EFFECT OF TRANSIENT THERMAL SHOCKS ON ALCOHOLIC FERMENTATION PERFORMANCE

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ABSTRACT

Stuck and sluggish fermentations are among the main problems in winemaking industry leading to important economic losses. Several factors have been described as causes of stuck and sluggish fermentations, being exposure to extreme temperatures barely studied. The objective of this study was to identify thermal conditions leading to stuck and sluggish fermentations, focusing on the impact of an abrupt decrease/increase of temperature on fermentation performance and yeast viability/vitality. Three different strains of S*accharomyces cerevisiae*, SBB11, T73, and PDM were evaluated in synthetic grape must fermentations with and without nitrogen supplementation. Cold shocks (9ºC and 1.5 ºC for 16 hour) carried out on different days during the fermentation process were unable to alter fermentation performance. Conversely, shock temperatures higher than 32 ºC, applied in early stages of the process, lead to sluggish fermentation showing a delay directly related to the temperature increase. Fermentation delay was associated with a decrease in cell vitality. The impact of the heat shock on fermentation performance was different depending to the strain evaluated and nitrogen supplementation. None of the conditions evaluated produced a stuck fermentation and importantly, in all cases must nutrition improved fermentation performance after a heat shock.

Key words: wine; sluggish fermentations; heat shock; nitrogen addition

1. INTRODUCTION

Wine fermentation is probably one of the most ancient biotechnological processes carried out by microorganisms ever described (Samuel 1996). During fermentation, sugars present in grape must, mainly glucose and fructose, are converted into ethanol and carbon dioxide mainly by the yeast *Saccharomyces cerevisiae* (Ribereau-Gayon et al. 2006).

Despite many advances have been done in the field of wine fermentation technology, stuck or sluggish fermentations remain an important problem faced annually by oenologist and winemakers all over the world (Maissonave et al. 2013, Bisson 1999, Malherbe et al. 2007). A fermentation is considered stuck or sluggish when sugar consumption stops or when its rate is too low for practical purposes (Bisson 1999). When fermentation stops early, high residual sugar in the wines makes them microbiologically unstable and more susceptible to spoilage, with possible losses of quality and wine value (Lonvaud-Funel 1999; Maissonave et al. 2013). Restarting stuck or sluggish fermentations challenges winemakers because, even when restarting procedures succeed and fermentation finishes, wine quality is often affected (Urtubia et al. 2012). Therefore, it is important to detect and identify the factors that could lead to problematic fermentations.

Many possible causes have been described of problematic fermentation, such as nutrient limitation, toxicity of agricultural residues present in grapes (fungicides, pesticides), grape sanity and extreme temperatures, among others (Malherbe et al. 2007). All these factors suppose stress conditions that could have an adverse effect on growth or viability/vitality of yeast cells leading to alterations in sugar rate consumption and hence a problematic fermentation (Ivorra et al. 1999, Malherbe et al. 2007). Thermal

shocks (rapid increases or decreases in temperature) have been barely studied as causes of stuck or sluggish fermentations (Malherbe et al. 2007; Valentine et al. 2018). On one hand, we have observed in our Experimental Winery that sudden drops in temperature, often occurring during the first days of autumn, occasionally produce problems in fermentation development of late harvest grapes. The synergy of a cold shock with others stressors such as high ethanol content and starved cells (by the end of alcoholic fermentation) has not been studied before. On the other hand, increasing initial grape must temperatures have been reported (Coulter et al. 2008). In the grape-growing regions with warm and very warm climate (heliothermal index: HI +2 and HI +3) (Tonietto and Carbonneau, 2004) the high environmental temperature during harvest could affect winemaking in different ways. For instance, harvest of grapes berries during hot days favours an increase in initial must temperature. Additionally, grape temperature can increase even more due to the waiting time of the trucks until the discharge into the cellar, as it has been frequently observed in large volume wineries (Coulter et al. 2008). To this regard, the use of stainless steel tanks with thermal regulation aims to overcome these issues in the industrial wineries. Although there are still many "garage" and/or large volume wineries that use other materials such as concrete tanks for fermentations with deficient thermal control (Coulter et al. 2008).

Increases in temperature also occur due to the fermentation process itself. Grape must temperature increases at a rate of 1.3 ºC for every 100 g of sugars consumed during fermentation due to the heat produced by yeast metabolism (Boulton et al. 1996, Bisson et al. 2007). Therefore, temperature increases around 12 ºC to 15 ºC can occur and consequently grape must temperature can reach more than 40 ºC (Schimd et al. 2009, Valentine et al. 2018). Additionally, red grape fermentation is conducted together with their skins that form the cap on top of the liquid (Schmid et al. 2009). In this way, heat

98 is not uniformly distributed and the highest temperatures occur in the cap (Bisson et al.
99 2007, Schmid et al. 2009, Guerrini et al. 2017, Valentine et al. 2018). The addition of
100 nitrogen during vinification has a direct impact on yeast metabolic activity, which may
101 as well favour temperature increases (Bisson 1999; Malherbe et al. 2007). Nitrogen
102 supplementation during the first half of the fermentation (must density of 1060 g/L) is a
103 widely used oenological practice since it enhances the kinetics and reduces fermentation
104 length (Beltran et al. 2005).

105 The aim of this study was to identify thermal conditions leading to sluggish or stuck
106 fermentations, focusing on the impact of a transient and abrupt increase/decrease of
107 temperature on fermentation performance and viability/vitality of yeasts during
108 alcoholic fermentation. Three different strains of S*accharomyces cerevisiae* were
109 evaluated and nitrogen supplementation was included as a variable.

110

111 ## 2. MATERIAL AND METHODS

112 ### 2.1 Yeast strains and inoculum preparation

113 Three strains of *Saccharomyces cerevisiae* were tested in this study: SBB11, T73 and
114 PDM. Strain SBB11 was selected from Syrah grapes from Mendoza, Argentina,
115 whereas T73 and PDM are commercial strains from Lallemand and Maurivin
116 Companies respectively. Yeast cells were plated and grown on Yeast Peptone Dextrose
117 (YPD) medium. Single colonies were spread into five YPD plates and incubated for 48
118 h at 28 °C in order to get confluent growth. Yeasts were collected in 100 mL YPD broth
119 and incubated with agitation at 150 rpm for 6 hours at 28 ºC. To determine the dilution
120 for inoculation in synthetic grape must in order to obtain an initial cell concentration of
121 2 x 10^6 cells/mL, cells were counted in Neubauer chamber. Synthetic grape must (SM)

was prepared with 120 g/L glucose and 120 g/L fructose (Bely et al. 1990). Nitrogen content adjusted to 140 mg N/L (42 mg N/L as ammonium and 98 mg N/L in amino acid form) and pH to 3.3 with NaOH. Finally, SM was sterilized through filtration by 0.2 μm membrane.

2.2 Microvinifications

Fermentations were performed in 500 mL Erlenmeyer flasks equipped with Müller valves containing 300 mL of SM at 26 °C ± 2 ºC with a daily manual shaking in order to simulate pumping over normally performed at industrial winemaking. Weight loss and must density were daily monitored for cold shock and heat shock assays respectively. Fermentation was considered to be finished when residual sugar concentration was below 4 g/L (Bisson et al. 1999). Must density was measured by densitometer (Densito 30 PX, Mettler Toledo, Switzerland). Residual sugar concentration was measured by a glucose/fructose enzymatic test (Roche, Darmstadt, Germany).

2.3 Experimental design

Different experimental approaches were designed in order to mimic the thermal changes that may occur during wine fermentation. Regarding cold shock assays, the objective was to evaluate the influence of an abrupt and transient decrease in ambient temperature on fermentation performance and yeast viability/vitality. Abrupt decreases of 9 ºC ± 2 ºC and 1.5 ºC ± 2 ºC for 16 hours were carried out at 2, 6, 10 and 14 days after initiation of alcoholic fermentation (D0 corresponds to the inoculation day). Also, a daily cold shock assay was included, by placing flasks at 1.5 ± 2 ºC in a cold chamber overnight, in order to produce periodic cold shocks in the same fermentation process. Every cold shock trial was performed for two yeast strains (SBB11 and T73) in triplicates and

146 control treatment was carried out at constant temperature (26 ± 2 ºC) Regarding heat
147 shock assays the objective was to evaluate the impact of an abrupt and transient increase
148 in temperature that may occur during the first days of fermentation due to yeast
149 metabolic activity and favoured by other factors as was previously described (high
150 temperature of the grape, transport delay, refrigeration problems, nutrition, others) on
151 fermentation performance and yeast viability/vitality. Heat shocks were assessed by
152 placing flasks on day 3 of fermentation (must density 1060 ± 5 mg/L) for 16 hours on
153 incubators set at different temperatures: 32 ºC ± 2 ºC; 36 ºC ± 2 ºC and 40 ºC ± 2 ºC.
154 Additionally, fermentations were carried out with or without nitrogen supplementation.
155 In the case of fermentations performed with nutrition, three hours before the heat shock
156 an addition of 200 mg/L of diammonium phosphate (DAP) was carried out. Internal
157 temperatures of each treatment were monitored with sensors (iButton®) placed inside
158 the Erlemeyer flasks. Experimental design is summarized in Figure 1.

159 2.4 Yeast viability and vitality determination by flow cytometry

160 Cell viability and vitality were determined by flow cytometry using a commercial kit
161 (Fungal Light™ CFDA,AM/Propidium Iodide Yeast Vitality Kit, Molecular Probes™,
162 USA). Cell viability was assessed by staining with propidium iodide (PI), which
163 diffuses into the cell when the membrane is damaged and where it can intercalate with
164 DNA showing red fluorescence (non-viable cells). Cell vitality was assessed by staining
165 with acetoxymethyl ester of 5-carboxyfluorescein diacetate (CFDA,AM) which can
166 permeate the membrane of metabolically active cells. Once inside it can cleave off
167 lipophilic diacetate groups by cytosolic non-specific esterases, yielding a charged green
168 fluorescent product (vital cells). Cells were analysed using a C6 cytometer (Accuri, BD
169 Biosciences, California, USA). Fluorescence measurements were collected in FL1
170 (530/30 nm BP filter) to determine cells stained with CFDA,AM; whereas PI stained

cells were collected in FL3 (>670 nm LP filter). According to cell staining, three different yeast populations were defined: (1) CFDA+ PI-, vital and viable cells, (2) CFDA+ PI+, vital cells with damaged membrane and (3) CFDA- PI+, non-vital non-viable cells. Yeast cells from each fermentation were suspended in phosphate buffered saline (PBS) and diluted to a final concentration of 1 x 10^6 cells/mL. One mL of cell dilution was stained at 37 °C for 15 minutes with PI and CFDA,AM according to supplier recommendations. For each flow cytometry analysis, samples from control treatments were used to define gates corresponding to viable cells and non-viable cells (treated at 90 ºC for 10 minutes). Fluorescence images were acquired and processed by software (BD CSampler). Random samples were also analysed by plating on YPD media in order to verify the cell viability. Results were expressed as vitality reduction, normalizing data with its respective control as described below:

$$\text{Vitality reduction (\%)} = 100 - \frac{(\text{CFDA+}_{\text{heat shock}} * 100)}{\text{CFDA+}_{\text{control}}} \quad (1)$$

2.4 Statistical analyses

The overall fermentation performance of the different trials was statistically analysed estimating the area under the curve in the density vs. time graph (AUC). This parameter was calculated using Riemann sum area under the curve (AUC) method from inoculation time until day 11, and results are expressed as arbitrary units. Day 11 was chosen to compare all treatments according to the shortest fermentation length. All analyses were carried out with statistical software (Infostat, FCA, Universidad Nacional de Córdoba, Argentina). The AUC data were statistically analyzed using two-way ANOVA and comparisons were performed with LSD Fisher test ($p \leq 0.05$).

2. RESULTS

The transient and abrupt decrease in temperature (cold shock) did not affect the fermentation in any of the treatments evaluated in both strains studied (SBB11 and T73) compared to the condition. Alcoholic fermentation had the same length in all the cases, suggesting that an abrupt decrease of 17 ºC (from 26 ºC to 9 ºC) was not able to affect the global performance of alcoholic fermentation, regardless the day the cold shock was applied. Furthermore, we reduced the temperature more drastically (from 26 ºC to 1.5 ºC) for 16 hours using the same approach previously described. Again, no effect was observed on general alcoholic fermentation performance in any of the cases studied. A slight but momentary arrest on fermentation rate was observed immediately after the cold shock, however regular rate was recovered straightaway once placing back the flasks at 26 ºC (Figure 3). In order to mimic environmental temperature variations (warm days and cold nights) we designed a third approach where cold socks (1.5 ºC) were daily applied. Fermentation rate showed to be clearly slower than the control, as expected. However, fermentation continued and finished consuming all sugars by day 35 (Figure 3). Thus, cold shock did not produce any stuck fermentation. Also, yeast viability/vitality was determined by flow cytometry and no difference was observed for any of the conditions assessed in comparison with the control.

To study transient and abrupt increase in temperature (heat shock) as a possible cause of stuck or sluggish fermentations, synthetic must inoculated with three different *S. cerevisiae* strains (SBB11, T73 and PDM) was subjected to an abrupt upshift on temperature (32 °C, 36 ºC and 40 °C) for 16 hours on day 3 of the fermentation process (Figure 2). These assays were performed in the presence or absence of DAP supplementation. Regarding the heat shock at 32°C, no differences were observed in the fermentation performance for all the studied strains compared to their respective controls. This small increase in temperature (6 ºC, from 26 ºC to 32 ºC) in the early

stages of fermentation was not able to affect yeast viability/vitality, regardless DAP supplementation (data not shown). In contrast, heat shock at higher temperatures (36 ºC and 40 ºC) did show an effect on alcoholic fermentation performance. Figure 4 shows the effect of heat shocks on fermentation performance expressed as AUC, where higher AUC values means higher impact of the shock on fermentation. Figure 4 also shows in which way this effect was modified in the presence of DAP supplementation, regardless the strain. As expected higher temperatures produced a stronger effect on fermentation performance (Figure 4A). Interestingly, DAP supplementation was able to reduce the effect on fermentation performance when heat shocks were applied (Figure 4B).

Each strain showed different responses to heat shocks and consequently different global fermentation performance. These observations may be associated to the specific characteristics of each strain, such as its thermotolerance and nitrogen requirements. In the case of SBB11, the thermal shocks of 36 °C and 40 °C led to sluggish fermentations in both nutritional conditions (Figure 5AB). A delay of 18 and 20 days on the fermentation length respect to the control was observed for 36 ºC and 40 ºC heat shock treatments respectively, in must without DAP supplementation (Figure 5A). Likewise, a delay on fermentation length of 11 and 26 days respect to the control condition was observed in DAP supplemented musts after heat shocks at 36 ºC and 40 ºC respectively (Figure 5B). DAP supplementation reduced AUC in all treatments assessed with this strain (Figure 5C). More than 40 days were necessary to finish alcoholic fermentation when a 40 ºC heat shock was applied, in both nutritional conditions (Figure 5AB). Regarding T73, heat shock treatments led to a clear sluggish fermentation, being more intense in the absence of DAP supplementation (Figure 5DE). In 40 ºC heat shock treatments, twice as much time was needed to consume all the sugars compared to the control in both nutritional conditions. A clear effect of nutrition was observed for this

247 strain. DAP supplementation was able to moderate the impact of a heat shock at 40 ºC
248 and 36 ºC, bringing similar AUC to that of the control without nutrition (Figure 5F).
249 Finally, in the case of PDM a strong effect on the fermentation performance was
250 observed after a 40 ºC heat shock treatment regardless DAP supplementation; whereas
251 no differences were observed for 36 ºC heat shock (Figure 5GI). Even when DAP
252 supplementation in 40 ºC treatments did not moderate the impact of heat shock on AUC
253 measurements, total fermentation length was clearly reduced when musts were
254 supplemented with DAP, suggesting that a possible fermentation reactivation after day
255 11 could be favoured by nutrition (Figure 5GH).

256 The percentage of viable/vital yeasts was higher than 98 % in all samples analysed
257 before the heat shock. The day after the heat shock (day 4) yeast viability/vitality was
258 determined. The percentages of vitality in the fermentations subjected to thermal shock
259 at 32 °C did not show differences with their respective control, suggesting no effect on
260 yeast vitality (data not shown). Conversely, at 36 ºC and 40 ºC the percentages of viable
261 cells with damaged membranes (CFDA+ PI+) were negligible compared to the other
262 populations (CFDA+ PI- and CFDA- PI-). Therefore, in further analyses vital cells were
263 quantified including all CFDA+ stained cells, regardless membrane integrity (PI- and
264 PI+). In figure 6, the effect of heat shock on cells vitality the day after the shock is
265 shown as “vitality reduction”. Reduction in yeast vitality was higher after a heat shock
266 at 40 ºC compared to 36 ºC for all strains studied (Figure 6). Vitality reduction directly
267 correlated with the delay in fermentation performance observed in figure 5. However,
268 DAP supplementation was not always able to protect cells immediately after the shocks,
269 showing variable percentages of vitality reduction in the different conditions, depending
270 on the strain and temperature of the shock (Figure 6). For SBB11 and PDM, DAP
271 supplementation before 36 ºC heat shock allowed a smaller vitality reduction compared

272 to treatments without nutrition. In contrast, in 40ºC heat shock assays, DAP
273 supplemented treatments showed higher vitality reduction compared to treatments
274 without DAP for those strains. T73 was the only strain where a positive effect of DAP
275 supplementation on cell vitality was observed after a 40 ºC heat shock (Figure 6). The
276 latter is in line with the previous observations regarding fermentation performance,
277 where DAP supplementation produced a significant improvement on fermentation
278 performance (Figure 5 DE). Cell vitality was evaluated the day after the heat shock (day
279 4), and 96 hours later (day 7) to determine cell vitality recovery (Figure 7). In none of
280 the heat shock treatments vitality was restored, since on day 7 vitality yeasts
281 percentages remained similar to those observed on day 4 for all strains, as shown in
282 Figure 7 for T73. The observed decrease on yeast vitality was maintained throughout
283 the whole process and was correlated with the observations on fermentative
284 performance since fermentation rate is neither completely recovered after the shock.

285

286 ## 3. DISCUSSION

287 It is widely known that thermal changes can occur during alcoholic fermentation due to
288 external factors such as ambient temperature or factors directly related to the
289 fermentation process itself, such as yeast metabolic activity and nutrition. In spite that
290 many improvements in winemaking technology have been incorporated in order to
291 maintain fermentation temperature in a security range, certain conditions exceed the
292 capacity to control the temperature, mainly in large volume fermentations with high
293 thermal inertia. Thermal changes as causes of sluggish or stuck fermentation have been
294 barely studied. The main goal of this study was to identify transient thermal conditions
295 leading to problematic fermentations.

296 Decreases in ambient temperature are highly frequent in nature and may take place
297 seasonally, daily or just unexpectedly, depending on the region, climate and
298 environment. Our experiment was designed in order to verify previous observations
299 occurring in our experimental cellar, where stuck fermentations were recorded days
300 after an abrupt decrease in ambient temperature. Cold shocks were carried out on
301 different days during alcoholic fermentations characterized by increasing ethanol
302 content. The results showed that an abrupt and transient reduction to a temperature near
303 1.5 ºC applied in a high ethanol concentration (D14: 11.7 ± 0.5 % v/v ethanol) did not
304 affect alcoholic fermentation. In line with our results, Valentine et al. (2018) studied
305 short-term temperature changes in one industrial wine yeast grown under conditions
306 resembling winemaking, trying to reproduce cooling of a warm fermentation by a heat
307 exchanger. A short cold shock from 34 ºC to 0 ºC during 20 seconds showed no adverse
308 effect on fermentation performance for up to 80 h after treatment (Valentine et al.
309 2018). Our results are consistent with the observations that a near-freezing temperature
310 reduces cell metabolism (enzyme kinetics) and membrane fluidity, and cells enter in a
311 quiescent state similar to starvation (Panadero et al. 2006, Price and Sowers 2004). This
312 later is evidenced by a temporal decrease in fermentation kinetics, which is immediately
313 recovered after returning to a higher temperature. No synergistic effect of ethanol and
314 cold shock on reducing the vitality of the yeast was evidenced.

315 As mentioned before, temperature increases are frequently recorded during alcoholic
316 fermentation. During the first days of alcoholic fermentation, increases in must
317 temperature reaches up to 39 ºC – 40 ºC which risks the end of fermentation (Guerrini et
318 al. 2017, Valentine et al. 2018). However, in red wine fermentations temperatures are
319 not a homogenous mixture in terms of both density (grape solids and liquid) and
320 temperature distribution (Schmid et al. 2009). The highest temperatures are registered in

321 the cap and are dissipated to the liquid fraction after every pump-over procedure. As
322 previously reported by Guerrini et al. (2017) temperature maximums are reached during
323 overnights on the first three days of fermentation. The experimental design of our study
324 aimed to reproduce these temperatures increases on must and its effect on fermentation
325 performance and cell viability and vitality. Must nutrition during alcoholic fermentation
326 is a widely used oenological practice. Consequently, DAP supplementation on day 3
327 was included as variable in our experimental design.

328 A heat shock at temperatures higher than 32 ºC significantly affected fermentation
329 performance and yeast viability/vitality, being the effect directly related with the
330 temperature. The latter confirms that sudden and transient increases in must temperature
331 are able to produce a sluggish fermentation, although no complete arrest was evidenced
332 under the conditions assessed in this study. It was recently reported that cells briefly
333 exposed during fermentation to higher temperatures than those applied in this study (i.e.
334 up to 50 ºC for 20 s), showed no impact on culture viability or fermentation progress
335 (Valentine et al. 2018). Importantly, heat shock length in the mentioned study is
336 radically lower than that applied in our study (20 s vs. 16 h), suggesting that the
337 combination of temperature and length of the heat shock treatment determined the final
338 effect on the fermentative process.

339 Despite most heat shock treatments produced an impact leading to sluggish
340 fermentations, the magnitude of the effect on fermentation performance was strain and
341 nutrition dependent. For instance, SBB11 shown to be the most sensitive strain to both
342 heat shock temperatures. Although DAP supplementation produced a slight reduction in
343 fermentation length, heat shock led to a clear sluggish in fermentation for this strain. In
344 the case of T73, 36 ºC and 40 ºC heat shocks produced a strong effect on fermentation
345 performance in absence of nutrition, which was practically reverted when DAP

supplementation was carried out. The latter suggests that T73 is strongly favoured by nutrition. Finally, PDM fermentation performance was not affected by heat shocks at 36 ºC, being only altered when a 40 ºC heat shock was applied. Also nutrition did not modify the immediate response of this strain, however total fermentation length was shorter when DAP is added. PDM seems to be the most thermotolerant strain assessed, since only heat shocks at the highest temperatures (i.e. 40 ºC) were able to significantly affect fermentation performance. This latter is in line with previous studies that indicate that PDM presents fitness advantage over other *S. cerevisiae* strains, being able to ferment in a wider temperature range, showing tolerance to temperatures within the range of 40 to 45°C (García-Ríos et al. 2014). Regarding nutritional requirements, PDM has been described as a great nitrogen demander (Gutierrez et al. 2012). However, in our experiments this was not evidenced in the AUC measurements until day 11. Nonetheless, a clear decrease in fermentation length was observed in DAP supplemented fermentations. It is generally accepted that nitrogen addition produces an increase in biomass and stimulates the rate of sugar consumption (Beltran et al. 2005). In our study an improvement in the metabolic activity may support the recovery of the fermentation rate after heat shocks in DAP supplemented treatments, since no increment in biomass was evidenced.

Traditionally, heat treatments have been widely employed in food industry due to its lethal effect on spoiler or pathogen microorganisms. Heat injury of vegetative cells is multi targeted. The site of damage can be some cell wall components, the cytoplasmic membrane, ribosomes and ribosomal RNA, as well as degradation and misfolding of proteins (Yamamoto et al. 2008, Smelt and Brul, 2014). In our study, yeast viability loss followed by no recovery after 96 hours of heat shock, showed to be the cause of the decrease in fermentation rate. In contrast, other authors have shown that a brief

371 exposure (i.e. 20 sec) to high temperatures causes the temporary loss of population
372 viability (Valentine et al. 2018). The authors reported that a delay in the reinitiation of
373 fermentation depends on the restoration of cell number, followed by a complete
374 recovery of fermentation rate. Additionally, cells exposed to a period of sublethal heat
375 can initiate a separate set of mechanisms that improves heat tolerance (Valentine et al.
376 2018, Jarolim et al. 2013). In contrast, our results showed no viability recovery,
377 suggesting that heat shocks assessed in our study had an intensity that overpassed cell
378 response capacity therefore fermentation rate was never completely recovered.
379 Moreover, in the conditions assessed in our study, temperature distribution was not
380 homogeneous (i.e. heat shock in static conditions). This later would result in cells
381 differently affected by the heat and fermentation could continue due to a residual viable
382 population less affected by the heat.

383 Despite none of the heat treatments assessed produced stuck fermentation, it is
384 important to highlight that sluggish fermentations as those observed in this study (taking
385 40-45 days) are a great struggle for winemakers, bringing huge operative and logistics
386 problems to the industry. In all cases DAP supplementation improved the global
387 performance after a heat shock, reducing fermentation lengths. This later suggest that
388 nutrition would be a recommendable practice since it has shown a positive effect against
389 heat shocks that may occur during the first days of fermentation. Consequently, an
390 adequate management of fermentation temperature is highly recommended to avoid
391 fermentative problems.

392 CONCLUSIONS

393 The purpose of this study was to evaluate thermal conditions able to produce
394 problematic fermentations. Specifically, the effect of an abrupt and transient reduction
395 or increase of must temperature on fermentation performance was evaluated. We

396 evidenced that a cold shock is not able to alter the fermentation performance, even if
397 produced in different moments of alcoholic fermentation. In contrast, heat shocks
398 occurring during the first days of fermentation can lead to sluggish fermentations whose
399 intensity is directly related to the temperature of the heat shock applied. Moreover,
400 fermentation delay was attributable to decreased cell vitality. Three *S. cerevisiae* strains
401 were studied and showed to be differently affected by the thermal shocks. Interestingly,
402 DAP supplementation showed to confer a positive effect against heat shocks, since
403 improvement in fermentation rate was observed for all treatments subjected to nutrition.

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FIGURE CAPTIONS

Figure 1: Schematic experimental design of the assay. Each treatment was carried out in independent triplicates for each yeast strain. Alcoholic fermentation was conducted at 26 ºC. Thermal shocks are represented in different colours and were applied for 16 hours. DAP: diammonium phosphate supplementation. ✓: flow cytometry analysis.

Figure 2: Inside temperature profile recorded during heat shock assay. Fermentations were carried out with DAP supplementation at 26 ºC. Thermal shocks (32 ºC, 36 ºC and 40 ºC) were applied for 16 hours.

Figure 3: Effect of cold shock on fermentation performance of two yeast strains: SBB11 (A) and T73 (B). Fermentations were conducted at 26 ºC and cold shocks applied for 16 hours on different days are indicated by arrows. Fermentation progress was monitored as cumulative mass loss (CO_2 g/L). Insets show the complete fermentation profile for the treatment "Daily T 1.5 ºC". The data represent the average of triplicate ± SD.

Figure 4: Global effect the temperature (A), and temperature*nutrition (B) on fermentation performance after heat shock. Results are expressed as area under the curve (AUC) measured until day 11 for all treatments with the three strains evaluated: SBB11, T73 and PDM. Treatments without DAP (stripped bars) and with DAP supplementation (filled bars). Different letters mean statistical differences between the treatments (LSD Fisher test $p<0.05$).

Figure 5: Effect of a heat shock on fermentation performance expressed as a decrease in must density (mg/L) and AUC statistical analysis for the three strains studied SBB11 (A-C), T73 (D-F) and PDM (G-I). Fermentations were carried out without DAP (open symbols) and with DAP supplemented (filled symbols). Mean values of three

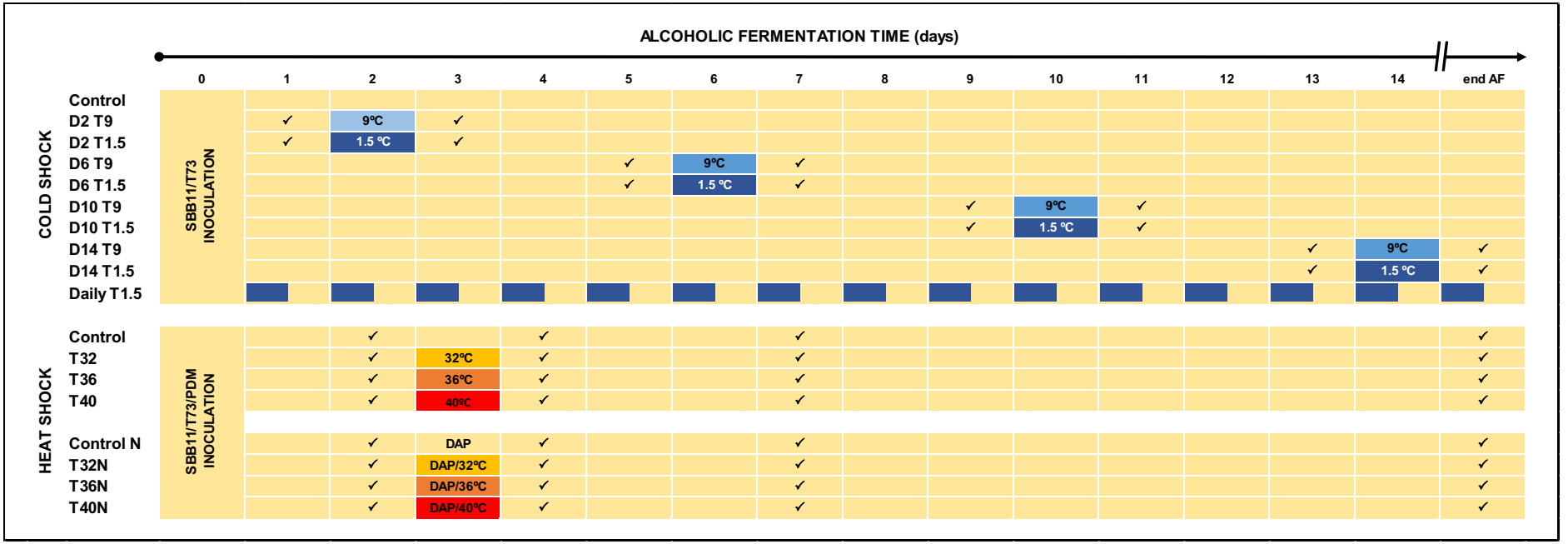
510 experimental data with standard deviation are represented. Different letters mean
511 statistical differences between the treatments (LSD Fisher test $p<0.05$).

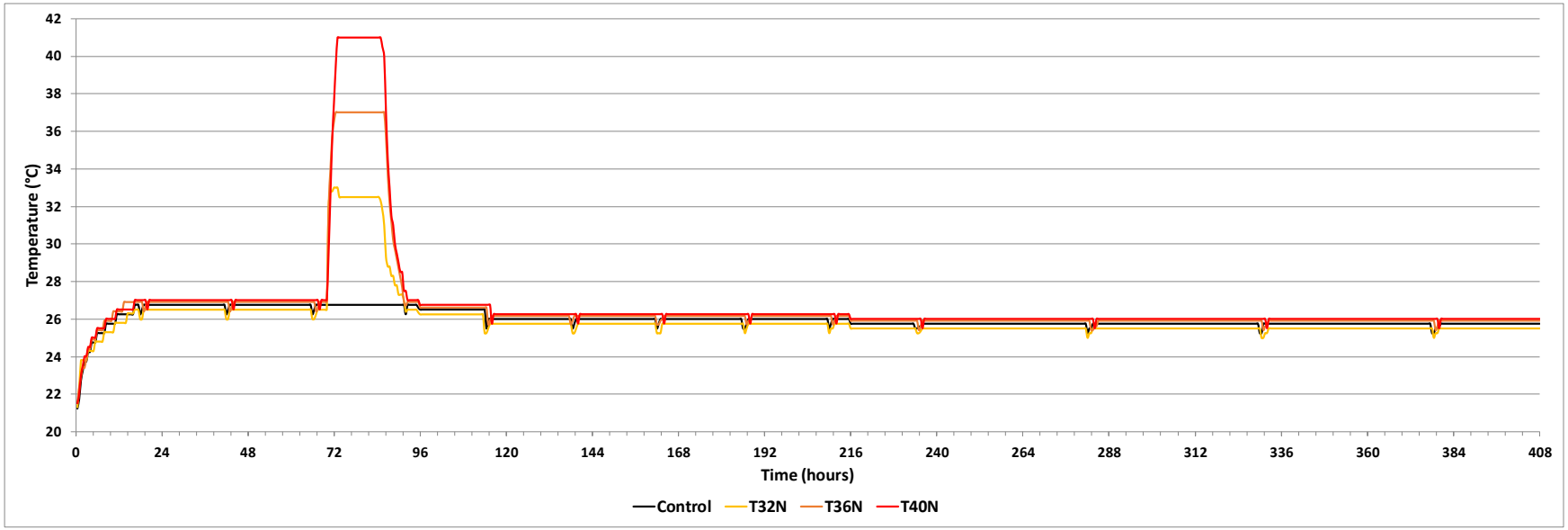
512

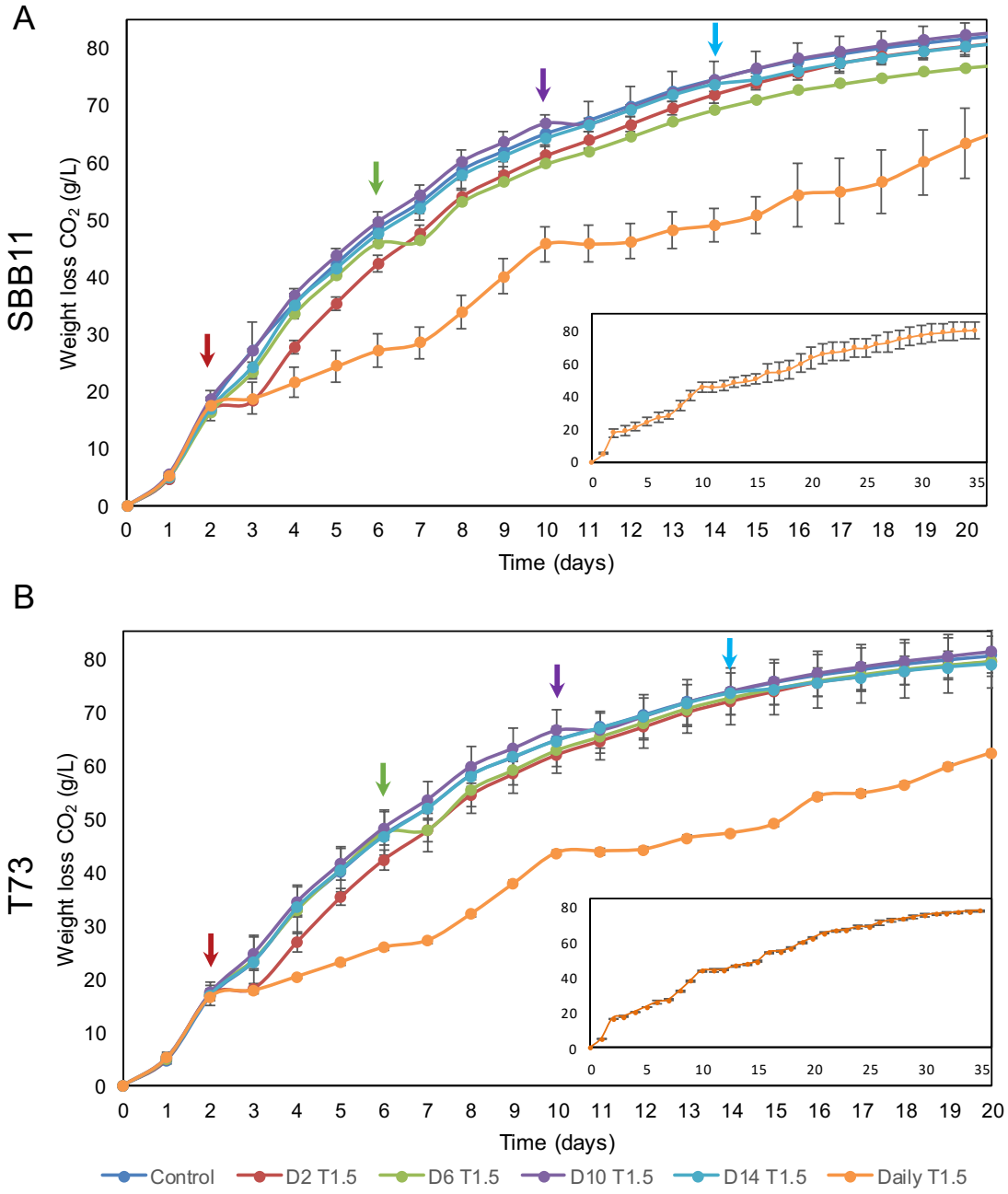
513 Figure 6: Vitality reduction the day after the heat shock. Cellular vitality evaluated by
514 flow cytometry in cells stained with CFDA for the three strains SBB11, T73 and PDM.
515 Results are expressed as vitality reduction, calculated as detailed in Material and
516 Methods section.

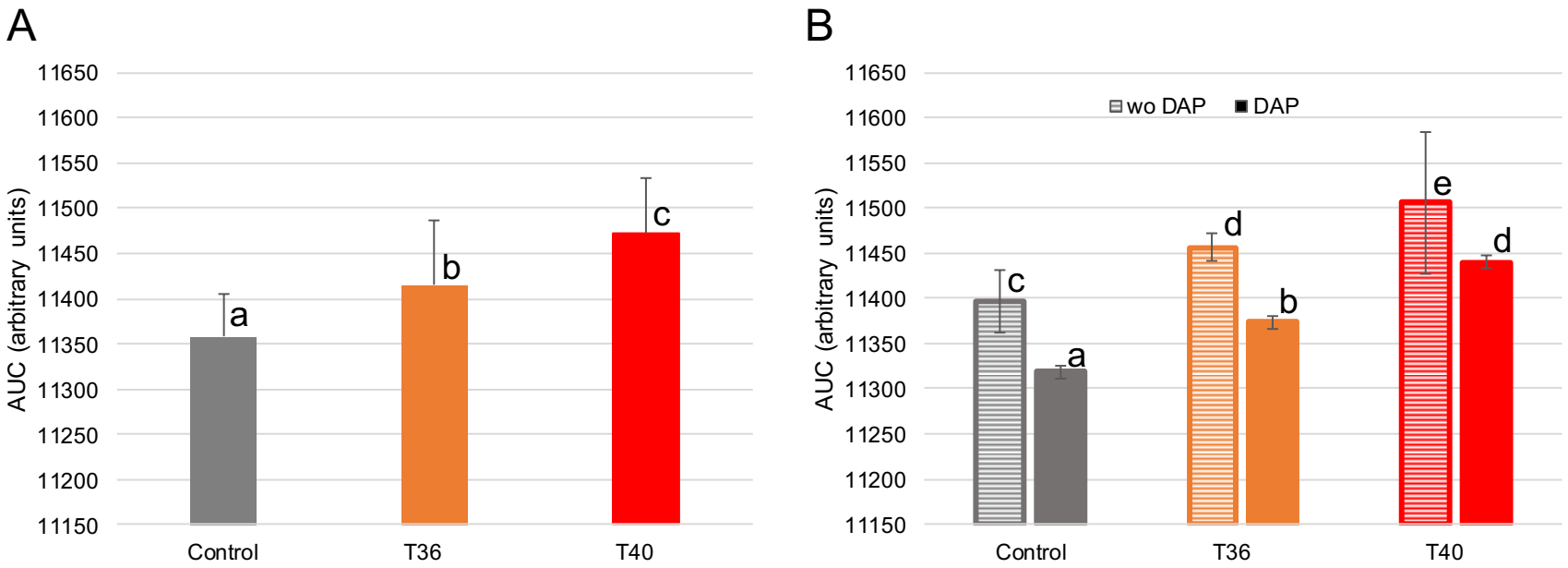
517

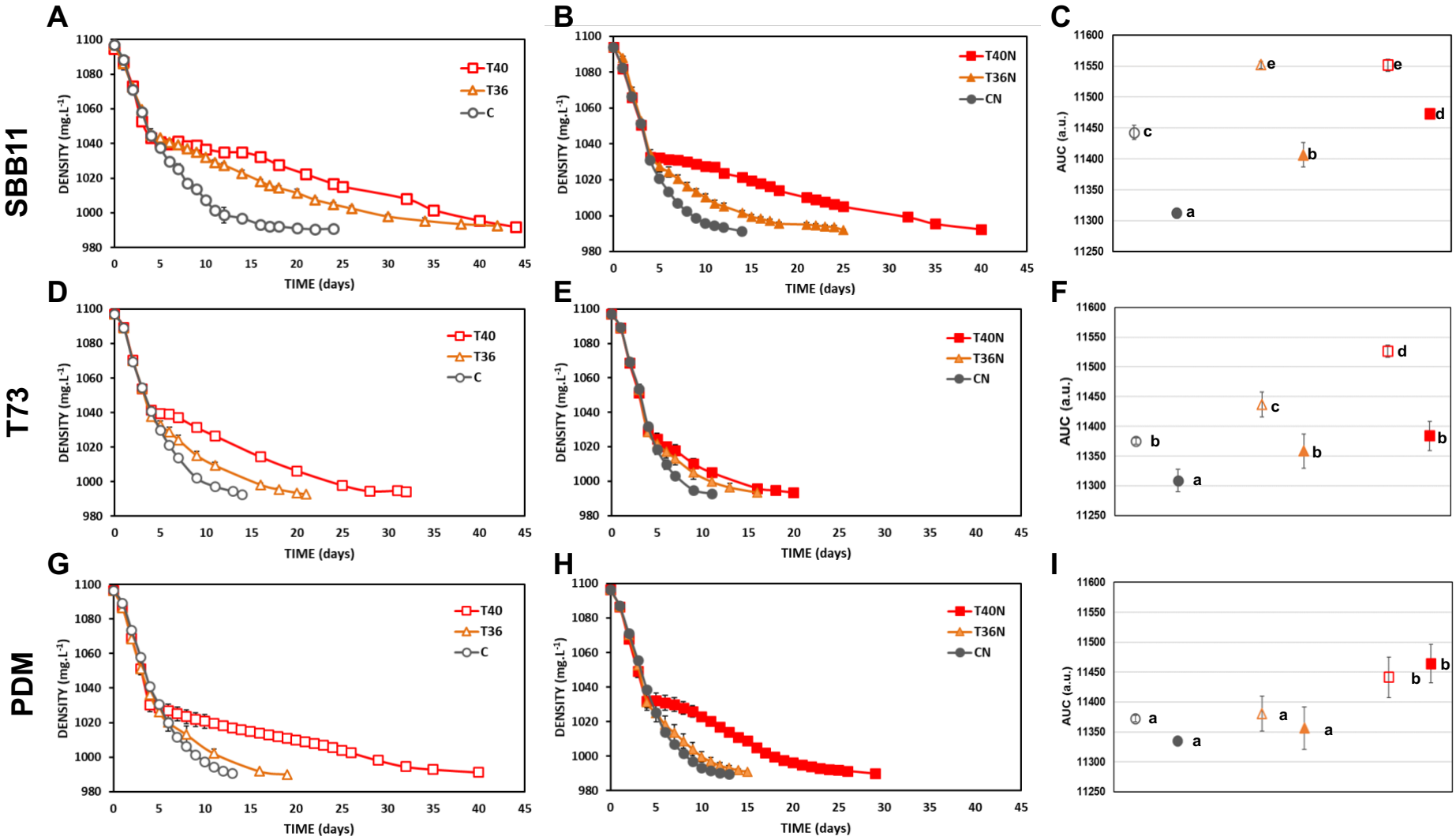
518 Figure 7: Cell vitality on day 4 and day 7 measured with flow cytometry from
519 treatments (control, 36 ºC and 40 ºC) performed with T73. Red: Non-vital cells (CFDA-
520 PI+), Green: vital cells (CFDA+ PI-) and Yellow: vital cells with compromised
521 membranes (CFDA+ PI+). Cells were gated based on viable and non-viable controls.
522 DAP: diammonium phosphate.

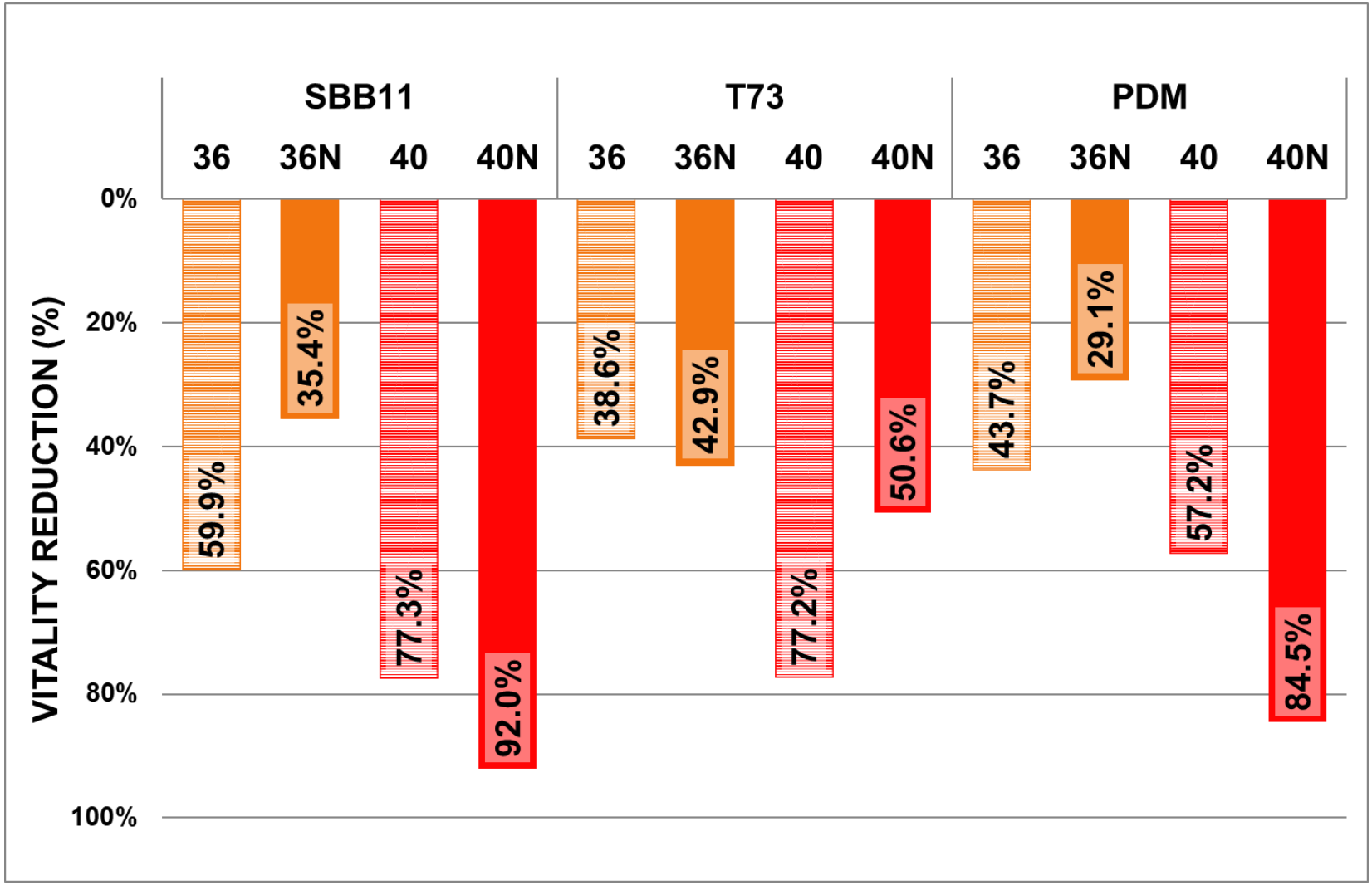

ALCOHOLIC FERMENTATION TIME (days)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
end AF
COLD SHOCK
SBB11/T73 INOCULATION
Control
D2 T9
D2 T1.5
D6 T9
D6 T1.5
D10 T9
D10 T1.5
D14 T9
D14 T1.5
Daily T1.5
9ºC
1.5 ºC
HEAT SHOCK
SBB11/T73/PDM INOCULATION
Control
T32
T36
T40
Control N
T32N
T36N
T40N
32ºC
36ºC
40ºC
DAP
DAP/32ºC
DAP/36ºC
DAP/40ºC

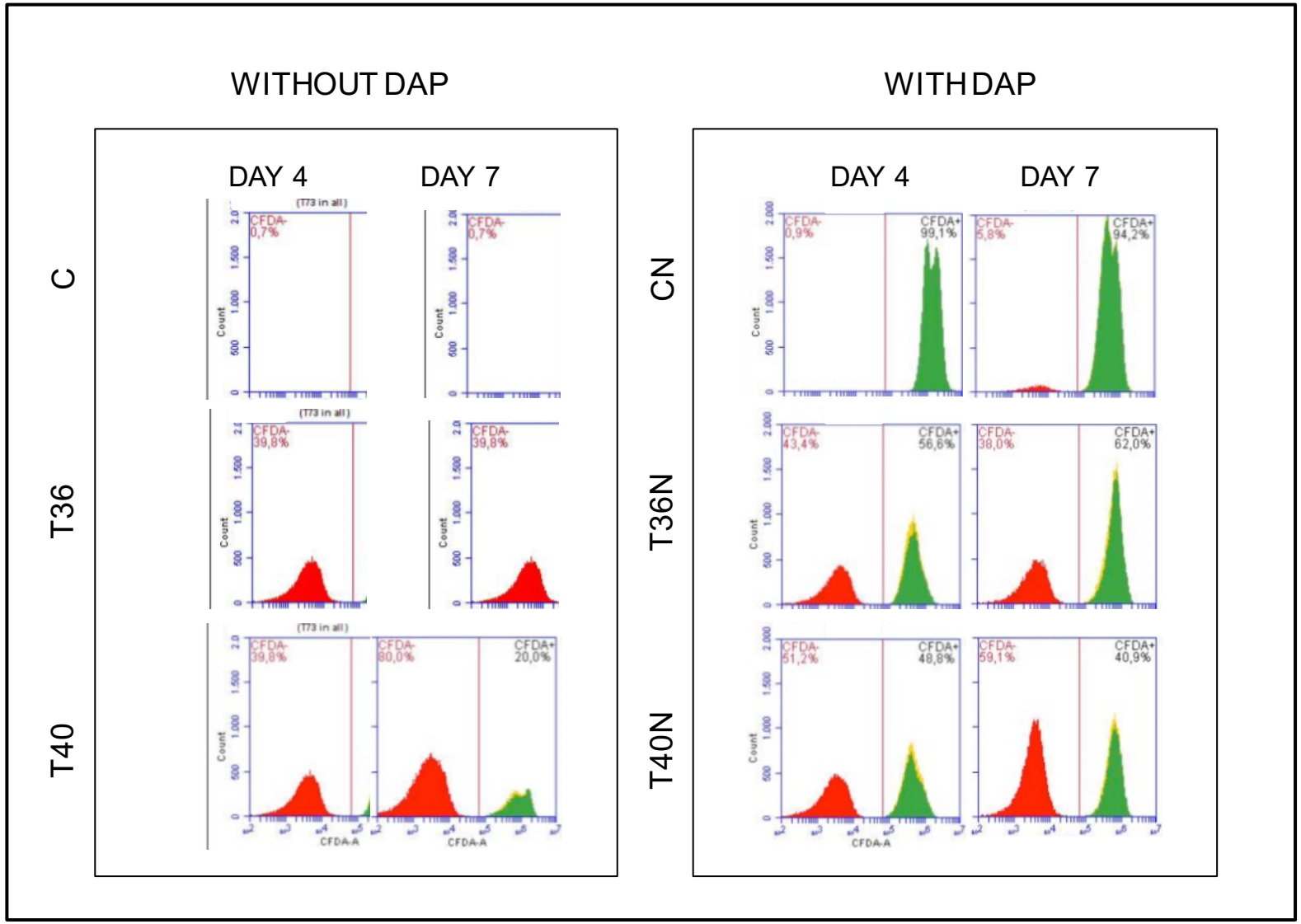

Temperature (°C)
42
40
38
36
34
32
30
28
26
24
22
20
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360
384
408
Time (hours)
Control
T32N
T36N
T40N


A
SBB11
Weight loss CO_2 (g/L)
Time (days)
B
T73
Weight loss CO_2 (g/L)
Time (days)
Control
D2 T1.5
D6 T1.5
D10 T1.5
D14 T1.5
Daily T1.5


A
11650
11600
11550
11500
11450
11400
11350
11300
11250
11200
11150
AUC (arbitrary units)
a
b
c
Control
T36
T40
B
wo DAP
DAP
c
a
d
b
e
d
Control
T36
T40


A
B
C
D
E
F
G
H
I
SBB11
T73
PDM
DENSITY (mg.L-1)
TIME (days)
AUC (a.u.)
T40
T36
C
T40N
T36N
CN
a
b
c
d
e


SBB11
T73
PDM
36
36N
40
40N
36
36N
40
40N
36
36N
40
40N
VITALITY REDUCTION (%)
0%
20%
40%
60%
80%
100%
59.9%
35.4%
77.3%
92.0%
38.6%
42.9%
77.2%
50.6%
43.7%
29.1%
57.2%
84.5%


WITHOUT DAP
WITH DAP
DAY 4
DAY 7
DAY 4
DAY 7
C
T36
T40
CN
T36N
T40N
CFDA-
0,7%
CFDA-
0,7%
CFDA-
39,8%
CFDA-
39,8%
CFDA-
39,8%
CFDA-
80,0%
CFDA+
20,0%
CFDA-
0,9%
CFDA+
99,1%
CFDA-
5,8%
CFDA+
94,2%
CFDA-
43,4%
CFDA+
56,6%
CFDA-
38,0%
CFDA+
62,0%
CFDA-
51,2%
CFDA+
48,8%
CFDA-
59,1%
CFDA+
40,9%
Count
CFDA-A