of melatonin in yeasts is
97 still unclear, although a recent paper showed that the compound demonstrated possible antioxidant
98 activity in response to oxidative damage by hydrogen peroxide in *S. cerevisiae* (Vázquez et al., 2017).
99 On the other hand, tryptamine has also been detected in red wines at mg/L concentrations after
100 malolactic fermentation (Wang et al., 2014). Serotonin appears to exert antifungal activity against
101 *Candida* and *Aspergillus* spp. in vitro (Lass-Flörl et al., 2002, 2003).

In review

102 Thus, the objective of this study was to evaluate the effects of different compounds derived from
103 aromatic amino acid metabolism and produced during alcoholic fermentation on the growth and
104 physiology of different wine yeast species. We first described an analysis of the growth parameters of
105 different yeast strains and species in the presence of increasing concentrations of specific compounds
106 of interest. Then, the effects of aromatic alcohols and ethanol, which are well-known morphogenesis
107 inducers in *S. cerevisiae*, were examined for their impact on the filamentous growth of different non-
108 *Saccharomyces* wine species.

109 ## 2. Materials and Methods

110 ### 2.1 Strains and growth media

111 Eight strains from *Saccharomyces* species and two strains from four species of non-*Saccharomyces*
112 yeast were used in the study. The *S. cerevisiae* strains included the laboratory strain ∑1278b, the wine
113 strains SB (Marullo et al 2007), QA23, T73, P5 and P24 (Lallemand, Canada), the animal nutrition
114 strain Sc20 and the hybrid *S. kudriavzevii / S. cerevisiae* Vin7 (Oenobrands SAS, France) (Borneman
115 et al., 2012). The non-*Saccharomyces* yeasts were *Starmerella bacillaris* (sym. *C. zemplinina*) (Cz4-
116 CECT13129, Cz11), *H. uvarum* (Hu4-CECT13130, Hu11), *M. pulcherrima* (Mpp-CECT 13131,
117 FLAVIA) and *T. delbrueckii* (Tdp-CECT 13135, BIODIVA). FLAVIA and BIODIVA are commercial
118 strains (Lallemand, Canada) whereas the other non-*Saccharomyces* strains were isolated from
119 grapes/wine media (Padilla et al., 2016). Yeasts were typically grown on YPD [2% (w/v) peptone, 1%
120 (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) agar] at 28ºC.

121 ### 2.2 Effects on yeast growth

122 Yeasts were pre-cultured for 48 h on minimal medium [(MM) 1x Yeast Nitrogen Base (YNB) without
123 (w/o) amino acids (aa) or ammonium, 2% (w/v) glucose and 10 mM $(NH_4)_2SO_4$ (280 mgN/L)] at 28ºC
124 and then inoculated into each medium, adjusting the initial optical density (OD_{600nm}) to 0.2. To evaluate
125 the effects of nitrogen concentration, yeasts were grown on MM and on low nitrogen medium [(LNM)

126 1x Yeast Nitrogen Base (YNB) w/o aa or ammonium, 2% (w/v) glucose and 1 mM $(NH_4)_2SO_4$ (28
127 mgN/L)]. Media were supplemented with increasing concentrations of melatonin (Mel), tryptamine
128 (Trpm), serotonin (Ser), tyrosol (TyrOH), phenylethanol (PheOH) and tryptophol (TrpOH), ranging
129 from 50 to 1000 mg/L. All assays were performed using a POLARstar Omega microplate reader (BMG
130 LABTECH, Germany) and were performed in triplicate at 28 °C for 48 h. Microplate wells were filled
131 with 250 µL of inoculated media. A control well containing medium without inoculum was used to
132 determine the background signal. Measurements were taken every 30 min after pre-shaking the
133 microplate for 30 sec at 500 rpm. For each growth curve, the variables generation time (GT) and
134 maximal growth (OD max) were calculated according to Warringer and Blomberg (2003). Briefly, for
135 the GT determination, a slope was calculated between every second consecutive measurement for the
136 whole growth curve (OD values were previously $\log_{10}$ transformed). Of the seven highest slopes, the
137 highest two were discarded, and the mean for the following five was defined as maximum division
138 rate. The GT was obtained dividing the $\log_{10} 2$ by the maximum division rate. The lag phase was
139 calculated using the program GrowthRates (Hall et al, 2014).

140 ### 2.1.1 Statistical data processing

141 All experiments were performed in triplicate. The data was subjected to one-way analysis of variance
142 (ANOVA), and Tukey's post-hoc test (XLSTAT Software) was used to evaluate significant differences
143 between the control condition (no addition) and the addition of each compound. The results were
144 considered statistically significant at $p < 0.05$. For each compound, relative values were calculated
145 using the condition in the absence of added compound (0 mg/L) as a control ((condition-
146 control)/control). To better understand the interactions between the calculated parameters and their
147 effects on yeast growth, Principal Component Analysis (PCA) was performed using XLSTAT
148 Software at a concentration of 1000 mg/L for each compound and under both nitrogen conditions (MM
149 and LNM) for all strains tested.

150 ## 2.3 Filamentous growth assays

151 ### 2.3.1 Yeast strains, media and growth conditions

152 For the filamentous growth assay, two strains of each non-*Saccharomyces* species were tested, using
153 the strain QA23 (*S. cerevisiae*) as a control (González et al., 2017). Yeasts were grown on minimal
154 medium [MM - 1x Yeast Nitrogen Base (YNB) w/o aa or ammonium, 2% (w/v) glucose, and 10 mM
155 $(NH_4)_2SO_4$)] with agitation (120 rpm) for 16 h at 28 °C before seeding on plates for filamentation
156 analysis. To evaluate invasive and pseudohyphal growth, three different media were used, with
157 variations in glucose and nitrogen concentrations: SAD - synthetic medium [1x YNB w/o aa or
158 ammonium, 2% (w/v) glucose and 37 mM $(NH_4)_2SO_4$ and 2% (w/v) agar], SALG - synthetic medium
159 with low glucose [1x YNB w/o aa or ammonium, 0.5% (w/v) glucose and 37 mM $(NH_4)_2SO_4$) and 2%
160 (w/v) agar] (González et al., 2017) and SLAD - synthetic low-ammonium dextrose medium [SLAD -
161 1x YNB w/o aa or ammonium, 2% (w/v) glucose and 50 µM $(NH_4)_2SO_4$) and 2% (w/v) agar]. To test
162 the effects of aromatic alcohols, the above media were supplemented with 500 µM of TyrOH (6,90
163 mg/L), TrpOH (8,06 mg/L) or PheOH (6,10 mg/L) or 2% (v/v) ethanol. Those concentrations were
164 chosen according our previous studies (González et al., 2017).

165 ### 2.3.2 Invasive and pseudohyphal growth assays

166 Cells pre-grown in MM for 16 h were harvested by centrifugation, washed once in sterile water and
167 adjusted to an OD_{600nm} of 2.0. Subsequently, 10 µl of cells were spotted in triplicate on semisolid agar
168 media. Plates were incubated at 30°C for 3 days, 5 days and 7 days depending on the experiment.
169 Invasive growth was determined in a plate washing assay (Roberts and Fink, 1994). Colonies were
170 photographed before and after the plates were washed in a stream of water, after which the colonies

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171 were rubbed from the surface with a gloved finger. ImageJ software (http://rsb.info.nih.gov/ij/) was
172 used to quantitate invasive growth in the plate-washing assay. The background intensity was
173 determined for each spot and subtracted from the densitometry of the invasive area. Densitometry
174 analysis was performed on invasive patches over multiple days. The data was subjected to one-way
175 analysis of variance (ANOVA) and Tukey's post-hoc test (XLSTAT Software) was used to evaluate
176 significant differences on invasion intensity between media. The results were considered statistically
177 significant at $p < 0.05$ The examination of pseudohyphae was determined as described by Gimeno et
178 al. (1992). Before washing the plates, the colony periphery was observed and photographed each day
179 under microscopy (Raman FT-IR).

180 3. Results

181 3.1 Effects of the presence of aromatic amino acid-derived compounds on yeast growth

182 To evaluate the effects of amino acid-derived compounds on yeast growth, five strains of *S. cerevisiae*
183 and one strain of each *non-Saccharomyces* species were grown in the presence of 1000 mg/L of Mel,
184 Ser, Trpm, TyrOH, PheOH or TrpOH. As these molecules are derived from nitrogen metabolism, and
185 QSMs are produced during nutrient limitation, we tested their effects under two different nitrogen
186 conditions: 1 and 10 mM $(NH_4)_2SO_4$ (Fig 1). As an example, the growth curves obtained with *S.*
187 *cerevisiae* QA23 (Fig 1A) and *S. bacillaris* Cz4 (Fig 1B) in the presence of 1000 mg/L of the different
188 compounds and 10 mM $(NH_4)_2SO_4$ are shown. In the QA23 strain, Ser completely inhibited cell
189 growth. In addition to this dramatic phenotype, other subtle phenotypes were observed. TrpOH caused
190 a reduction in growth rate and maximal growth, and Trpm increased the lag phase. The other
191 compounds tested did not significantly affect the growth profile. In comparison, the growth of strain
192 Cz4 was reduced by TrpOH and Trpm, but not by the other compounds. Therefore, different
193 compounds cause the growth inhibition of different species.

194 The relative values of OD max (Fig 1C) and generation time (Fig 1D) were calculated for each
195 compound, using the condition without addition as a control (absolute values can be found in Table
196 S1). Overall, the addition of these compounds (with the exception of Mel) exerted negative impacts on
197 the maximal growth obtained for most of the tested strains (Fig 1C). Ser decreased the OD max in all
198 yeast species, particularly under low nitrogen conditions, while Trpm and aromatic alcohols had a
199 major impact in non-*Saccharomyces* strains under both nitrogen conditions. On the other hand, Ser
200 caused growth reduction in all strains, increasing their GT (Fig 1D). In general, this increase was
201 significant for *Saccharomyces* strains under both nitrogen conditions but only under low nitrogen
202 conditions for most non-*Saccharomyces* strains. Increases in GT were also observed when the medium
203 was supplemented with TrpOH in all the non-S*accharomyces* strains under both nitrogen conditions.
204 The other two aromatic alcohols, PheOH and TyrOH, exerted no effects in *Saccharomyces* strains, and
205 at 1 mM, among non-*Saccharomyces* strains, only the Tdp strain was affected by PheOH, and *S.*
206 *bacillaris* by TyrOH. In general, the relative OD max or GT presented a similar trend under both
207 nitrogen conditions; the most relevant differences consisted of greater effects from Ser in the non-
208 *Saccharomyces* strains under low nitrogen concentration. The effects of these compounds were
209 impacted by exogenous nitrogen levels, although in a strain dependent manner. The impact of ethanol
210 on yeast growth was also analysed, but no significant differences were observed at 1000 mg/L for any
211 of the yeast species studied (data not shown). Based on these results, at high nitrogen concentration
212 *Saccharomyces* and non-*Saccharomyces* strains clustered into two different groups in a PCA (Fig
213 S1A), primarily attributable to the higher reduction in the OD max on non-*Saccharomyces* strains due
214 to the presence of aromatic alcohols and Trpm. Under low nitrogen conditions (Fig S1B), all strains of
215 *Saccharomyces* were included in the same cluster, but Non-*Saccharomyces* strains were plotted into

In review

216 two different groups because *T. delbrueckii* clustered separately from the other non-*Saccharomyces* 217 species, because of their higher GT in PheOH.

218 **3.2 Effects of the concentrations of aromatic amino acid-derived compounds on yeast growth**

219 According to our previous results, the effects of certain aromatic amino acid-derived compounds were 220 slightly greater under low nitrogen conditions than under high nitrogen conditions. For this reason, we 221 investigated how the increasing concentrations of these compounds (from 50 to 1000 mg/L) affect the 222 growth of a larger collection of wine yeast in nitrogen-limiting conditions (absolute values of 223 generation time and maximal growth obtained for each strain and condition can be found in Tables S2 224 and S3).

225 When different concentrations of the metabolites were tested, we observed again that Ser (Fig 2 and 226 S2), TrpOH (Fig 3 and S2) and Trpm (Fig 4 and S2) exerted higher impacts on the cell growth of yeast 227 strains and in some cases in the lag phase. On the other hand, TyrOH and PheOH only affected to the 228 growth of non-*Saccharomyces* strains (Fig S3), even at low concentrations (50 mg/L) in the case of 229 PheOH.

230 The effects of different Ser concentrations on the *S. cerevisiae* Sc20 strain are shown as an example 231 (Fig 2A). Clear inhibition of yeast growth was observed at concentrations of Ser above 500 mg/L, 232 increasing GT and decreasing the OD max. Interestingly, GT and OD max values obtained in the 233 presence of Ser were strongly correlated (R^2 0.8204), indicating that this compound influenced both 234 growth parameters for most strains (Fig 2B). As shown in Fig 3C, all wine strains were affected by the 235 presence of Ser in the medium, and the increase in GT was directly proportional to the Ser 236 concentration, illustrating a dose-dependent effect. Instead, the lab strain ∑1278b was barely affected 237 by this compound. Most *S. cerevisiae* strains showed growth inhibition starting from 250 mg/L, 238 primarily in Vin7, T73, P5 and P24. Conversely, the strains of *S. bacillaris*, *H. uvarum* and *M.* 239 *pulcherrima* appeared to be more tolerant to this compound. On the other hand, *T. delbrueckii* presented 240 a specific profile, as growth was only affected above 750 mg/L, but they exhibited the highest growth 241 inhibition at 1000 mg/L. The effects of Ser on the relative OD max of the strains showed a profile 242 similar to GT (Fig S2).

243 For most strains, the addition of TrpOH caused a decrease in growth in a dose-dependent manner (see 244 Fig 3A and S2). The presence of TrpOH had a greater impact on GT than on maximal growth (Fig 3B), 245 particularly in non-*Saccharomyces* strains (Fig 3C). Among them, the most tolerant strain was *M.* 246 *pulcherrima* Mpp, which was only slightly affected at high doses of TrpOH. Conversely, the other *M.* 247 *pulcherrima* strain, FLAVIA, was one of the most heavily affected, indicating that sensitivity to TrpOH 248 is strain-dependent. In general, the *S. cerevisiae* strains were less affected by TrpOH.

249 Trpm influenced differently the growth of yeast strains, resulting in increases in the lag phase or in the 250 GT, decreases in the OD max, and even no inhibitory effects at all (see two examples in Fig 4A). Thus, 251 within the same species, we observed different responses to the presence of Trpm. For example, among 252 *S. cerevisiae* strains, Vin7 only showed an increase during the lag phase, and there were no significant 253 effects on the other growth parameters; QA23 primarily increased its generation time and decreased 254 the OD max, while the other *S. cerevisiae* strains were barely affected by Trpm (Fig 4B, 4C and S2). 255 On the other hand, non-*Saccharomyces* strains were more affected by the presence of this biogenic 256 amine, even at low concentrations, modifying all the growth parameters. Interestingly, in *M.* 257 *pulcherrima* strains, the effects of Trpm on the OD max and GT were not dose-dependent, 258 demonstrating similar inhibition from 100 to 1000 mg/L (Fig 4 and S2).

259 **3.3 Effects of culture medium composition on filamentous growth in non-*Saccharomyces*** 260 **species**

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261 The aromatic alcohols and ethanol have been described as molecules signalling morphological changes 262 in different yeasts, primarily in *S. cerevisiae* and *C. albicans*; therefore, we analysed their effects on 263 the non-*Saccharomyces* strains. We first studied invasive growth on rich (SAD) and nutrient-limiting 264 [glucose (SALG) and nitrogen (SLAD)] media for all strains using *S. cerevisiae* QA23 as a control 265 (Fig 5). Interestingly, all strains exhibited a certain degree of invasive growth. Moreover, media limited 266 for glucose or nitrogen resulted in enhanced invasive growth for most of them. Specifically, on SLAD 267 plates, most strains showed invasive growth that was significantly higher than on SAD, with the 268 exception of *H. uvarum* strains. *M. pulcherrima* and *T. delbrueckii* strains were the most invasive non-269 *Saccharomyces* yeasts in the absence of nitrogen. Carbon source limitation (SALG) had a similar effect 270 as nitrogen; most strains presented significant invasive growth compared to rich media, with the 271 exception of the two *T. delbrueckii* strains.

272 We also determined the ability of these yeasts to form pseudohyphae by analysing the morphology of 273 their colonies on SAD, SLAD and SALG media. Figure 6 shows the morphology of the colony 274 peripheries at day 7. *H. uvarum* strains exerted the highest pseudophyphal phenotype, mainly in 275 limitation of nitrogen (SLAD), similarly to the control strain. Surprisingly, these *H. uvarum* strains 276 were also able to produce pseudohyphae on rich media. *M. pulcherrima* and *S. bacillaris* strains formed 277 few filaments only in SLAD medium, and none of the tested strains underwent pseudohyphae in SALG 278 medium. Thus, the lack of glucose was not a limiting factor to trigger this aspect of the filamentous 279 growth response in non-*Saccharomyces* yeast.

280 **3.4 Effects of alcohols on filamentous growth in non-*Saccharomyces* species**

281 The effects of alcohols on invasive growth were assayed on SAD, SALG and SLAD plates, both with 282 and without supplementation with different alcohols. In general, the effects of alcohols varied 283 depending on the medium and the species (Fig 7A). On SAD medium (Fig 7B), TrpOH and PheOH 284 promoted invasive growth in the *S. cerevisiae* strain. Among non-*Saccharomyces* species, PheOH only 285 stimulated invasive growth in *H. uvarum,* while ethanol and TrpOH only in *T. delbrueckii*. 286 Furthermore, no significant effects were observed in *S. bacillaris* or in *M. pulcherrima*. On SALG 287 plates (Fig 7C), aromatic alcohols significantly decreased the invasive growth of the commercial QA23 288 strain. Among non-*Saccharomyces* strains, TrpOH and PheOH significantly promoted invasive growth 289 on *H. uvarum* Hu4 and *T. delbrueckii* Tdp, respectively. Ethanol appeared to strengthen invasive 290 growth in *S. bacillaris, M. pulcherrima* and *T. delbrueckii*, while TyrOH presented similar effects in 291 the two strains of *M. pulcherrima* and in the commercial *T. delbrueckii* BIODIVA strain. On SLAD 292 plates (Fig 7D), ethanol induced invasive growth in the QA23 strain, as well as in both strains of *S.* 293 *bacillaris* and *T. delbrueckii. H. uvarum* and *S. bacillaris* increased their invasive growth in the 294 presence of PheOH. On the other hand, TyrOH significantly reduced the invasive growth of *M.* 295 *pulcherrima* strains.

296 To study the effects of alcohols in pseudohyphal growth, we focused on SLAD medium (Fig 8). 297 Ethanol and PheOH stimulated pseudohyphal formation in *S. cerevisiae*. However, the addition of 298 alcohols to agar plates resulted in a reduction in filamentation in both strains of *H. uvarum*. Similar to 299 *S. cerevisiae*, ethanol changed growth patterns to a more filamentous form in *S. bacillaris*, but the 300 aromatic alcohols tested did not affect pseudohyphae development. TyrOH considerably increased 301 filament formation in *M. pulcherrima*. Moreover, the two strains of *T. delbrueckii* tested did not form 302 pseudohyphae when starved for nitrogen in the presence of any alcohol tested.

303 **4. Discussion**

In review

304 No organisms exist in isolation, all species share common environments and compete for nutrients.
305 Interactions between organisms are commonplace and may be diverse. Although there are many
306 examples of cooperation and symbiotic relationships among organisms, many interactions are
307 combative, with one species profiting from another's detriment. An excellent example of this is seen
308 on rotting fruit, where yeast and other microorganisms compete for sugar food sources. Non-
309 *Saccharomyces* yeasts are predominant in grape must, even during the first stages of spontaneous
310 fermentations, but are rapidly replaced by *S. cerevisiae,* which completes the process (Fleet, 2003).
311 Recently, some findings have associated interactions between species with the secretion of certain
312 compounds by yeast during alcoholic fermentation (Albergaria and Arneborg, 2016; Ciani and
313 Comitini, 2015, Wang et al, 2015a), such as some alcohols which are produced at high density by *S.*
314 *cerevisiae* (Zupan et al., 2013). Our results showed that aromatic alcohols reduced yeast cell growth,
315 especially in non-*Saccharomyces,* where the three fusel alcohols exerted negative effects on GT and
316 maximal growth in most strains, even at low concentrations (100-250 mg/L). Instead, in *S. cerevisiae*
317 strains, only TrpOH exhibited growth inhibition. These aromatic alcohols are produced by wine yeast
318 and are found in alcoholic beverages at concentrations ranging from 4-197 mg/L PheOH, 100-450
319 mg/L TrpOH, and 5-40 mg/L TyrOH (Swiegers et al., 2005). Non-*Saccharomyces* strains are able to
320 produce these aromatic alcohols, but at lower concentrations than *S. cerevisiae* (Zupan et al., 2013;
321 González, 2017), however, the negative effects on the growth of these alcohols were more pronounced
322 in non-*Saccharomyces*. Thus, the production of aromatic alcohols may play a role in certain yeast
323 interactions, inhibiting the growth of non-*Saccharomyces* strains and even directing the replacement
324 of these species during alcoholic fermentation by the major producer species, *S. cerevisiae*.
325 Nevertheless, in this study, we tested the effects of these alcohols individually, but mixtures of them
326 may have greater impact on yeast growth.

327 Mel is synthesized from tryptophan and exhibits various biological activities in humans, such as
328 antioxidant activity (Anisimov et al., 2006; Reiter et al., 2001). It has been proved that yeasts generate
329 low concentrations of Mel during alcoholic fermentation (Rodriguez-Naranjo et al., 2012); however,
330 its role in yeast regulation is still unknown. In our study, the presence of Mel in the media did not affect
331 the growth of the yeast strains tested. In contrast to Mel, its precursor, Ser, considerably reduced the
332 maximal growth and doubling time of all strains tested, and was the most inhibiting compound tested,
333 which indicates that Ser has toxic effects in yeast. Indeed, Ser has previously shown antifungal activity
334 against *Candida* and *Aspergillus* spp. in vitro (Lass-Flörl et al., 2002, 2003). On the other hand, Trpm
335 mostly affected the lag phase, being reduced at low concentrations but increased at high concentrations.
336 Trpm levels in wines are usually very low (0.02 – 0.2 mg/l), and its synthesis largely depends on
337 fermentation temperature but not on supplementation with its precursor amino acid (Lorenzo et al.,
338 2017), Ser is found at very lower concentration at the end of alcoholic fermentation (Fernández-Cruz
339 et al., 2017). Therefore, although Trpm and Ser appear to significantly affect different growth
340 parameters, this does not occur at concentrations usually found in wines.

341 Recently, the death of non-*Saccharomyces* yeasts in mixed fermentation with *S. cerevisiae* was
342 associated with mechanisms mediated through cell-to cell contact as well as high cell densities (Nissen
343 et al., 2003, 2004; Pérez-Nevado et al., 2006; Renault et al., 2013). However, the role of cell-to-cell
344 communication through QSM in inhibiting the growth of certain yeast strains during mixed-culture
345 fermentation remains unclear (Avbelj et al., 2016, Wang et al, 2015b). QS in yeasts involves a
346 morphological transition from a filamentous to a yeast form, or vice versa (Sprague and Winans, 2006).
347 Yeasts undergo this transition from a unicellular to a filamentous form in response to environmental
348 cues, which may arise from alterations in nutrient concentrations or in the presence of auto-inductive
349 molecules that are secreted by cells (Chen and Fink, 2006). Stimuli that trigger filamentous growth
350 include nitrogen limitation (Gimeno et al., 1992) and glucose limitation (Cullen and Sprague, 2000).
351 Filamentation is well established in *Saccharomyces* (Chen and Fink, 2006; Cullen and Sprague, 2012)

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and the dimorphic fungal human pathogen *C. albicans* (Chen et al., 2004; Hornby et al., 2001), but little is known about this type of growth in other genera and species of yeasts (Gori et al., 2011; Pu et al., 2014; van Rijswijck et al., 2015). In our study, we tested two strains each of the major genera involved during wine fermentation to test their ability to penetrate surfaces (invasive growth) or to form pseudohyphae. All strains tested were wild yeasts isolated from wine environments and were able to invade, even in rich media. Indeed, natural yeast isolates exhibit high levels of invasion (Casalone et al., 2005), allowing them to colonize natural niches, such as grapes. According to Pitoniak et al., (2009), yeasts require the filamentous growth pathway and *FLO11* to be able to fully colonize this environment. Nutrient limitation also promotes increased invasive growth in non-*Saccharomyces* species. The *S. bacillaris* and *M. pulcherrima* strains increased their invasive growth both under glucose and nitrogen limitation, but they only formed small pseudohyphae with nitrogen limitation. Indeed, the ability to form pseudohyphae and invade agar upon nutrient deprivation provides a selective advantage to yeast cells, facilitating foraging for scarce nutrients at a distance from their initial position (Casalone et al., 2005). On the other hand, *H. uvarum* exhibited a striking behaviour because its cells primarily invade the agar under glucose limitation but form a large number of pseudohyphae under nitrogen limitation and, to a lesser extent, in rich media. The ability of these strains to form pseudohyphae in rich media may be an advantage to colonize fruits by adhesion and a possible reason for the wide distribution of this species on natural fruit surfaces; in some studies, *H. uvarum* is the main species found in grape habitats (Beltran et al., 2002; Cadez et al., 2002; Ocón et al., 2010; Padilla et al., 2016; Pretorius, 2000), Finally, *T. delbrueckii* was the only species that did not form pseudohyphae in any of the tested media. Nevertheless, this species was able to invade under nitrogen limitation. This suggests the differential regulation of both phenotypes in this species. A possible explanation for this lack of pseudohyphal growth may be related to its ability to flocculate in liquid medium, especially in YPD medium. Both phenotypic traits are mediated by the same family gene and a recent study demonstrated that variations in the amino acid sequence of the adhesion domain of Flo11 causes different flocculation activities (Barua et al., 2016).

Overall, the two strains of each species tested presented similar behaviors, indicating that filamentous growth is a similar trait in several species. Aromatic alcohols have been reported to possess QS activity, and their effects together with ethanol on *S. cerevisiae* morphology have been thoroughly described (Chen and Fink, 2006; González et al., 2017). In this study, the effects of aromatic alcohols and ethanol were analysed in three different media, which differed in their glucose and ammonium content. As previously described, PheOH and TrpOH exerted effects on filamentous growth in *S. cerevisiae*. However, these results are not completely in concordance with Chen and Fink (2006), since they observed that PheOH and TrpOH both exerted effects on pseudohyphal growth but only PheOH affected invasive growth, and in our study we observed the opposite. Moreover, we also observed inhibitory effects on pseudohyphae with all aromatic alcohols in low glucose medium. In *H. uvarum*, the sole aromatic alcohol that promoted invasive growth was PheOH, both in rich and nitrogen-limiting media. A reduction in pseudohyphae formation was observed in the presence of aromatic alcohols, which also occurred with farnesol in *C. albicans* (Hornby et al., 2001). In a recent study, Pu et al. (2014) described the involvement of PheOH in filamentous growth, adhesion and biofilm formation in *H. uvarum*. On the other hand, TyrOH has been described as an inducer of filamentous growth in *C. albicans* (Chen et al., 2004). However, TyrOH did not affect significantly *S. bacillaris* growth in any of the conditions tested, as it might be expected due to its greater proximity to *C. albicans*. Anyway, this species produced very low concentration of aromatic alcohols, even in a previous study no synthesis was detected (Zupan et al., 2013; González, 2017) Therefore, in this species, other molecules may be the signals that initiate changes in morphogenesis, similar to *C. albicans* with farnesol (Kruppa, 2009). The effects of TyrOH on morphological changes were also observed in *M. pulcherrima,* suggesting a possible signalling role also in this species. Ethanol has been extensively reported to

In review

400 stimulate pseudohyphal growth in *S. cerevisiae* (González et al., 2017; Lorenz et al., 2000). In our
401 study, ethanol affected all species to varying degrees, with the exception of *T. delbrueckii*. However,
402 even in this species ethanol promoted invasive growth under all tested conditions. As we have
403 previously shown, *T. delbrueckii* did not undergo pseudohyphal growth under any of the tested
404 conditions, but these strains presented flocculent growth in liquid media, which may suppress
405 filamentation, as both responses are controlled by the same gene family (Soares, 2011).

406 Therefore, the aromatic alcohols appear to be species-specific signalling molecules because different
407 species manifest different responses to these auto-regulatory molecules. This finding was previously
408 observed for *S.cerevisiae* and *C.albicans*: Chen and Fink (2006) demonstrated that different aromatic
409 alcohols exert different effects on the morphogenesis of these two yeast species.

410 To conclude, we demonstrated that aromatic amino acid-derived compounds produced during alcoholic
411 fermentation by yeast, and at the concentrations found in fermented beverages, modulate the growth
412 of certain yeast species. Among these compounds, aromatic alcohols appear to be the most interesting
413 because yeasts synthesize these compounds at levels that have physiological effects, suggesting a
414 possible role in microbial interaction during wine fermentation. Our study reinforces the idea that these
415 molecules play roles as QSM on both *Saccharomyces* and non-*Saccharomyces* species, as they appear
416 to be able to induce or repress their filamentous and vegetative growth.

417 **Conflict of Interest**

418 The authors declare that the research was conducted in the absence of any commercial or financial
419 relationships that could be construed as a potential conflict of interest.

420 **Author Contributions**

421 BG design and perform experiments, analyze and discuss results, and writing the manuscript. JV
422 perform experiments, analyze and discuss results. PC design experiments, discuss results and
423 manuscript. AM, MJT and GB design experiments, analyze and discuss results, writing the manuscript
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626

In review

627 **Figures legends**

628 **Figure 1. Effects of aromatic amino acid-derived compounds on the growth of wine yeast species**
629 **at different nitrogen concentrations**. The effects of Mel, Ser, Trpm, TrpOH, PheOH and TyrOH on
630 the growth of four strains of *S. cerevisiae* and four of non-*Saccharomyces* were determined. Yeast were
631 grown for 48 h at 28 °C in minimal medium with two different nitrogen concentrations (10 mM or 1
632 mM $(NH_4)_2SO_4$) and supplemented with 1000 mg/L of each compound. Non-supplemented cultures
633 were used as controls. Experiments were carried out in triplicate. Growth curves of *S. cerevisiae* QA23
634 **(A)** and *S. bacillaris* Cz4 **(B),** with the different compounds added at 10 mM $(NH_4)_2SO_4$ medium are
635 shown. For each nitrogen condition and compound, maximal growth **(C)** and generation time **(D)** was
636 calculated. The fold-change for each growth parameter was determined in relation to its control
637 condition. Statistical analysis was performed using Tukey's test by comparing the effects of each
638 compound in the different strains; asterisk denotes a p-value < 0.05.

639 **Figure 2. Effects of increasing serotonin (Ser) concentrations on yeast growth.** Ser was added to
640 minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and 1000
641 mg/L). **(A)** Growth curves obtained with *S. cerevisiae* Sc20. **(B)** Correlation between the generation
642 time and maximal growth fold-changes obtained with different yeast species. **(C)** Generation time fold-
643 change for each strain at different Ser concentrations. Statistical analysis was performed, using Tukey
644 test and comparing the effects of Ser concentrations in each strain; asterisk denotes a p-value < 0.05.
645 The fold-change for each growth parameter was determined in relation to the control (no-supplemented
646 condition, w/o).

647 **Figure 3. Effects of increasing tryptophol (TrpOH) concentrations on yeast growth.** TrpOH was
648 added to minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and
649 1000 mg/L). **(A)** Growth curves obtained with *H. uvarum* Hu4. **(B)** Correlation between the generation
650 time and maximal growth fold-changes obtained with different yeast species. **(C)** Generation time fold-
651 change for each strain at different TrpOH concentrations. Statistical analysis was performed using the

652 Tukey test and comparing the effects of TrpOH concentrations in each strain; asterisk denotes a p-
653 value < 0.05. The fold-change for each growth parameter was determined in relation to the control (no-
654 supplemented condition, w/o).

655 **Figure 4. Effects of increasing tryptamine (Trpm) concentrations on yeast growth.** Trpm was
656 added to minimal medium (1 mM $(NH_4)_2SO_4$) at increasing concentrations (50, 100, 250, 500, 750 and
657 1000 mg/L). **(A)** Growth curves obtained with *S. cerevisiae* Vin7 and *M. pulcherrima* FLAVIA. **(B)**
658 Lag phase fold-change for each strain at different Trpm concentrations. **(C)** Maximal growth fold-
659 change for each strain at different Trpm concentrations. Statistical analysis was performed comparing
660 the effects of Trpm concentrations in each strain, using Tukey test statistical method; asterisk denotes
661 a p-value <0.05. The fold-change for each growth parameter was determined in relation to the control
662 (no-supplemented condition, w/o).

663 **Figure 5**. **Invasive growth phenotypes of different wine yeast species**. **(A)** In a plate washing assay
664 (PWA), equal concentrations of cells were spread on media with different nutrient contents and
665 incubated for 5 days at 28 °C. **(B)** Quantification of invasive growth was performed after washing the
666 plate via densitometry analysis. Cells were spotted in triplicate, and the average values are shown.
667 Statistical analysis was carried out by comparing each strain with respect to rich media (SAD), using
668 Tukey test statistical method; asterisk denotes a p-value < 0,05.

669 **Figure 6**. **Pseudohyphal growth phenotypes of different wine yeast species**. Cells were spotted on
670 rich medium (SAD) and nutrient limitation media (SALG and SLAD). Colony peripheries were
671 photographed after incubation for 5 days at 28 °C. Scale bar is 50 µm. Arrows mark examples of
672 pseudohyphae.

673 **Figure 7. Invasive growth of wine yeast species in the presence of aromatic alcohols and ethanol**.
674 **(A)** In a plate washing assay (PWA), equal concentrations of cells were spread on SAD, SALG and
675 SLAD media in the presence of the aromatic alcohol (TyrOH, PheOH or TrpOH) at 500 µM or 2%
676 (v/v) EtOH and incubated for 3 days at 28 °C. Panel **A** shows the results from the washed plate. The
677 invasive growth obtained with different wine yeast species in SAD **(B)**, SALG **(C)** and SLAD **(D)** was
678 obtained via densitometry. Cells were spotted in triplicate, and the average agar invasion values were
679 calculated. Relative invasion values were obtained by dividing the agar invasion in presence of each
680 compound and the one of the control (no-supplemented condition, w/o). Statistical analysis was
681 performed comparing the effects of the alcohols in each strain relative to the control, p-value < 0.05.

682 **Figure 8. Pseudohyphal growth phenotypes of different wine yeast species in response to ethanol**
683 **and aromatic alcohols**. Cells were spotted on SLAD medium. Colony peripheries were photographed
684 after incubation for 3 days at 28 °C. Scale bar is 50 µm.

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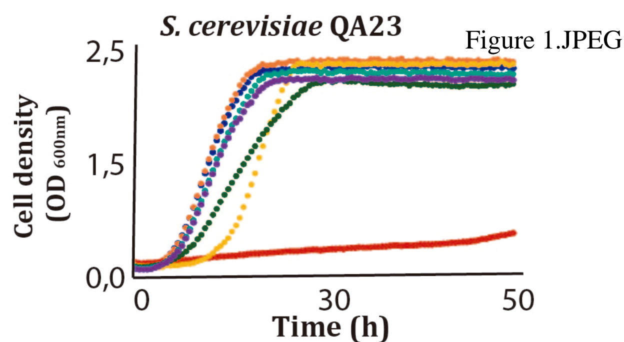
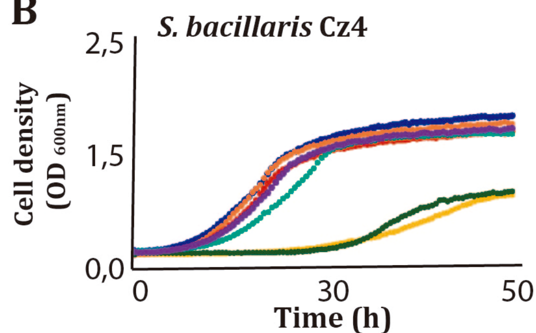
In review

Figure 1.JPEG





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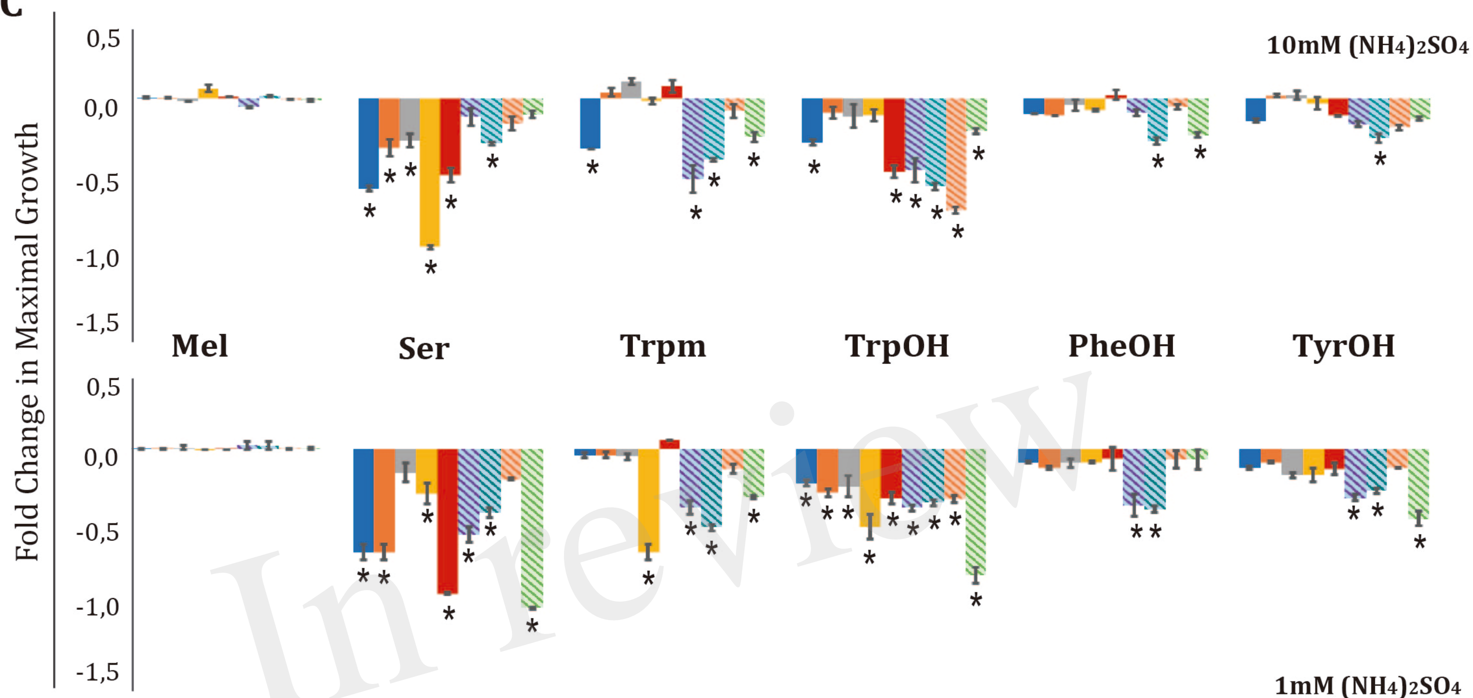

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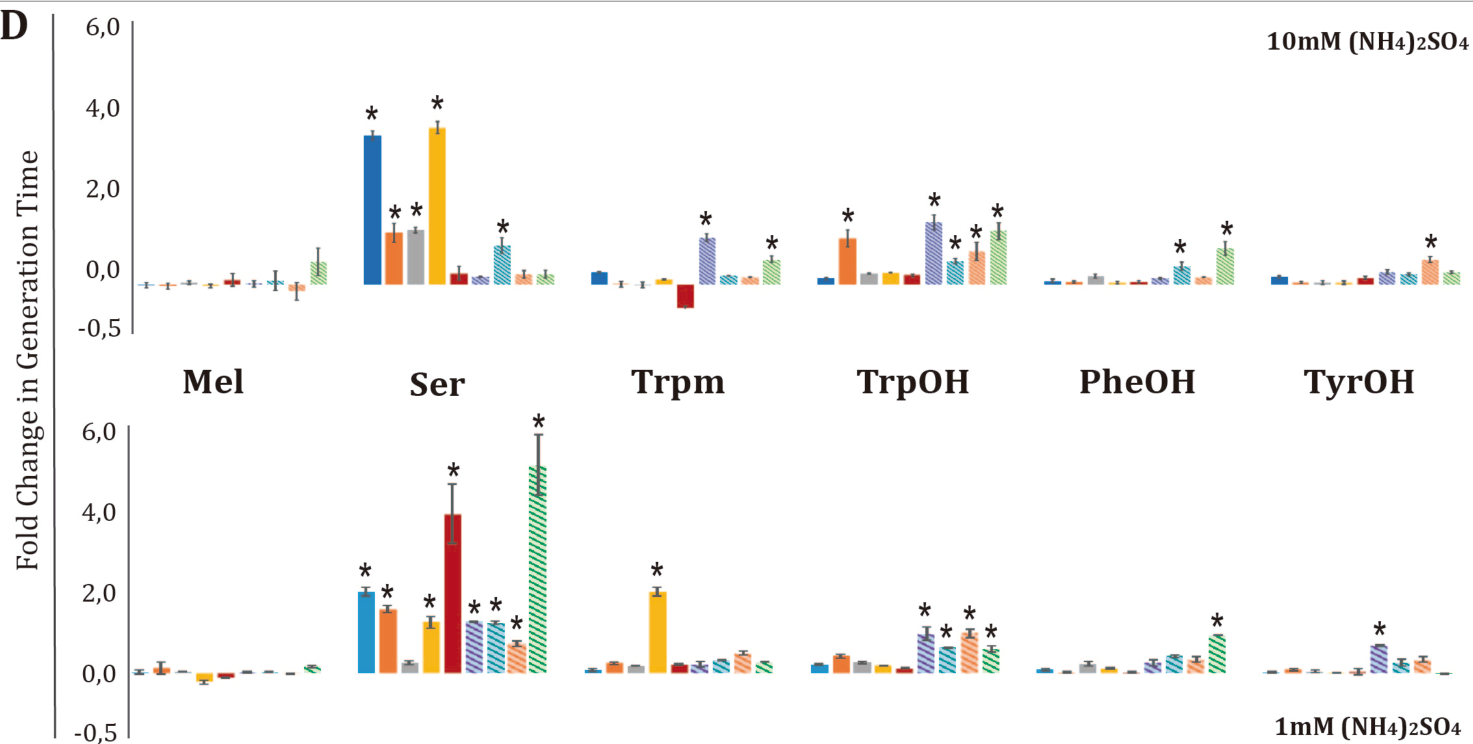
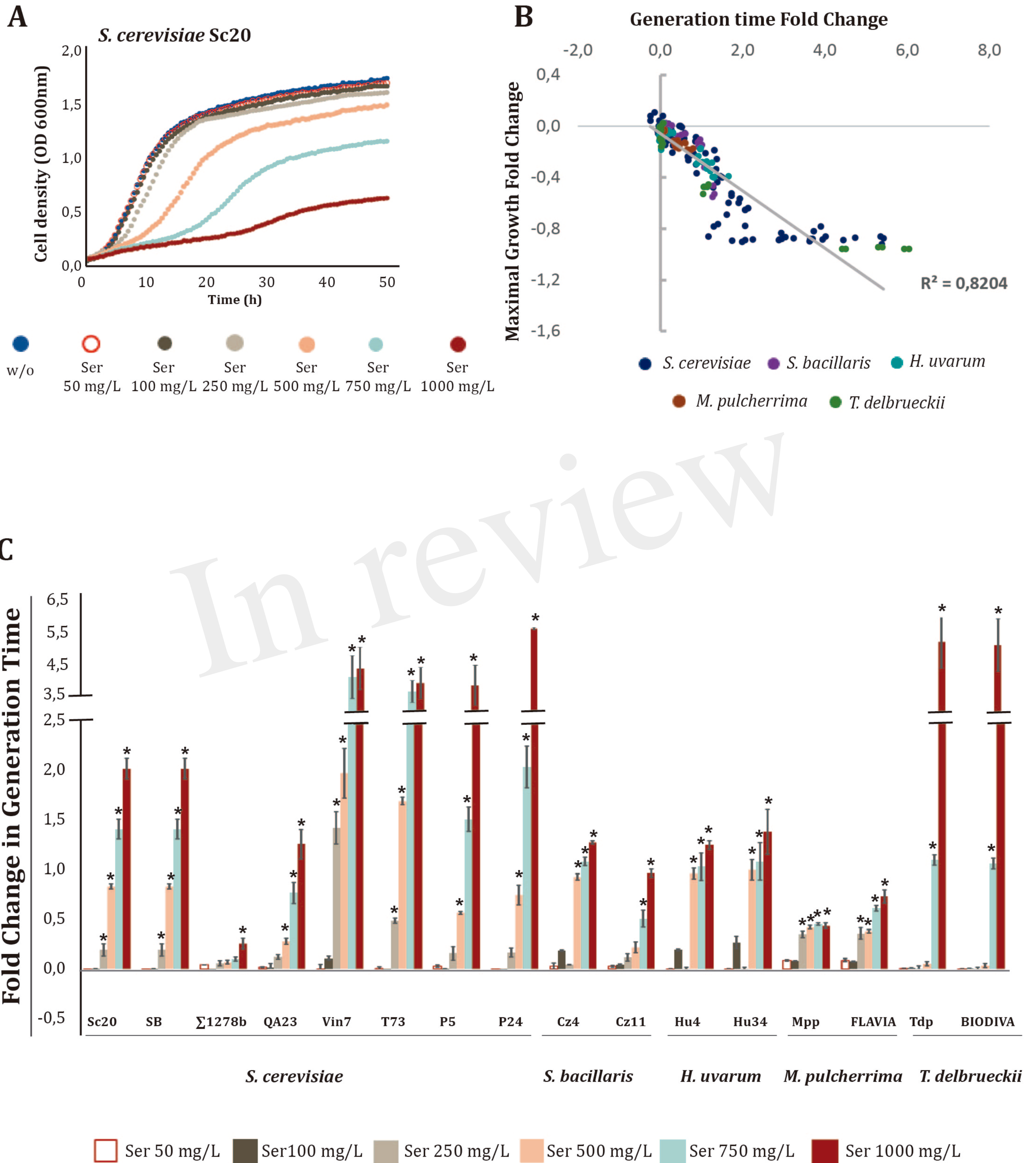


Sc20 SB ∑1278b QA23 Vin7

S. cerevisiae

S. bacillaris Cz4 *H. uvarum* Hu4 *M. pulcherrima* Mpp *T. delbrueckii* Tdp

Figure 2.JPEG



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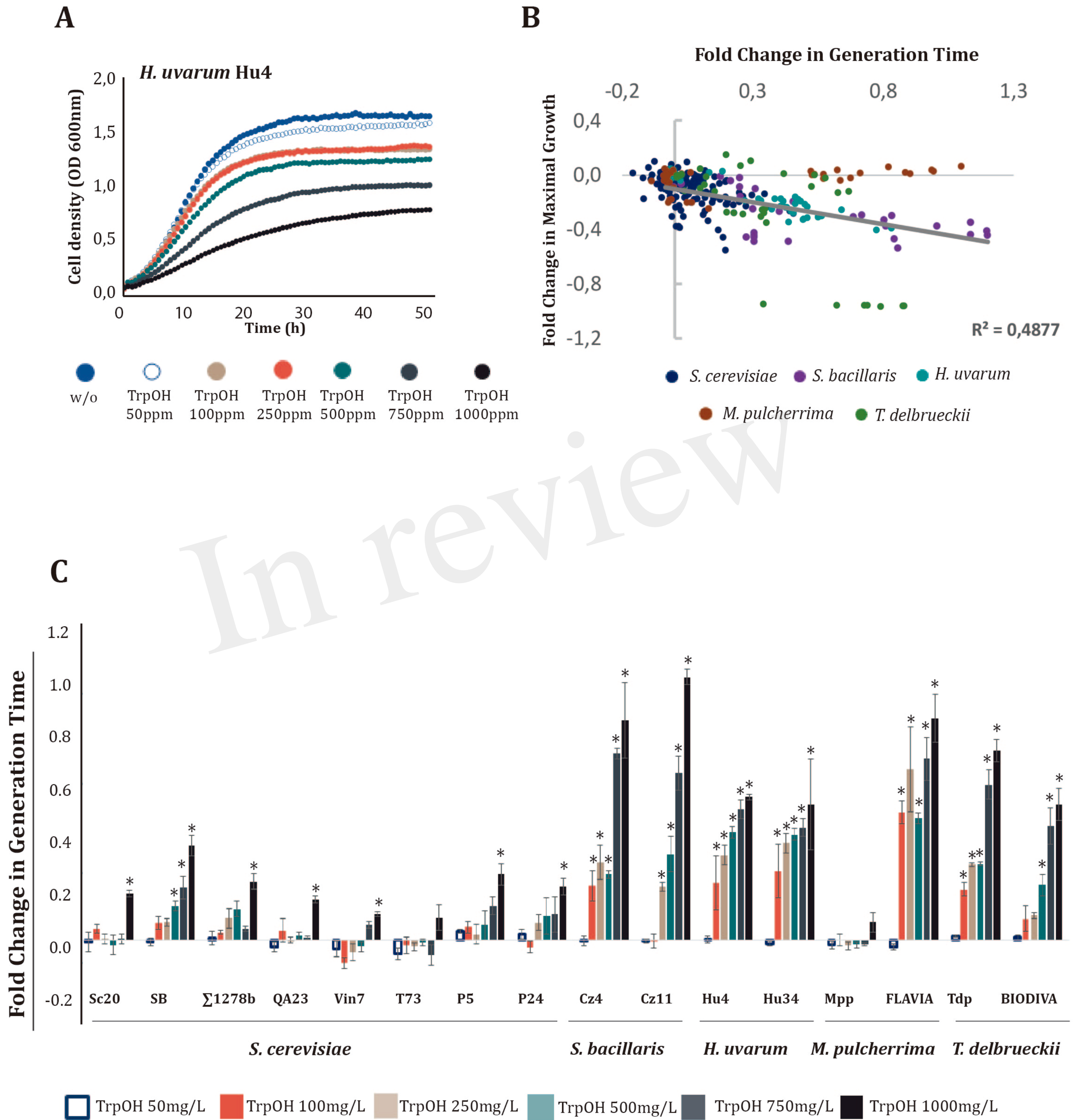

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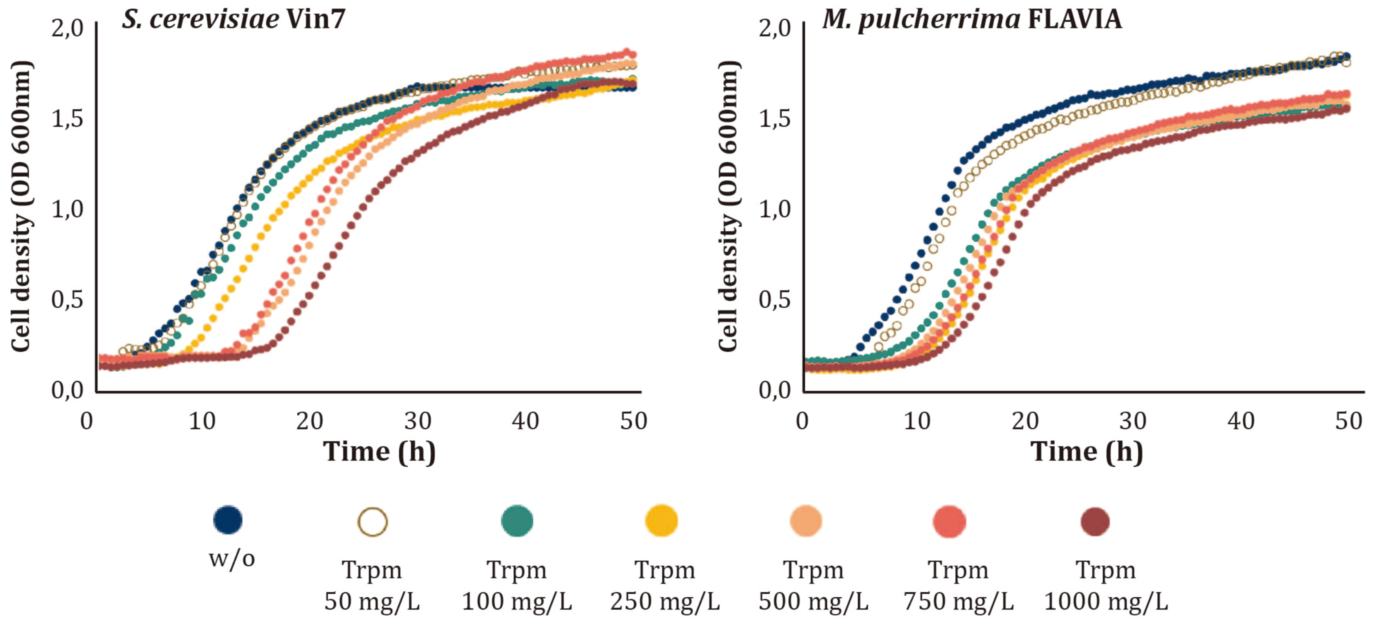

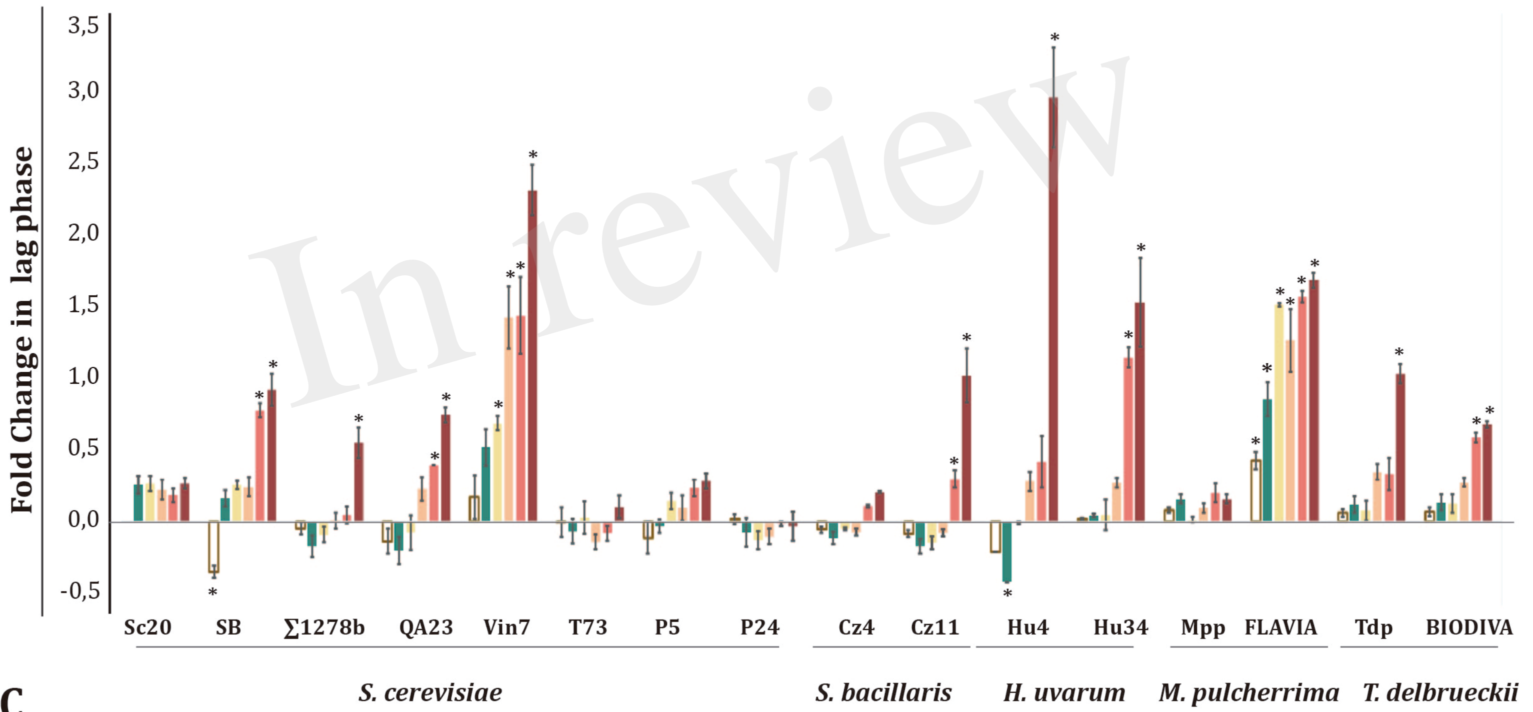

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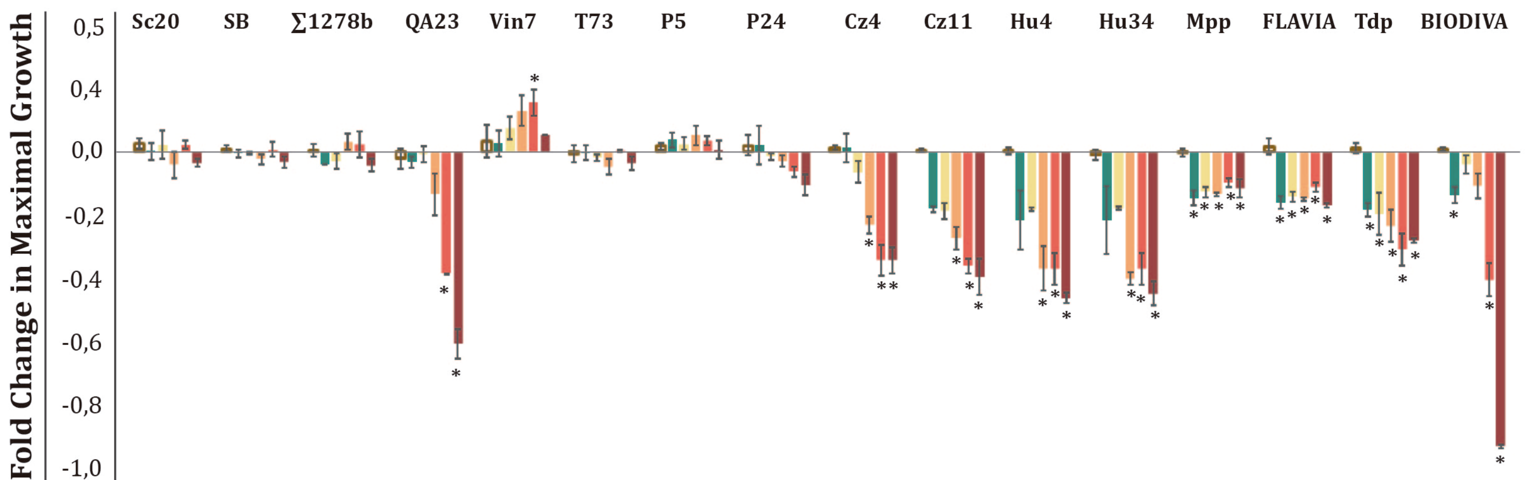


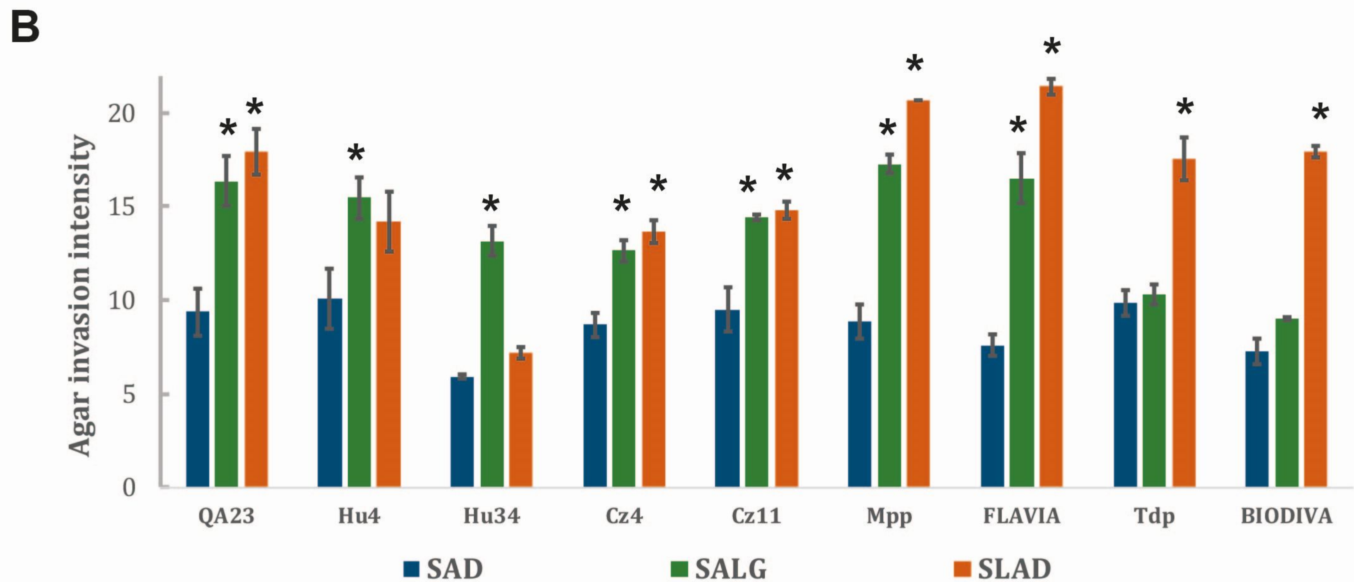
Figure 5.JPEG

A

		SAD	SAD After washing	SALG	SALG After washing	SLAD	SLAD After washing
Saccharomyces cerevisiae	QA23						
Hanseniaspora uvarum	Hu4						
	Hu34						
Starmerella bacillaris	Cz4						
	Cz11						
Metschnicowia pulcherrima	Mpp						
	FLAVIA						
Torulaspora delbrueckii	Tdp						
	BIODIVA						

In review

B



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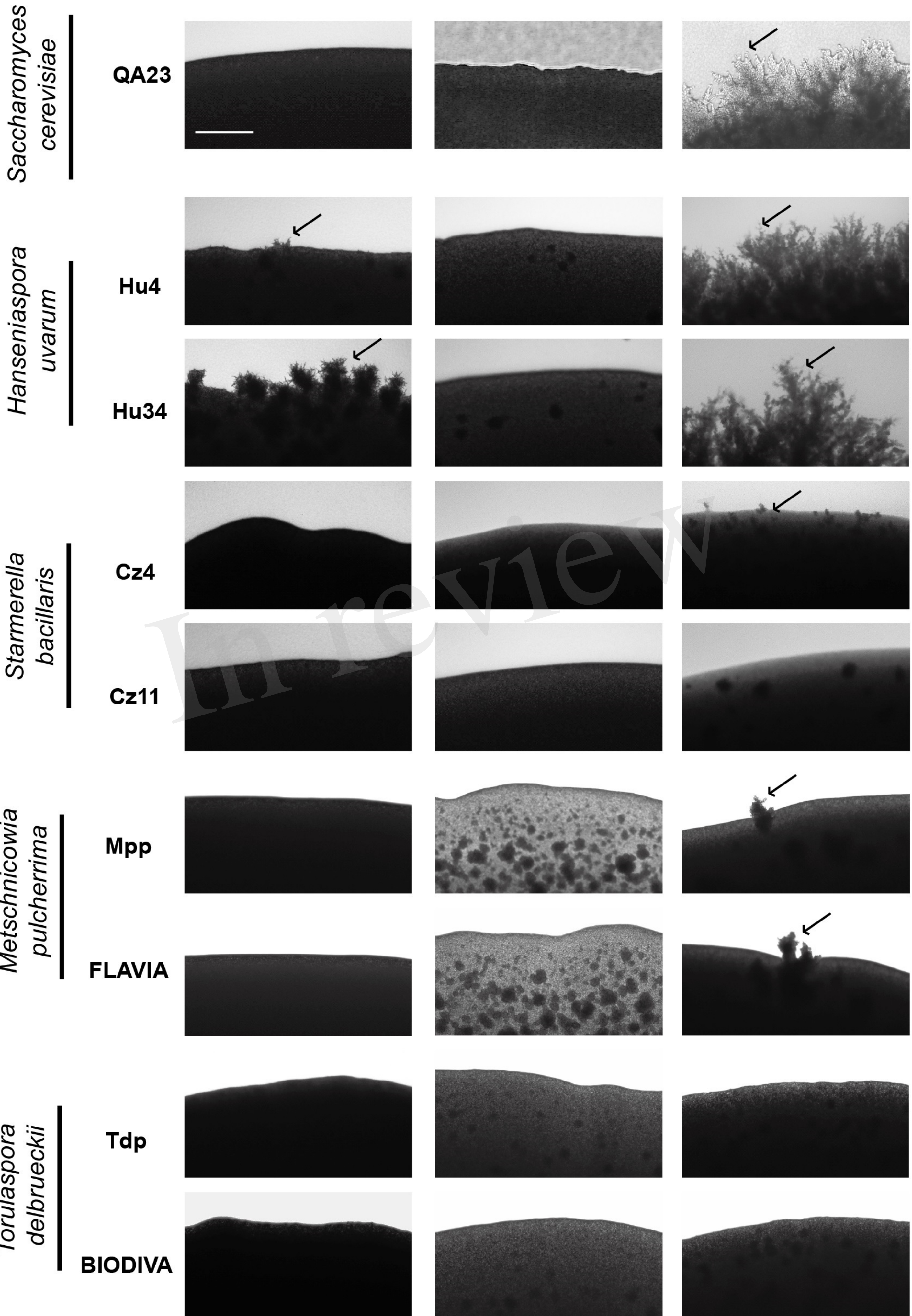

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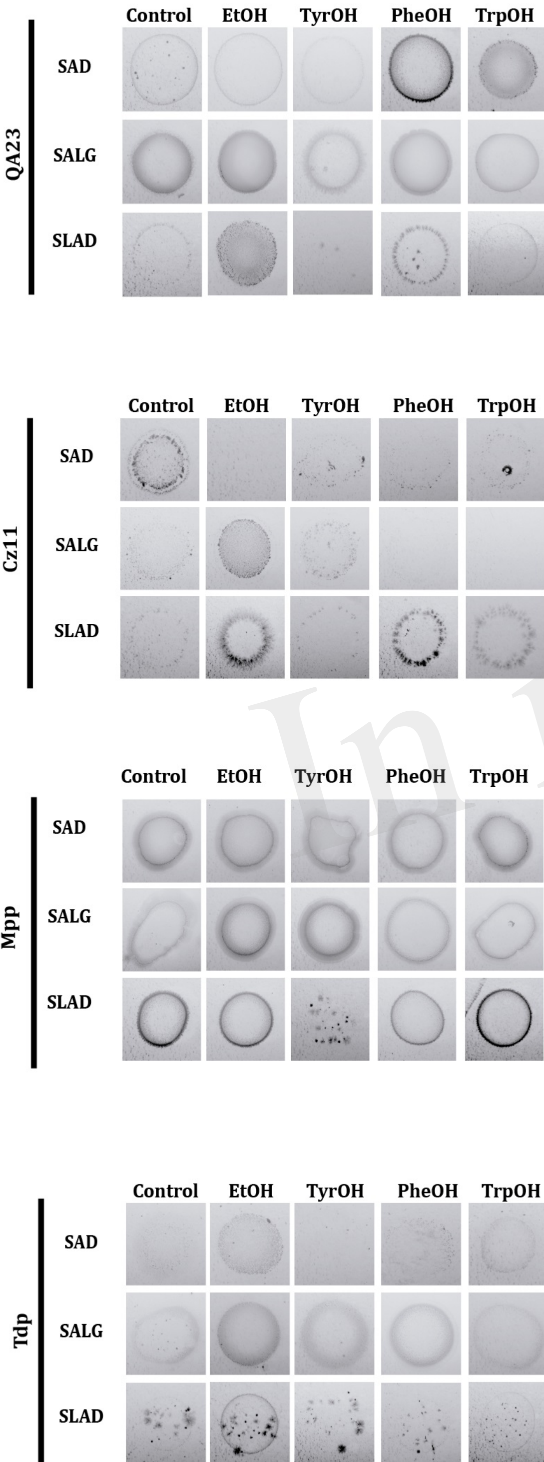

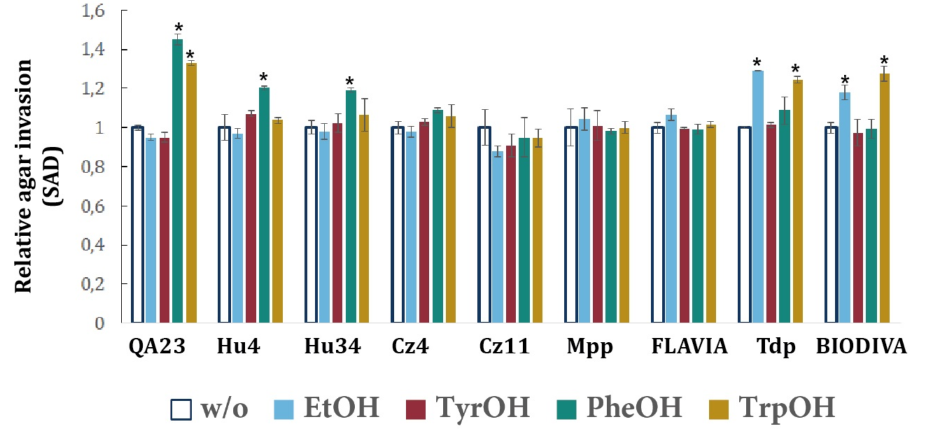

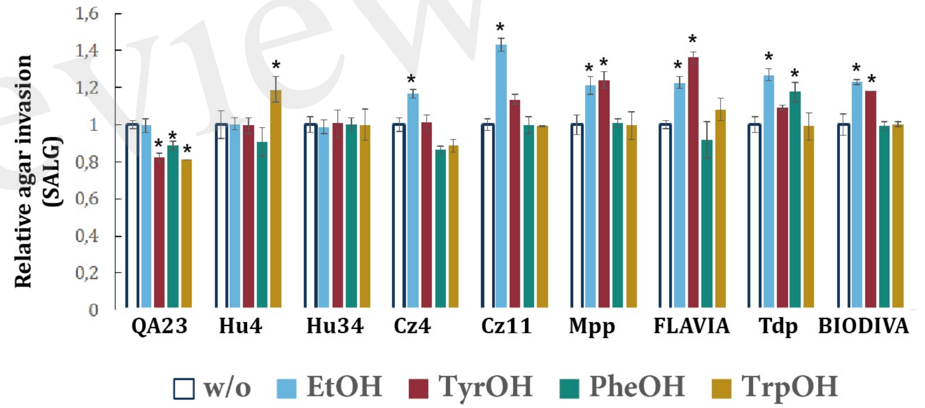

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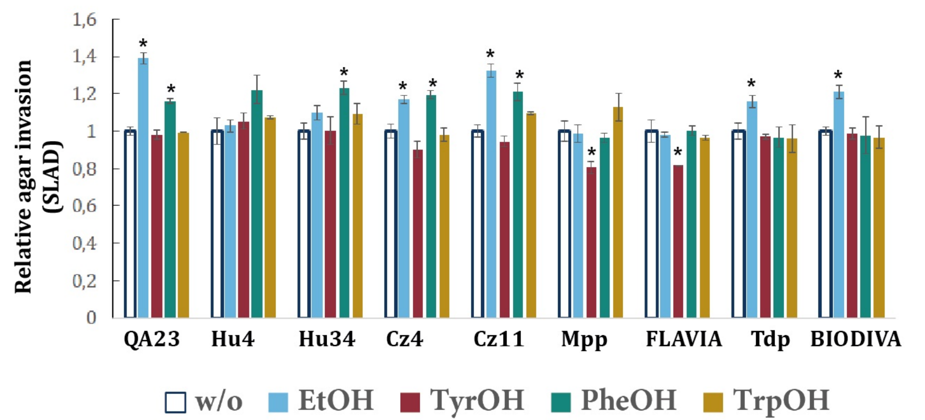

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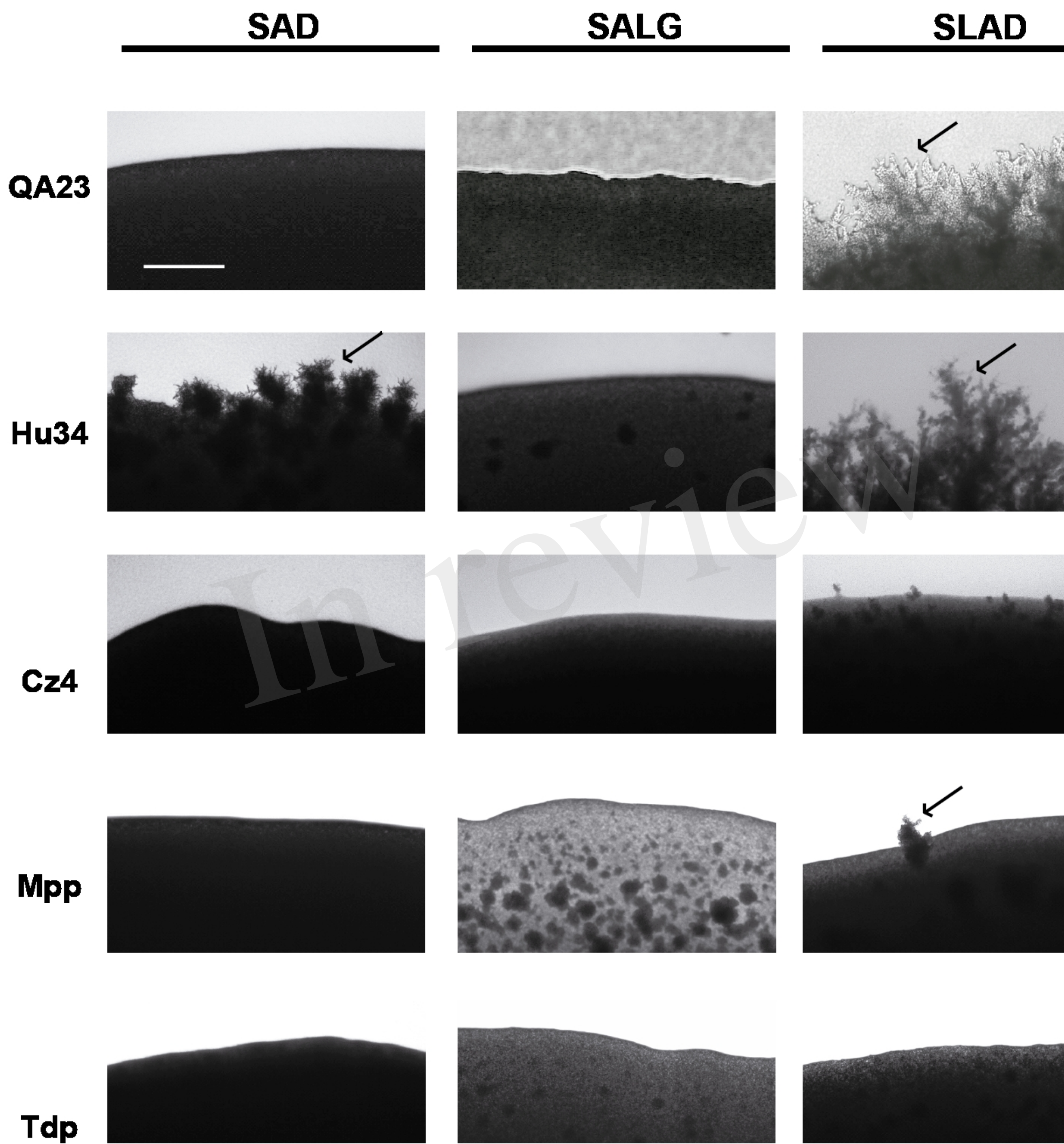

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