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1 TO BE OR NOT TO BE REQUIRED: YEAST VITAMINIC REQUIREMENTS IN 2 WINEMAKING

3 Marie Sarah Evers, Louise Ramousse, Chloé Roullier-Gall, Christophe Morge, Celine
4 Sparrow, Antoine Gobert, Hervé Alexandre

5 ABSTRACT

6 Although vitamins are prime actors in yeast metabolism, the nature and the extent of their
7 requirement in *Saccharomyces cerevisiae* in winemaking remains little understood. To fill this
8 gap, the evolution of 8 water-soluble vitamins and their diverse vitamers during its alcoholic
9 fermentation in a synthetic must medium was monitored, providing the first evidence of the
10 consumption of vitamers by five commercial *S. cerevisiae* strains, and highlighting the
11 existence of preferential vitameric sources for its nutrition. The vitamins **required by the**
12 **yeast**, B1, B5, and B8, were then identified, and the nature of their requirement characterized,
13 strongly asserting the **required** trait of B1 for fermentation, B8 for growth, and B5 for both
14 processes. The extent of the requirement for B5, that with the most impact of the three
15 vitamins, was then quantified in three *S. cerevisiae* strains, resulting in the conclusion that 750
16 $\mu\text{g.L}^{-1}$ should prove sufficient to cover the yeast's requirements. This investigation offers the
17 first insight into *S. cerevisiae* vitaminic requirements for winemaking.

18 HIGHLIGHTS

- 19 • *S. cerevisiae* is shown to consume vitamins B1, B3, B5, B6, B8 and B9 vitamers, and
20 to have preferential vitameric sources.
- 21 • Three vitamins appear **required** by *S. cerevisiae* in winemaking: B1 in its
22 fermentation, B8 in its growth, and B5 for both processes.
- 23 • B5 deficiencies appear to have the most dramatic consequences on *S. cerevisiae* in
24 winemaking.
- 25 • *S. cerevisiae* requirement levels have been determined as 750 $\mu\text{g.L}^{-1}$.

26

27 1. INTRODUCTION

28 While enological yeast requirements for certain nutritional resources, such as nitrogen, are
29 now better understood (Brice et al., 2014; Crépin et al., 2017; Gobert et al., 2017; Gutiérrez et
30 al., 2012; Tesnière et al., 2015), some nutrients have received little attention up to now.

31 Amongst them are vitamins which are involved as cofactors in numerous major metabolic
32 pathways, such as those of carbon, fatty acids, amino acids, nitrogen and sulfur (Evers et al.,
33 2021; Perli et al., 2020), or proved relevant in the production of wine volatile compounds
34 (Bohlscheid et al., 2007; Evers et al., 2023b; Labuschagne et al., 2021), but have remained
35 unexplored in recent decades.

36 Consequently, while their relevance in winemaking and for wine yeasts appears quite strongly
37 (Bataillon et al., 1996; Bohlscheid et al., 2007; Edwards & Bohlscheid, 2007; Labuschagne et
38 al., 2021; Labuschagne & Divol, 2021), the extent to which they are necessary to enological
39 yeasts has been given little attention. The considerable diversity of existing vitaminic
40 resources, whether between vitamins themselves or between their different forms, called
41 vitamers, renders their investigation complex, since not all vitamers are assimilated by yeasts,
42 or are metabolically active (Evers et al., 2021). The few existing estimations for yeast
43 vitaminic requirements in processing alcoholic fermentation have not been determined in
44 grape must-like cultivation media (Jones et al., 1981; Shinohara et al., 1996), and as such,
45 might not reflect the reality of winemaking.

46 The lack of knowledge regarding vitamin requirements leads to difficulties in mastering the
47 course of alcoholic fermentation during wine production. As such, vitamin deficiencies in
48 yeasts have been shown to result in decreased growth rates, sluggish fermentations, lower
49 resistance to various stresses, and the accumulation of undesirable compounds, such as H₂S
50 (Bataillon et al., 1996; Ferreira et al., 2017; Ough et al., 1989; Ough & Kunkee, 1967;
51 Trevelyan & Harrison, 1954; Xing, 2007). However significant they may be, and despite the
52 fact that an analytical method to quantify vitamins and vitamers in grape musts has been
53 developed (Evers et al., 2023a), the absence of knowledge regarding yeast requirements for
54 wine production does not allow identifying musts at risk of inducing limitations.

55 Consequently, here we investigate the vitaminic requirements of enological yeasts, their
56 preferred sources, and their deficiency thresholds.

57

58 2. MATERIALS AND METHODS

59 2.1. Chemicals and reagents

60 Phosphate buffer saline (PBS) was purchased from Sigma (Merck, Darmstadt, Germany). 5-6-
61 carboxyfluorescein diacetate (cFDA) was purchased from Molecular Probes (Thermo Fisher
62 Scientific, Waltham, MA, United States of America).

63 2.2. Fermentation media

64 Fermentations were conducted in different variations of a synthetic grape must formulated
65 from carbohydrate, nitrogen, mineral, trace elements, and vitamin sources, modified from the
66 MS300 developed by Bely and colleagues (Bely et al., 1990) and modified as described
67 before (Evers et al., 2023b).

68 2.2.1. Synthetic grape must adaptation

69 A synthetic grape must based on a composition similar to MS300, MSvitamins, but prepared
70 to reflect the vitaminic contents of natural grape musts, was implemented for model alcoholic
71 fermentations of enological yeasts and compared to MS300

72 Vitaminic forms were chosen according to the synthetic must as defined by Bely and
73 colleagues, but modified to the following concentrations representative of musts (Evers et al.,
74 2023a) (as expressed per liter): thiamine hydrochloride, 1750 µg; riboflavin, 600 µg; nicotinic
75 acid, 1250 µg; pantothenic acid hemicalcium salt, 750 µg; pyridoxine, 2750 µg; biotin, 1 µg;
76 ascorbic acid, 3 mg; myo-inositol, 20 mg.

77 2.2.2. Yeast vitaminic consumption profile assessment

78 In order to assess the evolution of all the individual vitamers during alcoholic fermentation by
79 enological yeasts, the synthetic must medium MSvitamers in this assay was prepared with all
80 the known vitamers in grape musts. Vitamins in the medium were added in the following
81 concentrations expressed per liter and chosen to reflect the vitamer contents that can be found
82 in a natural grape must (Evers et al., 2023a): thiamine hydrochloride, 500 µg; thiamine
83 monophosphate, 800 µg; thiamine pyrophosphate, 1300 µg; riboflavin, 600 µg; nicotinic acid,
84 300 µg; nicotinamide, 500 µg; nicotinamide adenine dinucleotide, 300 µg; nicotinamide
85 adenine dinucleotide phosphate, 800 µg; pantothenic acid hemicalcium salt, 600 µg;
86 coenzyme A, 300 µg; pyridoxal, 500 µg; pyridoxal-5'-phosphate, 900 µg; pyridoxamine, 700
87 µg; pyridoxamine-5'-phosphate, 300 µg; pyridoxine, 200 µg; pyridoxine-5'-phosphate, 600
88 µg; biotin, 1 µg; folic acid, 3 µg; ascorbic acid, 3 mg; myo-inositol, 20 mg.

89 2.2.3. Determination of required vitamins

90 The MSvitamins medium was used as control in the assay. In order to determine the **required**
91 character of the selected vitamins (thiamine (B1), niacin (B3), pantothenic acid (B5),
92 pyridoxine (B6), biotin (B8) and folic acid (B9)), each modality in this assay corresponded to
93 the complete removal of one of these vitamins from the medium while the other vitamins
94 were kept unchanged.

95 2.2.4. Requirement threshold evaluation

96 In a similar way to what was performed in the previous assays, only **required** vitamin B5
97 (pantothenic acid), that having the most impact, was investigated to determine its requirement
98 levels. The MSvitamins medium was used as control in the assay ($1750 \mu\text{g.L}^{-1}$ B5), and only
99 the B5 concentrations varied in it: this entailed testing four other doses (250, 150, 100, and 0
100 $\mu\text{g.L}^{-1}$ of B5).

101 2.3. Yeasts and inoculation procedures

102 Five commercial strains of *Saccharomyces cerevisiae* were used: Selectys[®] La Marquise
103 (hereafter named “strain A”), Selectys[®] Thiols (“strain B”), Selectys[®] Italica CR1 (“strain
104 C”), SO.DELIGHT[®] (“strain D”) and VIALATTE FERM[®] W28 (“strain E”) (Sofralab SAS,
105 Magenta, France) were assessed. All the yeasts were streaked from frozen cryotubes on YPD
106 plates (0.5% (w/v) yeast extract, 1% (w/v) bactopectone, 2% (w/v) glucose, and 0.02% (w/v)
107 chloramphenicol) and incubated at 28 °C for 48 hours. One colony was then used to inoculate
108 100 mL liquid synthetic must medium, incubated at 28°C for another 48 hours as a
109 precultivation broth.

110 In order to reflect the field reality of winemaking, the other assays (synthetic must adaptation,
111 required vitamin determination, and requirement level assessment) were inoculated with *S.*
112 *cerevisiae* strains A, B and C in active dry yeast (ADY) form. These strains were selected to
113 account for possible differences in regards to their vitamin requirements. The ADY for each
114 strain were rehydrated according to the manufacturer’s specifications.

115 The density of viable cells in the inoculation broth was then determined by flow cytometry on
116 a BD Accuri[™] C6 Plus apparatus (Becton, Dickinson and Company, Franklin Lakes, NJ,
117 United States), as previously described (Evers et al., 2023b).

118 2.4. Fermentation and growth monitoring

119 All fermentations were carried out in **250 mL and 100 mL Erlenmeyer flasks containing 200**
120 **mL or 50 mL, respectively, of varying synthetic must media**, according to the variations

121 described in 2.2., previously sterilized by vacuum filtration on a 0.22 µm pored membrane-
122 based apparatus (Millipore Steritop, Merck, Darmstadt, Germany). The fermentation
123 temperature was set at 20°C without shaking. All the assays were triplicated.

124 During the course of alcoholic fermentation, viable cell concentration was determined by flow
125 cytometry as previously described, thus allowing for the monitoring of biomass growth.
126 Fermentation progress was monitored as previously described (Evers et al., 2023b; Seguinot
127 et al., 2020).

128 2.5. Analytical methods

129 Endpoint fermentation samples were centrifuged at 13.000 rpm for 3 min, and the collected
130 supernatants were stored at - 20°C until analysis.

131 2.5.1. HPLC-UV quantification of vitamins and vitamers

132 During alcoholic fermentation, sampling was done for vitamin and vitamer analysis using an
133 HPLC system (1260 Infinity II, Agilent, Santa Clara, CA, United States of America)
134 combined with a pump, an auto-sampler, and a diode array detector (1260 Infinity II, Agilent,
135 Santa Clara, CA, United States of America). Also, data analysis software (OpenLab CDS,
136 Agilent, Santa Clara, CA, United States of America) was used. The analysis was performed
137 using a binary solvent system eluted on a 4.6 x 250 mm, 5 µm Zorbax SB-Aq column
138 (Agilent, Santa Clara, USA), preceded by a Zorbax SB-Aq 4,6 x 12.5 mm, 5 µm guard
139 column (Agilent, Santa Clara, USA), at a temperature of 25°C, according to the method
140 detailed by Evers and colleagues (Evers et al., 2023a).

141 2.5.2. Enzymatic determination of central carbon metabolites

142 Acetic acid, acetaldehyde, D-lactic acid and glycerol were enzymatically quantified using a
143 Y15 enzymatic autoanalyzer (Biosystems, Barcelona, Spain), calibrated and configured for
144 the associated enzymatic kits (references 12930, 12820, 12812, 12801; Biosystems,
145 Barcelona, Spain). The complete consumption of sugars was similarly verified enzymatically
146 using the D-glucose/D-fructose kit (reference 12800; Biosystems, Barcelona, Spain) on the
147 Y15 enzymatic autoanalyzer.

148 2.6. Statistical analyses

149 Data analyses and graphical representations were performed using R software (version 4.0.3).
150 Nonparametric Kruskal-Wallis and Dunn tests from the rstatix package (Kassambara, 2021)

151 were used for statistical comparisons of samples. p-value < 0.05 was used as the acceptance
152 criterion for rejecting the null hypothesis.

153

154 3. RESULTS AND DISCUSSION

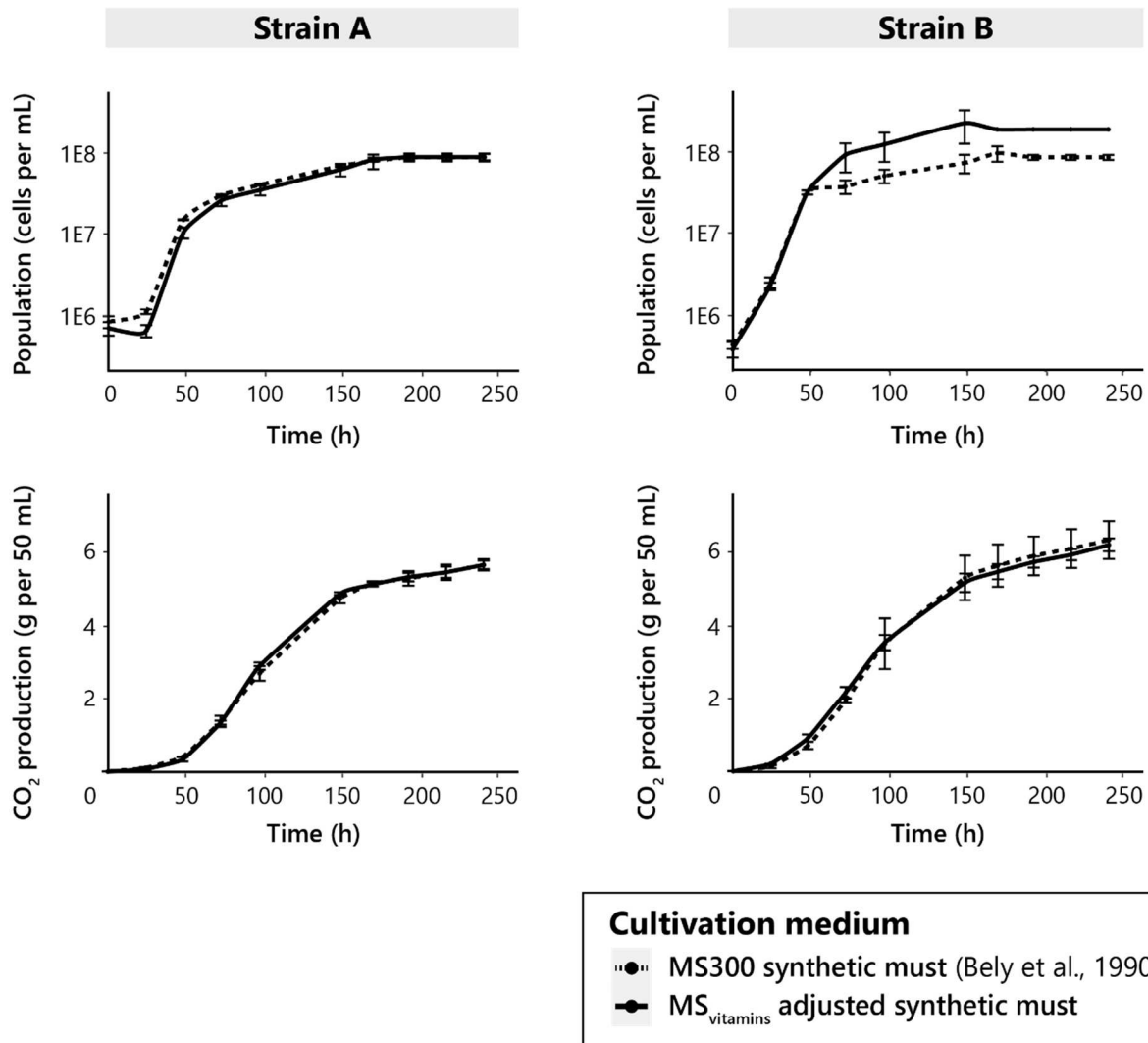
155 3.1. Synthetic grape must optimization

156 Synthetic grape must media are common models for fermentations in enological matrixes, and
157 exist in numerous variations (Beltran et al., 2005; Kinzurik et al., 2015; Viana et al., 2014);
158 however, their vitaminic composition most often fails to replicate those that may be found in
159 real grape must, due to a lack of knowledge regarding vitamins. Consequently, based on the
160 vitaminic composition of grape musts as determined in 85 white musts (Evers et al., 2023a), a
161 vitamin-accurate synthetic grape must MSvitamins has been developed from the medium
162 elaborated by Bely and colleagues (Bely et al., 1990), and described previously (Evers et al.,
163 2023b). This MSvitamins synthetic must has therefore been tested.

164 Interestingly, no significant difference ($p > 0.05$) was found between the two tested media for
165 either strain in regards to their growth and fermentation kinetics (Figure 1; Supplementary
166 data Table S1), suggesting that the vitaminic requirements of the yeasts were covered in the
167 same way by both media.

168 3.2. *S. cerevisiae* preferential vitaminic sources in wine-like alcoholic fermentation

169 Although vitamins are primary actors in regards to yeast metabolism, and are therefore
170 important nutrients for *S. cerevisiae* in winemaking, little is known of their essential nature, or
171 the extent to which they are required. Understanding these requirements is, furthermore, made
172 even more complex by the diversity of vitamins, each of them existing in several varying and
173 closely-related forms, under the name vitamers, which are not all taken up exogenously
174 (Combs & McClung, 2017; Perli et al., 2020).



175

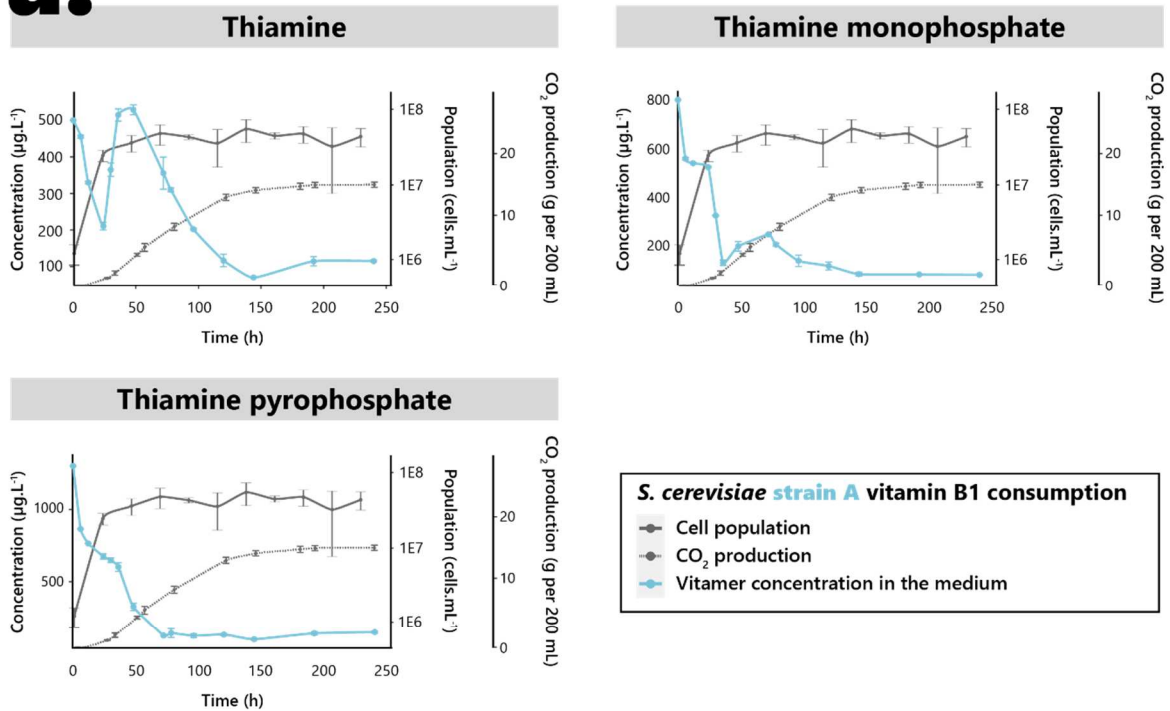
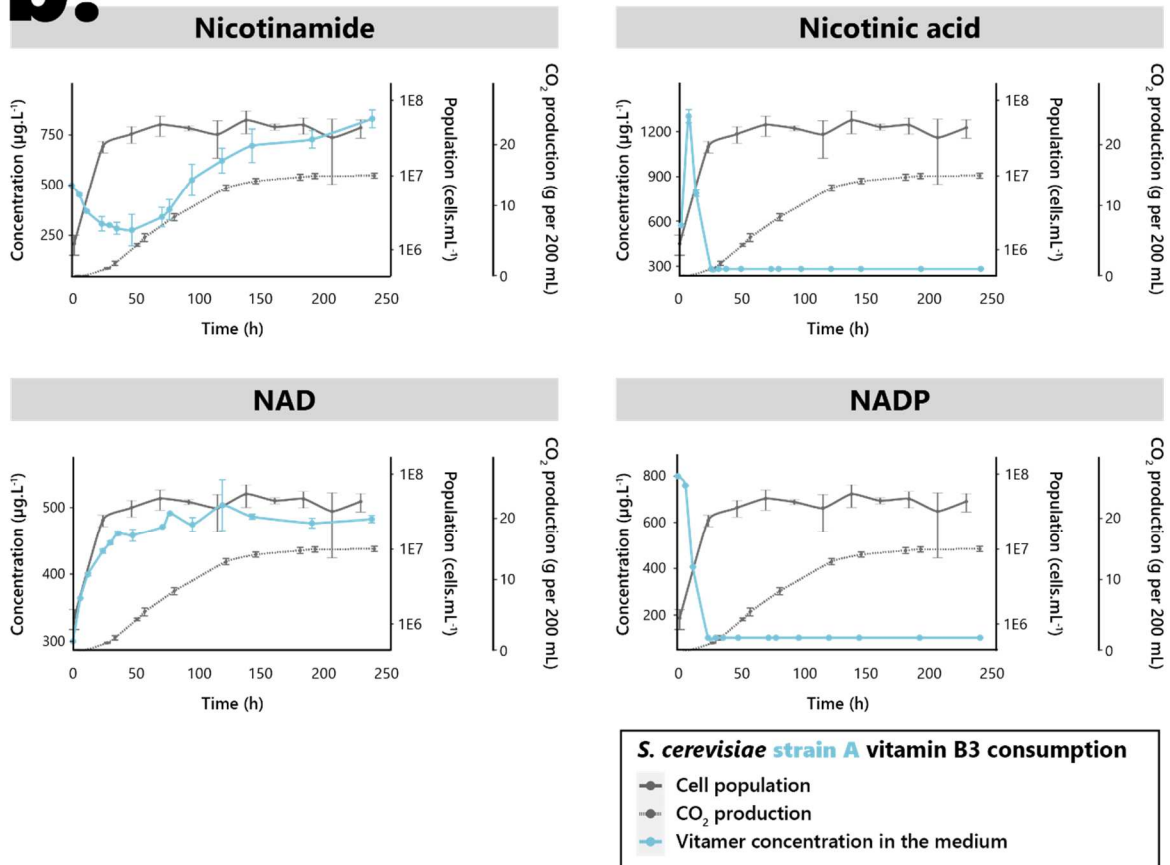
176 **Figure 1 - Growth and fermentation kinetics of two *S. cerevisiae* commercial ADY strains in two different synthetic**
 177 **must media at 20 °C (in triplicate).**

178 The preferential vitamers of wine yeasts, however, have never been investigated, and have
 179 therefore been studied here in five different commercial *Saccharomyces cerevisiae* strains.

180 This led to the first evidence of the consumption of the different vitamers by yeast in
 181 enological contexts, and to initial understanding of its preferred vitaminic sources.

182 Here, the alcoholic fermentation process performed using *S. cerevisiae* strain A, was marked
 183 by a striking decrease in the contents of all B1 vitamers in the medium (Figure 2a), showing
 184 evidence that the yeast consumed the different vitamers accessible to it. It furthermore
 185 appeared that thiamine pyrophosphate (TPP) might be the preferred B1 source, since its
 186 specific maximal consumption rates $Q_{S_{max}}$ were the highest of the three vitamers
 187 (Supplementary data Table S2).

188

a.**b.**

189

190

191

192

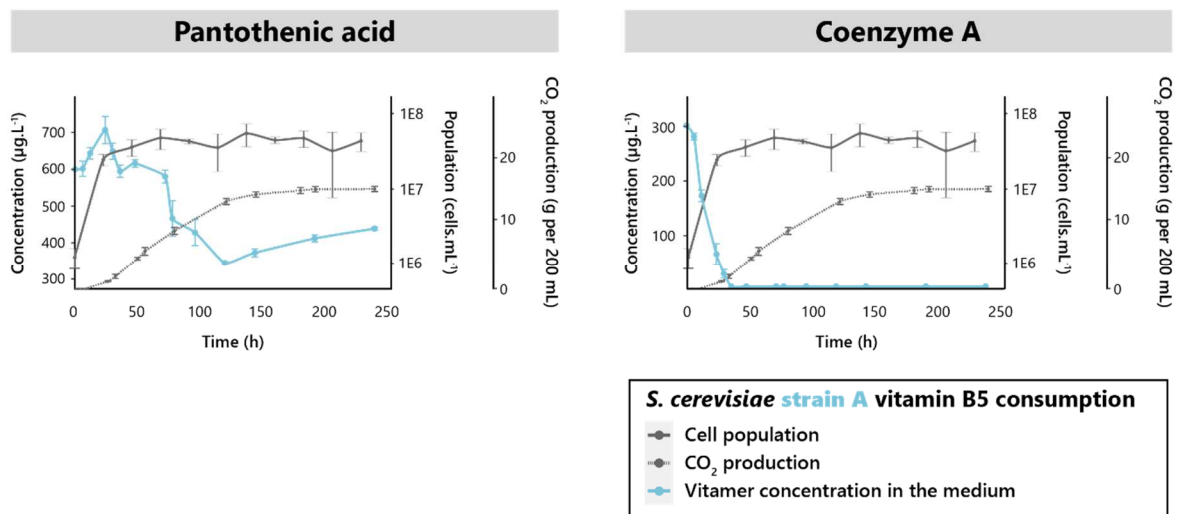
Figure 2 – *S. cerevisiae* strain A population, CO₂ production, and vitaminic consumption during its alcoholic fermentation in a synthetic must medium at 20 °C (in triplicate). a. B1 vitamers concentration in the medium. b. B3 vitamers concentration in the medium.

193 This suggests that, although the free thiamine (T) form is the only one to act as a substrate of
194 transporter Thi10 (Enjo et al., 1997; Perli et al., 2020), certain mechanisms involved in the
195 assimilation of the other B1 vitamers might occur in *S. cerevisiae*, possibly relying on
196 periplasmic acid phosphatase Pho3, which hydrolyzes thiamine phosphate esters before
197 uptake (Enjo et al., 1997; Nosaka, 1990; Nosaka et al., 1989). Furthermore, it appears that this
198 consumption took place during the yeast exponential growth phase, suggesting a marked
199 requirement of *S. cerevisiae* during this phase. It is noteworthy, however, that the exhaustion
200 of TPP in the medium appeared concomitant to the deceleration of the fermentation process,
201 possibly indicating its role as a cofactor with pyruvate decarboxylase Pdc (Perli et al., 2020),
202 and of the supposedly **required** character of vitamin B1 in the course of fermentations
203 (Bataillon et al., 1996). The overall decrease in vitamin B1 agrees with previous findings
204 (Hall et al., 1956), although this is the first evidence of the consumption of all B1 vitamers, a
205 behavior that appeared to be shared by all five *S. cerevisiae* strains investigated
206 (Supplementary data Figure S1, Figure S2).

207 In addition, *S. cerevisiae* strain A, exhibited a striking consumption of B3 vitamer nicotinic
208 acid (NA) and nicotinamide adenine dinucleotide phosphate (NADP), as their contents fell
209 sharply during the yeast exponential growth phase (Figure 2b). This suggests that both
210 vitamers might be yeast preferential B3 sources, since their $Q_{S_{max}}$ was slightly higher
211 (Supplementary data Table S3). While total vitamin B3 had been found to decrease during
212 wine fermentations (Castor, 1953; Lafourcade et al., 1956), the nature of such a decrease was
213 in our case emphasized by the apparent decrease in the preferred vitamers NA and NADP
214 that might therefore be **required** by *S. cerevisiae*. The rapid drop in NA contents might reflect
215 the uptake of the vitamer through *S. cerevisiae* transporter Tna1 before intracellular
216 conversion towards the cofactor form nicotinamide adenine dinucleotide (NAD) (Belenky et
217 al., 2011; Llorente & Dujon, 2000). This appears to have been the case with the continuous
218 increase in NAD concentrations in the medium (Figure 2b). A slower, and less marked
219 consumption of nicotinamide (NM) by strain A seemed to have taken place during the yeast
220 exponential growth phase, before a possible release induced rises in contents (Figure 2b). The
221 decrease in NM appeared to occur simultaneously with the very sharp increase in NA at the
222 very beginning of yeast growth, which might reflect the extracellular NM Pnc1-dependent
223 conversion to extracellular NA after NM intake by an unidentified transporter (Belenky et al.,
224 2011), before NA enters the NA/NM NAD-producing salvage pathway (Croft et al., 2020).
225 Interestingly, NM was the sole B3 vitamer to exhibit differences in behavior between the *S.*

226 *cerevisiae* strains assessed, since neither strain C, nor strain E, led to further increases of the
 227 vitamer in the medium (Supplementary data Figure S3, Figure S4). It should also be noted
 228 that, while no overshoot of NAD has been described in *S. cerevisiae*, to our knowledge, this
 229 yeast has been shown to continuously release and uptake NAD precursors (Croft et al., 2020),
 230 such as NM and NA, as observed here over varying timespans (Figure 2).

231 While pantothenic acid (PA) underwent limited and rather slow decreases in contents during
 232 strain A alcoholic fermentation (Figure 3), the B5 cofactor form coenzyme A (CoA) appeared
 233 to be markedly consumed by the yeast during its exponential growth phase, rapidly
 234 plummeting to exhaustion in the medium. This strongly confirmed its preferential character
 235 towards *S. cerevisiae* for B5 uptake, indicated by its higher $Q_{S_{max}}$ (Supplementary data Table
 236 S3). Such a preference was further suggested by the delayed decrease in PA contents,
 237 occurring only after CoA depletion (Figure 3), and most likely taken up by transporter Fen2
 238 (Stolz & Sauer, 1999) to serve as precursor for CoA synthesis. These consumption behaviors,
 239 interestingly, appeared to be shared by all the *S. cerevisiae* strains assessed (Supplementary
 240 data Figure S5).

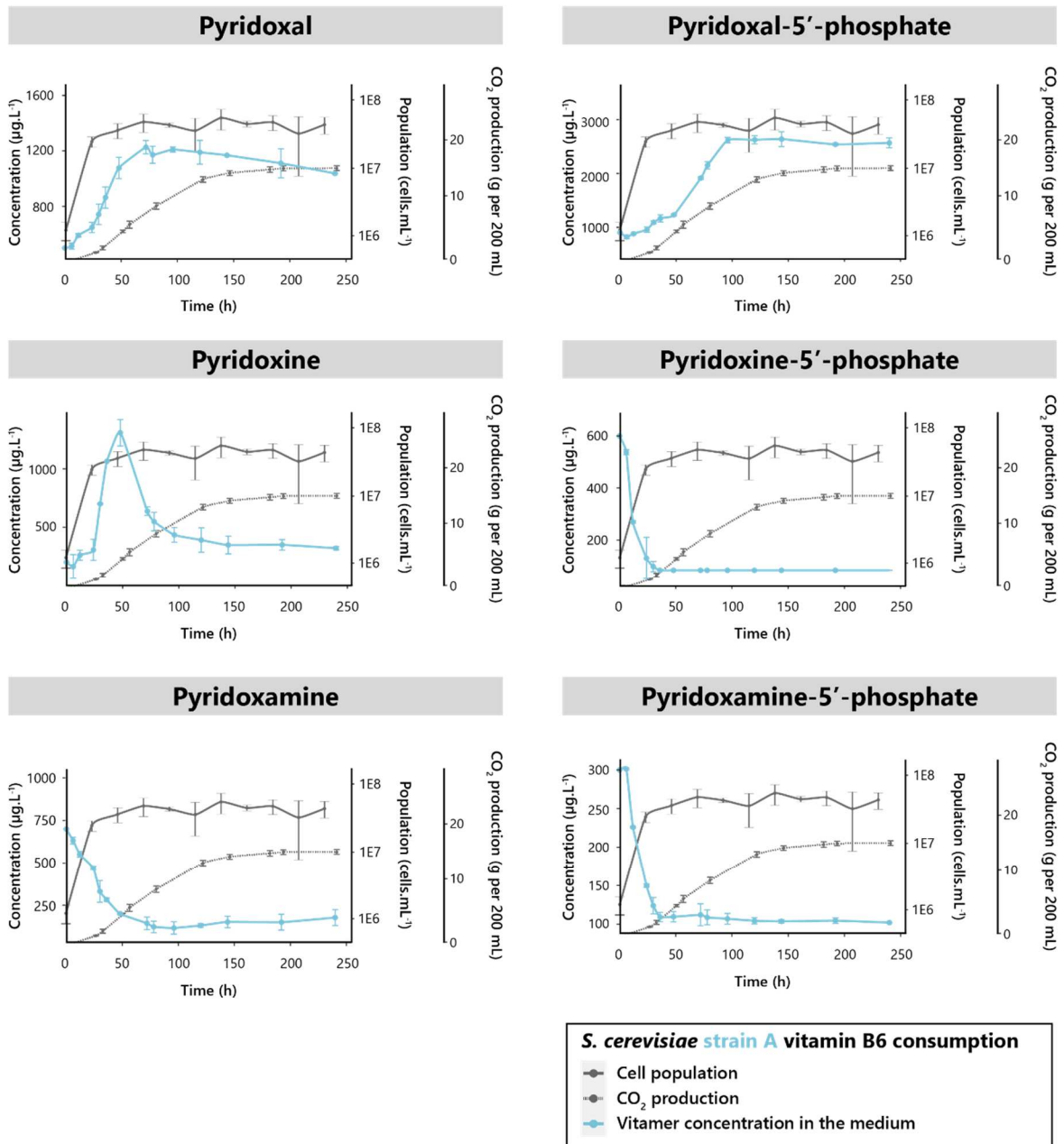


241
 242 **Figure 3 - *S. cerevisiae* strain A population, CO₂ production, and B5 vitaminic consumption during its alcoholic**
 243 **fermentation in a synthetic must medium at 20 °C (in triplicate).**

244 Evidence of the consumption of vitamin B6 complex by *S. cerevisiae* strain A appeared
 245 through marked decreases in contents of vitamers pyridoxine-5'-phosphate (PNP),
 246 pyridoxamine (PM) and pyridoxamine-5'-phosphate (PMP) in the medium (Figure 4), as it
 247 fell dramatically during the yeast exponential growth phase, suggesting that these three forms
 248 might be its preferred B6 sources, confirmed by their higher $Q_{S_{max}}$ values (Supplementary
 249 data Table S3). Pyridoxine (PN) might be a secondary nutritional source, since it was

250 consumed by the yeast after release, although this PN uptake took place only after PM
 251 exhaustion from the medium. Pyridoxal (PL) was not taken up by strain A, increasing only
 252 during fermentation, similarly to its phosphorylated cofactor form pyridoxal-5'-phosphate
 253 (PLP), which most likely increased due to intracellular synthesis through the B6 salvage
 254 pathway (Perli et al., 2020; Stolz & Vielreicher, 2003).

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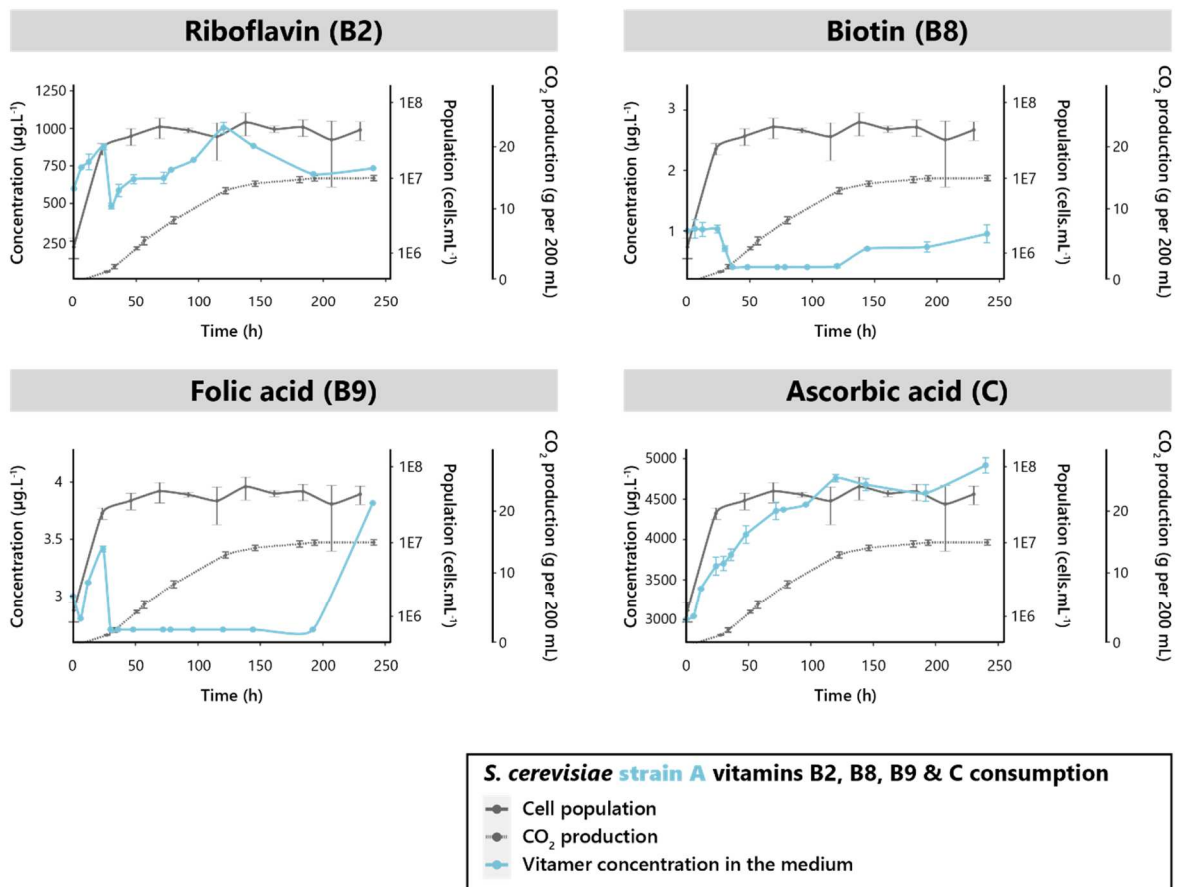


256

257 **Figure 4 - *S. cerevisiae* strain A population, CO₂ production, and B6 vitaminic consumption during its alcoholic**
 258 **fermentation in a synthetic must medium at 20 °C (in triplicate).**

259 All the *S. cerevisiae* strains investigated appeared to display rather similar behaviors in
 260 regards to their B6 consumption during alcoholic fermentation (Supplementary data Figure
 261 S6, Figure S7, Figure S8, Figure S9).

262 While riboflavin (vitamin B2) contents have been known to increase during alcoholic
 263 fermentation, due to biosynthesis by *S. cerevisiae* (Santos et al., 1995), following a rise
 264 proportional to yeast growth (Tamer et al., 1988), here, the riboflavin contents in strain A
 265 cultivation medium generally appeared constant between the beginning and the end of the
 266 fermentation process (Figure 5). While the other strains displayed riboflavin profiles
 267 resembling those from strain A (Supplementary data Figure S10, Figure S11), it should be
 268 noted that strain C induced a decrease in B2 during its exponential growth, most likely
 269 reflecting uptake through transporter Mch5 (Reihl & Stolz, 2005; Spitzner et al., 2008).



270

271 **Figure 5 - *S. cerevisiae* strain A population, CO₂ production, and B2, B8, B9 and C vitaminic consumption during its**
 272 **alcoholic fermentation in a synthetic must medium at 20 °C (in triplicate).**

273 Biotin (vitamin B8, or B7) presents a singular trait in *S. cerevisiae*, since the yeast's capacity
 274 of synthesizing the vitamin, contrary to other groups, has been established as strain-dependent
 275 (Ohsugi & Imanishi, 1985). In addition, it has been deemed the sole vitamin **required** by the

276 *Saccharomyces* genus (Castor & Archer, 1956). Here, the vitamin was evidently consumed by
277 strain A (Figure 5), as its contents fell during the yeast exponential growth phase. This agrees
278 with previous conclusions that found a significant fall in the B8 contents of musts (Castor,
279 1953). A late increase of biotin appears to have taken place during the late fermentation
280 stages. Similar rapid consumptions of B8 appeared in the other four *S. cerevisiae* strains
281 (Supplementary data Figure S10, Figure S11).

282 A similar evolution of strain A intake and release of folic acid (vitamin B9) seemed to take
283 place over the course of the alcoholic fermentation process, the vitamin initially increasing
284 during yeast exponential growth, before rapidly decreasing as it reached its stationary phase
285 (Figure 5). However, B9 appeared to increase in the medium during the final stages of
286 fermentation, most likely released by strain A, resulting in a mostly unchanged final B9 titer,
287 in accordance with previous conclusions (Hall et al., 1956). Although all the strains evaluated
288 appeared to consume B9 during the fermentation process, a notable difference in behavior
289 appeared in strain E, which immediately led to decreases in B9 titers, rather than being
290 characterized by an initial rise in the vitamin (Supplementary data Figure S10, Figure S11).

291 Finally, ascorbic acid (vitamin C) seemed to increase continuously over the course of
292 fermentation by strain A (Figure 5), leading to rises in titers up to 1.7 times its initial
293 concentration. Such a trend, however, did not seem to agree with previous conclusions that
294 found ascorbic acid to drop by half during alcoholic fermentation (Ournac, 1966), although
295 this behavior was shared by all the strains evaluated (Supplementary data Figure S10, Figure
296 S11).

297 Therefore, this investigation highlights the high consumption of vitamins by *S. cerevisiae*, and
298 provides initial evidence of the assimilation of the different vitamins by the yeast. The evident
299 consumption of vitamins B1, B3, B5, B6, B8 and B9 suggested the possibility for *S.*
300 *cerevisiae* to exhibit requirements towards those groups, and proved the existence of
301 preferential vitamins assimilated by the yeast in B3 (NA and NADP), B5 (CoA) and B6 (PM,
302 PMP and PNP).

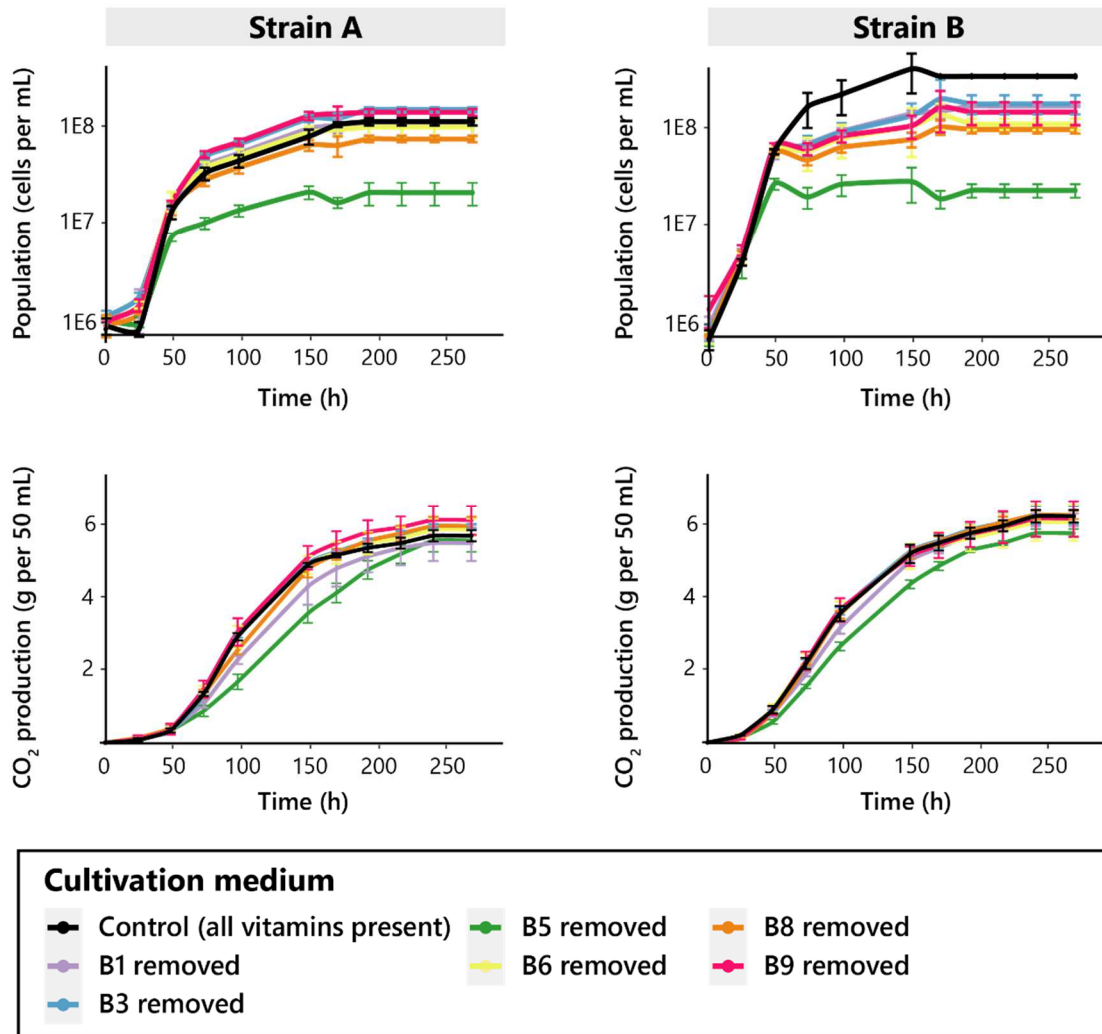
303 3.3. Determination of required vitamins

304 After establishing evidence of the consumption of vitamins B1, B3, B5, B6, B8 and B9, these
305 six groups were further investigated to characterize the existence and extent of their possible
306 requirement by *S. cerevisiae* in winemaking. Two strains, strains A and strain B, were
307 selected to account for possible differences in their required vitamins, although all five strains

308 consumed the same vitamins and vitamers, only to varying extents. Thus, each of them was
309 retrieved, one at a time, from the MSvitamins control synthetic must medium in which either
310 strain A or strain B were inoculated, and monitored in regards to their growth and
311 fermentation kinetics. The final concentrations in acetic acid, as a central carbon metabolite
312 (CCM), notably connected to acetyl-coenzyme A (Deroite et al., 2018; Mormino et al., 2021),
313 were also determined.

314 The required character of vitamin B1 (thiamine) in the yeast fermentation kinetics appeared
315 strongly (Figure 6; Supplementary data Table S3) here, significantly impacting ($p < 0.05$) the
316 maximal fermentation rate r_{CO_2max} in both of the yeasts evaluated when the vitamin was
317 omitted from the medium. Thus, decreases of 17% were observed in the fermentation rates for
318 both strains (Supplementary data Table S3). In addition to this influence on *S. cerevisiae*
319 fermentation, the absence of B1, here, also significantly reduced ($p < 0.05$) the maximal cell
320 population y_{max} reached by strain B. This evidence of the required character of B1 for *S.*
321 *cerevisiae* fermentation processes concurs with various previous works that drew similar
322 conclusions (Bataillon et al., 1996; Evers et al., 2023b; Labuschagne et al., 2021), and
323 suggests that the influence of this vitamin on yeast growth capacity, which led to
324 contradictory findings in previous works (Evers et al., 2023b; Labuschagne et al., 2021), is
325 most likely strain- or preculture-dependent. Interestingly, in addition to the impact of thiamine
326 on the yeast fermentation and growth kinetics observed, we also noted the influence of the
327 vitamin on *S. cerevisiae* CCM, due to the significant ($p < 0.05$) decreases in the acetic acid
328 titers found for both strains in the absence of thiamine, dropping from 1.32 ± 0.07 and $1.10 \pm$
329 0.06 g.L^{-1} to 0.71 ± 0.06 and $0.70 \pm 0.23 \text{ g.L}^{-1}$ in strains A and B, respectively. Such decreases
330 are consistent with previous findings that concluded that higher concentrations of acetic acid
331 are associated with higher initial thiamine contents (Eglinton & Henschke, 1993; Evers et al.,
332 2023b). Such variations in acetic acid titers therefore suggest that the removal of B1 has a
333 notable influence on yeast CCM, since acetic acid, closely interconnected with acetyl-
334 coenzyme A, is one of its major components (Deroite et al., 2018; Mormino et al., 2021).

335 Likewise, the required character of vitamin B8 (biotin) for yeast growth stands out here
336 (Figure 6; Supplementary data Table S3), as its absence led to a significant decrease ($p <$
337 0.05) in the maximal population reached, by 44% and 64% in strains A and B, respectively.
338 This appears consistent with previous studies that found the vitamin is required for yeast
339 growth (Bohlscheid et al., 2007; Evers et al., 2023b; Ough et al., 1989; Oura & Suomalainen,
340 1978).



341

342 **Figure 6 - Growth and fermentation kinetics of two *S. cerevisiae* commercial ADY strains in single-vitamin omission**
 343 **synthetic must media at 20 °C (in triplicate).**

344 Biotin, however, did not impair the yeast's fermentative capacity, as no significant differences
 345 ($p > 0.05$) could be seen regarding the maximal fermentation rate r_{CO_2max} of either strain,
 346 contrary to other works that concluded on the **required** presence of B8 for fermentation,
 347 although no consensus seems to exist on the matter (Bohlscheid et al., 2007; Evers et al.,
 348 2023b; Ough et al., 1989; Oura & Suomalainen, 1978). This difference might point to a strain-
 349 dependent aspect of this essential trait, or reflect the variations in the pre-cultivation processes
 350 chosen before the inoculation of the yeasts in the medium, since pre-starving the yeasts might
 351 lead them to directly consume external resources. Additionally, a clear influence of biotin on
 352 *S. cerevisiae*'s central carbon metabolism appeared here, significantly increasing ($p < 0.05$)
 353 the acetic acid titers in the synthetic wine when absent from the medium for both strains.
 354 These increases were from $1.19 \pm 0.16 \text{ g.L}^{-1}$ to 1.42 ± 0.11 , and from 1.13 ± 0.06 to $1.21 \pm$

355 0.10 g.L⁻¹ in strains A and B, respectively. Such findings appear consistent with previous
356 results (Evers et al., 2023b), further attesting the role of the vitamin in *S. cerevisiae* CCM.

357 In addition to further confirming the **required** traits of B1 and B8 for yeast fermentation and
358 growth, respectively, a striking influence of vitamin B5 (pantothenic acid) regarding both
359 aspects was observed here for both strains tested (Figure 6; Supplementary data Table S3).
360 Withdrawing B5 from the medium led to the most drastic reductions in the maximal
361 populations reached y_{\max} **by 82% and 91%**, and in the maximal fermentation rates $r_{\text{CO}_2\max}$ **by**
362 **33%** in both strains. Such findings were consistent with conclusions drawn in previous works,
363 that found that the absence of exogenous pantothenic acid triggered decreases in the
364 fermentation rates and cell counts of *S. cerevisiae* strains (Ough et al., 1989). Here, B5
365 appeared to be the most determinant vitamin for *S. cerevisiae*, since its absence led to a risk
366 of inducing the most marked consequences, providing new understanding of *S. cerevisiae*'s
367 fermentative behavior, and which might have major consequences in winemaking. In
368 accordance with these considerable impacts on yeast growth and fermentation kinetics, the
369 removal of B5 from the yeast cultivation medium appeared to have huge consequences on *S.*
370 *cerevisiae* acetic acid titers, that rose dramatically from 1.19 ± 0.16 to 2.12 ± 0.09 g.L⁻¹ and
371 1.13 ± 0.06 to 1.67 ± 0.10 g.L⁻¹ in strains A and B, respectively. Although, to our knowledge,
372 there has been no investigation as yet of the impact of B5 on acetic acid in wine-like matrixes,
373 reductions in the vitamin titers of anaerobic batch cultivations of *S. cerevisiae* have been
374 shown to lead to increases in its yields (Taherzadeh et al., 1996). This most likely reflects an
375 accumulation of acetic acid by the yeast, since its conversion to acetyl-CoA by synthetase Acs
376 requires CoA to take place.

377 Removing three vitamins, B3 (nicotinic acid), B6 (pyridoxine) and B9 (folic acid), from the
378 medium led to significant reductions ($p < 0.05$) in the yeast's y_{\max} values, as well as in its μ_{\max}
379 when grown in the absence of B3 strains (Figure 6; Supplementary data Table S3). These
380 findings attest to the effect of these vitamins on both the celerity and intensity of growth of
381 this specific strain, although this influence appeared to be highly strain-dependent, as strain A
382 was not affected. However, nicotinic acid, pyridoxine, and folic acid did not affect the yeast's
383 fermentation capacity. Similarly, previous findings did not show any relevant influence of B6
384 on either the growth or fermentation of pre-starved *S. cerevisiae* UCD 522 (Montrachet) at a
385 given yeast assimilable nitrogen (YAN) concentration (Xing & Edwards, 2019). In contrast,
386 investigations on three different ADY strains demonstrated various effects of removing B6 on
387 yeast fermentation rates, either increasing or diminishing it, while it had no influence on the

388 viable cell count of any of the strains (Ough et al., 1989), similar to what was observed in
389 strain B. In previous findings, NA was not found to be **required** by *S. cerevisiae* EC1118
390 growth, and additions of NA even slightly impaired cell mean life span. Also, it did not affect
391 its metabolic rate (Orozco et al., 2012), a phenomenon similar to that observed for strain A.
392 None of these three vitamins, however, were found to have any significant effect ($p > 0.05$)
393 on the acetic acid titers of the strains, although previous works observed increases as a
394 function of the available NA contents of the medium (Eglinton & Henschke, 1993; Monk &
395 Cowley, 1984). This contradiction might, however, reflect differences in the pre-cultivation
396 processes adopted before inoculation.

397 All in all, this investigation provided evidence of the considerable influence of certain
398 vitamins on *S. cerevisiae* during wine-like alcoholic fermentation, and established that
399 amongst all the vitamins, vitamins B1, B5 and B8 are **required** to ensure that these processes
400 occur efficiently, impacting growth, fermentation, and the yeast central carbon metabolism.

401 3.4. B5 vitaminic requirements

402 **The required character of vitamin B5 was obvious in the selected *S. cerevisiae* strains, while**
403 **its absence had the most detrimental effects on the yeast, leading us to investigate the extent**
404 **of its requirement in *S. cerevisiae*. The aim here was to provide thresholds at which the yeast**
405 **might be at risk of deficiencies, and therefore, of impairing wine in enological contexts. Since**
406 **strains A and B had requirements of similar natures, needing the same required vitamins, a**
407 **third *S. cerevisiae* strain, strain C, was assessed to obtain a more precise estimation of *S.***
408 ***cerevisiae* vitaminic requirements. The initial contents of B5, ranging between 750 and 0**
409 **$\mu\text{g}\cdot\text{L}^{-1}$, were therefore evaluated and compared regarding their capacity to cover those**
410 **requirements.**

411 While the effect of the vitamin appeared to be rather dose-dependent, significant decreases (p
412 < 0.05) in all growth parameters μ_{max} , G , and y_{max} at B5 doses lower than $750 \mu\text{g}\cdot\text{L}^{-1}$ were
413 found in strain A (Figure 4; Supplementary data Table S4), strongly emphasizing this
414 concentration as its requirement threshold for growth, and further demonstrating the **required**
415 character of B5 for *S. cerevisiae*. A far lower requirement for the maintenance of B5
416 fermentative capacity was highlighted in strain A, however, since significant decreases ($p <$
417 0.05) in its $r_{\text{CO}_2\text{max}}$ could be found only below $100 \mu\text{g}\cdot\text{L}^{-1}$ of B5 (Figure 4; Supplementary data
418 Table S4).

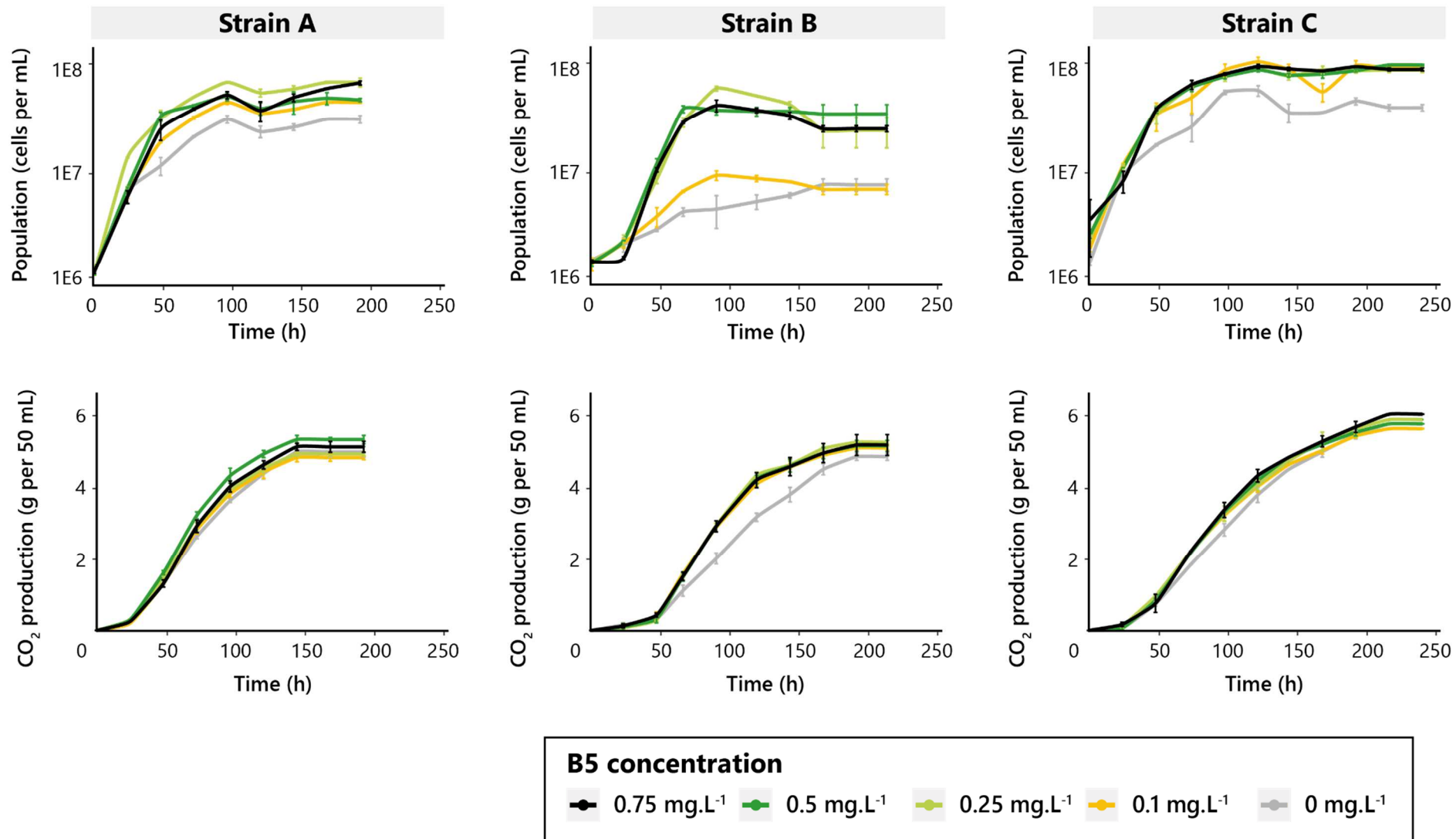
419 The effect of B5 on yeast CCM was also assessed here, since significant rises ($p < 0.05$) in the
420 acetic acid and acetaldehyde titers produced by strain A occurred at doses lower than 250
421 $\mu\text{g.L}^{-1}$ (Figure 4; Supplementary data Table S5). Glycerol metabolism, however, seemed to be
422 a higher B5-requiring process, since its contents appeared to increase significantly ($p < 0.05$)
423 when strain A was grown in less than 750 $\mu\text{g.L}^{-1}$ (Figure 4; Supplementary data Table S5).

424 Interestingly, strain B was characterized by a slightly lower B5 requirement level, while strain
425 C was not affected by it for its growth and fermentation (Figure 4; Supplementary data Table
426 S4, Table S5). Such differences in behaviors between the three strains suggest that B5
427 requirements in *S. cerevisiae* might be strain-dependent.

428 It can however be assumed that B5 contents above 750 $\mu\text{g.L}^{-1}$ might be sufficient to cover
429 most of the requirements of *S. cerevisiae* strains for wine-like alcoholic fermentation and
430 processes. This requirement level, however, was determined only from these three selected
431 strains, and it is, consequently, not impossible that, given the strong strain-dependent
432 characters observed here, that other strains might have even higher requirement levels. This
433 estimated requirement might, though, give rise to numerous issues in winemaking, since only
434 a quarter of 85 quantified white grape musts exhibited B5 contents above this threshold
435 (Evers et al., 2023a), thus many of them are at risk of inducing deficiencies.

436 Consequently, this investigation provided information regarding the extent of *S. cerevisiae*
437 requirements for growth, fermentation, and for the proper maintenance of its CCM in
438 winemaking contexts.

439



440

441

Figure 7 – Growth and fermentation kinetics of *S. cerevisiae* La Marquise, Italica and Thiols in a synthetic grape must containing decreasing B5 concentrations at 20°C (in triplicate).

442 4. CONCLUSION

443 Although they play major roles in yeast metabolism, the nature and extent of vitaminic
444 requirements of wine yeasts, and, notably, of *S. cerevisiae* still remain poorly described in
445 enological contexts. However, the monitoring of vitamins over the course of alcoholic
446 fermentation provides initial evidence of the consumption of varying vitamins, and highlights
447 the existence of preferential vitaminic sources in *S. cerevisiae*. Furthermore, the required
448 character of several vitamins, B1, B5, and B8, was clearly demonstrated regarding yeast
449 growth, fermentation, and CCM, underlining their evident relevance in winemaking contexts.
450 The requirement level of *S. cerevisiae* for B5, the vitamin having the most impact of the three
451 investigated, was also quantified. All in all, this investigation provides the first estimation of
452 the extent and consequences of vitaminic deprivations in wine yeasts, although the still-
453 limited number of strains evaluated and the strain-dependent character of these requirements
454 opens paths for further evaluations in winemaking. In addition, these requirements should be
455 further confirmed in natural grape musts in order to provide a more accurate depiction of true
456 winemaking conditions.

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