1 **Effect of pure and mixed cultures of the main wine yeast species on grape must**
2 **fermentations**

3 Imma Andorrà[1], María Berradre[2], Nicolás Rozès[1], Albert Mas[1], Jose M. Guillamón[3*]
4 Braulio Esteve-Zarzoso[1]

5

6 [1] Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i
7 Virgili. Marcel.lí Domingo s/n, 43007, Tarragona, Spain

8 [2] Laboratorio de Alimentos, Departamento de Química, Facultad Experimental de
9 Ciencias, Universidad del Zulia, Estado Zulia, Venezuela

10 [3] Departamento de Biotecnología de los Alimentos, Instituto de Agroquímica y
11 Tecnología de Alimentos (CSIC), P.O. Box 73, E-46100 Burjassot, València, Spain

12

13 * Corresponding author:
14 José Manuel Guillamón

15 Departamento de Biotecnología de los Alimentos. Instituto de Agroquímica y
16 Tecnología de los Alimentos (CSIC). Apartado de Correos 73, E-46100-Burjassot
17 (Valencia) Spain. Tel: 34-96-3900022; Fax: 34-96-3636301, E-mail:
18 guillamon@iata.csic.es

19

20 Keywords: *Saccharomyces*, *Candida*, *Hanseniaspora*, wine fermentation, volatile
21 compounds, amino acids.

22 **Abstract**

23 Mixed inoculation of non-*Saccharomyces* yeasts and *S. cerevisiae* are of interest for the
24 wine industry for technological and sensory reasons. We have analysed how mixed
25 inocula of the main non-*Saccharomyces* yeasts and *S. cerevisiae* affect fermentation
26 performance, nitrogen consumption and volatile compound production in a natural
27 Macabeo grape must. Sterile must was fermented in triplicates and under the following
28 six conditions: 3 pure cultures of *S. cerevisiae*, *Hanseniaspora uvarum* and *Candida*
29 *zemplinina* and the mixtures of *H. uvarum:S. cerevisiae* (90:10), *C. zemplinina:S.*
30 *cerevisiae* (90:10) and H. *uvarum:C. zemplinina:S. cerevisiae* (45:45:10). The presence
31 of non-*Saccharomyces* yeasts slowed down the fermentations and produced higher
32 levels of glycerol and acetic acid. Only the pure *H. uvarum* fermentations were unable
33 to finish. Mixed fermentations consumed more of the available amino acids and were
34 more complex and thus better able to synthesize volatile compounds. However, the
35 amount of acetic acid was well above the admissible levels and compromises the
36 immediate application of mixed cultures.

37 **Introduction**

38 The fermentation of grape juice into wine is a complex microbial reaction
39 involving the sequential development of various species of yeast. Traditionally, wine
40 has been produced by the natural fermentation of grape juice by yeasts that originate
41 from grapes and winery equipment [1]. Yeasts with low fermentation activity, such as
42 *Candida spp*., *Hanseniaspora spp*., *Kluyveromyces spp*., *Pichia spp*. and *Rhodotorula*
43 *spp*., are predominant in grape musts and during the early stages of fermentation.
44 Subsequently, *S. cerevisiae* proliferates, dominating and completing the wine
45 fermentation [2, 3]. Generally, these non-*Saccharomyces* species were considered to be
46 of secondary significance or undesirable to the process. However this trend is changing.
47 In a recent review, Fleet [4] discussed the possibilities of using yeasts other than those
48 from the *Saccharomyces* genus for future wine fermentations and the commercial
49 viability of mixed cultures. These species have great potential to introduce appealing
50 characteristics to wine which may improve its organoleptic quality.

51 The major non-*Saccharomyces* yeasts present during alcoholic fermentation are
52 *Candida stellata*, currently classified as *Candida zemplinina* [5], and *Hanseniaspora*
53 *uvarum* (anamorph *Kloeckera apiculata*). Although the population size of these species
54 reduced throughout the wine fermentations, several quantitative ecological studies have
55 indicated that their growth was not completely suppressed, either in spontaneous or in
56 inoculated fermentations [2, 6-8]. Similar studies have shown their capacities to
57 improve wine flavour [9-11] or have evaluated the biotechnological nature of their
58 enzymatic activities [12, 13]. *Candida stellata* is frequently associated with overripe
59 and botrytized grape berries [14-18]. The most interesting oenological characteristic of
60 this species is that it is highly fructophilic [14]. Ciani and Ferraro [19] demonstrated
61 that mixed fermentations containing *C. stellata* and *S. cerevisiae* consumed sugars more

62 completely and postulated that this was due to the preferential use of fructose by *C.*
63 *stellata*. This yeast may be used in mixed cultures with *S. cerevisiae* for stuck
64 fermentations, where the proportion of fructose is usually higher than glucose.
65 However, more controversial results have been reported about this species' contribution
66 to wine aroma. Some authors have reported the production of high levels of acetic acid
67 [20, 21], glycerol [20, 22] and succinic acid [23] whereas others have found low acetic
68 acid production [24] and low glycerol production [16]. Csoma and Sipiczki [25]
69 asserted that these contradictory results were because *C. stellata* is easily confused with
70 other yeast species that colonize the same substrates. This hypothesis is supported by
71 the recent finding that the strain DBVPG 3827, frequently used to investigate the
72 oenological properties of *C. stellata*, belongs to *Starmerella bombicola* [5] and by the
73 description of a new species, *Candida zemplinina* that was previously considered *C.*
74 *stellata* [5, 26, 27]. Such findings raise doubts about the precise taxonomic position of
75 the oenological *C. stellata* strains described in the literature [25]. *Hanseniaspora*
76 species have been considered great producers of esters, most of them contributing to the
77 flowery and fruity aroma of wines. However, the main ester is ethyl acetate, which in
78 high concentrations produces an unpleasant aroma of glue, solvent, etc. Another
79 characteristic of the excessive growth of *Hanseniaspora* during wine fermentation is the
80 increase in volatile acidity as a result of the synthesis of acetic acid and ethyl acetate.
81 Ciani et al. [28] have recently confirmed the unacceptable increase in ethyl acetate
82 content in a mixed culture of *H. uvarum*/*S. cerevisiae*. *H. uvarum* strains also possess
83 enzymatic characteristics of interest to winemaking because of their technological
84 effects and their contribution to aroma formation. Pectinases, proteases and
85 glycosidases are some of the enzymes secreted by *H. uvarum* which improve the
86 clarification, stabilisation and aroma of wines. Moreira et al. [29] analysed the

87 production of alcohols, esters and heavy sulphur compounds by pure and mixed cultures
88 of apiculate wine yeasts. *H. guilliermondii* produced high levels of 2-phenylethyl
89 acetate, 2-phenylethanol, acetic-acid-3-(methylthio)propyl ester (cooked potatoes
90 aroma) and 3-methylthiopropionic acid. Concentrations of heavy sulphur compounds
91 were also higher in a pure culture of *H. uvarum* than in a pure culture of *S. cerevisiae*.

92 Consequently, the impact of non-*Saccharomyces* yeasts on wine fermentation
93 cannot be ignored. They introduce an element of ecological diversity to the process that
94 goes beyond *Saccharomyces* species and they require specific research and
95 understanding to prevent any unwanted consequences from their use and to exploit their
96 beneficial contributions [4]. In this study we report the impact of pure and mixed
97 populations of *C. zemplinina*, *H. uvarum* and *S. cerevisiae* on fermentation behaviour,
98 nitrogen consumption and aroma production.

99

100 **Material and methods**

101 *Experimental fermentations*

102 Fermentations were conducted using several combinations of the commercial
103 strain of *Saccharomyces cerevisiae* QA23 (Lallemand, Inc. Canada) and the strains
104 *Candida zemplinina* CszB4 and *Hanseniaspora uvarum* HuB10 previously isolated
105 from wine fermentations. Both strains were selected on the basis of a preliminary
106 experiment which consisted of a multiple co-inoculation of several strains of the same
107 species in grape must. The strains selected were those with a higher presence at the end
108 of fermentation (data not shown).

109 Fermentations were conducted on Macabeo must coming from the experimental
110 cellar of the Faculty of Oenology in Tarragona (Spain) during 2007 vintage. This must
111 was sterilised by the addition of 250 mg l^{-1} of dimethyldicarbonate (Sigma-Aldrich,

112 Steinheim, Gemrany). After settling, 400 ml of must was put in 500 ml bottles. This
113 must contained 180 g l^{-1} of sugar content, which corresponded to 10 % of the probable
114 alcohol degree, a pH of 3.1 and 4.8 g l^{-1} of total acidity of tartaric acid. A total 114.57
115 mg N l^{-1} of Yeast Assimilable Nitrogen (YAN) was found, 57.16 mg of which was in
116 organic form (amino acids) and 57.41 mg was ammonium. All experiments were done
117 in triplicate fermentations at a controlled temperature of 20 ºC and 150 rpm of stirring
118 on an orbital shaker. Sugar consumption was monitored daily by measuring the density
119 (g l^{-1}) of the fermenting must and by enzymatic assay (Roche Applied Science;
120 Germany). Fermentations were considered to be finished when the level of reducing
121 sugars was below 2 g l^{-1}.

122 The musts were inoculated with 10^6 cells ml^{-1} in all cases. The inocula were *S.*
123 *cerevisiae* (S), *C. zemplinina* (C), *H. uvarum* (H), *C. zemplinina*/*S. cerevisiae* (CS), *H.*
124 *uvarum/S. cerevisiae* (HS) and *C. zemplinina*/*H. uvarum*/*S. cerevisiae* (CHS) always at
125 the ratio of 90:10 for non-*Saccharomyces vs. Saccharomyces* (45:45:10 for the triple
126 culture). The total yeast populations were enumerated on plates with YPD medium (2%
127 glucose, 2% Bacto peptone, 1% yeast extract, 2% agar, W/v; Cultimed, Barcelona,
128 Spain). The selective lysine-agar medium (Oxoid, Barcelona, Spain), which is unable to
129 support the growth of *S. cerevisiae* [30], was used to enumerate non-*Saccharomyces*
130 populations.

131

132 *Nitrogen content analysis*

133 YAN was analysed by the formol index method [31], and the ammonium content was
134 quantified using an enzymatic method (Roche Diagnostics, Germany). The individual
135 amino and imino acids were analysed by OPA and FMOC derivatizations, respectively,
136 using the Agilent 1100 Series HPLC as described by Beltran et al. [32]. Several

137 dilutions of each sample were analysed and averaged using the analysis software. The
138 concentration of each amino acid was calculated using external and internal standards
139 and expressed as mg l^{-1}. The software used was Agilent ChemStation Plus (Agilent
140 Technologies, Germany).

141

142 *Volatile compound analysis*

143 The concentrations of the different volatile compounds were analyzed at the end of each
144 fermentation. The protocol followed by Ortega et al. [33] was modified to determine
145 volatile fatty acids, ethyl esters of fatty acids, higher alcohol acetates and other volatile
146 compounds. The following were added to 15-ml screw-capped tubes: 1.5 ml of wine,
147 3.5 ml of $(NH_4)_2SO4$ (45%, w/v), 200 µl of dichloromethane and 20 µl of internal
148 standard. This internal standard was made up by 4-methyl-2-pentanol (176 µg ml^{-1}), 1-
149 nonanol (160 µg ml^{-1}) and heptanoic acid (150 µg ml^{-1}) in ethanol. The tube was shaken
150 for 30s (3x) and then centrifuged at 4000 rpm for 10 min. Once the phases were
151 separated, the bottom phase (dichloromethane) was transferred to a glass vial insert. The
152 extract (3 µl) was injected in split mode (10:1, 30 ml min^{-1}) into an Agilent 6850
153 equipped with a flame ionisation detector (FID), (Agilent Technologies, Böblingen,
154 Germany) and a HP-FFAP column of 30 m x 0.25 mm, 0.25 µm phase thickness. The
155 temperature program was as follows: 35ºC for 5 min, then raised at 3ºC min^{-1} up to
156 200ºC and then at 8ºC min^{-1} up to 220ºC. Injector and detector (FID) temperatures were
157 180ºC and 280ºC, respectively. The carrier gas was helium at 3 ml min^{-1}. Volatile
158 compounds were identified and quantified by comparison with standards.

159

160 *Organic acid analysis*

161 The values of different organic acids were analyzed at the end of the fermentations of
162 the wine samples. Organic acids were determined by HPLC using an Agilent 1100
163 Series connected to an Agilent multiple wavelength detector (Agilent Technologies,
164 Wilmington, DE). The samples (450 µl) were mixed with 50µl of formic acid (Internal
165 Standard, 46.84 g l^{-1}) and 50 µl were injected into a 300 mm x 7.8 mm AMINEX HPX-
166 87H columm (BioRad, Hercules, CA). The solvent used was sulphuric acid 2.5 mM at
167 0.5 ml min^{-1}. The analysis temperature was 70ºC. The concentration of each metabolite
168 was calculated using external and internal standards.

169

170 *Oenological parameters*

171 The glucose, fructose, glycerol and ethanol content of the wines were analyzed using
172 commercial enzymatic kits (Roche Diagnostics, Germany). Acetic and succinic acids
173 were determined by HPLC as described above. The pH was determined by using a pH-
174 meter Crison MicropH 2000 (Crison, Barcelona, Spain).

175

176 *Statistical treatment*

177 The data were analyzed with SPSS 15.0 software for Windows (SPSS Inc., Chicago,
178 IL). Analysis of variance was carried out by an ANOVA Tukey test to determine
179 significant differences between the samples. The statistical level of significance was set
180 at $P \leq 0.05$.

181

182 **Results**

183 *Kinetics and main fermentation products*

184 As expected, the fastest fermentation was with the pure culture of *S. cerevisiae*, 185 considered as control, whereas the slowest fermentations were those inoculated either 186 with a pure culture of *Hanseniaspora uvarum* or *Candida zemplinina* (Figure 1). The 187 pure *H. uvarum* culture was the only condition that did not finish the fermentation (20 g 188 l^{-1} of glucose left in the medium) (Table 1). All the fermentations reached a similar 189 ethanol concentration (around 9.5-10%) with the exception of the *H. uvarum* pure 190 culture, which only reached 4%. Regarding other oenological parameters, the greatest 191 differences among the different cultures were detected in the glycerol and acetic acid 192 concentrations. All the fermentations showed a higher concentration of glycerol and 193 acetic acid than the control *S. cerevisiae* fermentation, with the exception of the 194 unfinished *H. uvarum* fermentation, which produced less glycerol but much more acetic 195 acid.

196 *Microbial populations*

197 Total yeast population was very similar in all fermentations and reached a 198 population around 10^8 cfu ml^{-1} (Fig 2). This population level was reached after 72 hours, 199 except in the case of *H. uvarum* pure culture which reached this maximum population 200 on the fifth day of fermentation. The presence of *S. cerevisiae* in the mixed cultures 201 meant that the maximum total yeast populations were quickly reached. These maximum 202 populations were kept stable during the process, that is, there was no decline phase in 203 last stages of fermentation, and even the population of *C. zemplinina* increased steadily 204 throughout the fermentation. The only exception was the pure *H. uvarum* culture which 205 showed a clear decline during the last stages in accordance with its stuck fermentation.

206 The non-*Saccharomyces* counts were similar to the total yeast populations (the 207 same order of magnitude) at the beginning of fermentation. However, in the mixed

208 fermentations, these numbers decreased as fermentation proceeded. The comparison
209 between the counts obtained in both culture media (non-selective YPD and selective
210 lysine-agar) clearly proved that most of the yeast population was non-*Saccharomyces* at
211 the beginning of the process but that *Saccharomyces* population took over the process in
212 the middle and at the end of the fermentation. Non-*Saccharomyces* yeasts represented
213 less than 1% of total yeast population at the end of the fermentation.

214 The counts of the pure non-*Saccharomyces* cultures (C and H) should have been
215 the same in YPD and lysine-agar. This was the case with *C. zemplinina*; however, *H.*
216 *uvarum* counts were smaller in lysine-agar than in YPD in some samples. This result
217 could be because YPD is a richer medium which supports better growth than lysine-
218 agar, especially when cells are stressed by the presence of ethanol.

219 *Ammonium and amino acid consumption*

220 We analysed the ammonium and amino acid content in the media at different
221 stages of the fermentation. We detected the maximum consumption in the middle of the
222 fermentation because nitrogen release, as consequence of yeast autolysis, was observed
223 in the final phases of the fermentation. This maximum consumption of both individual
224 amino acids and ammonium is shown in the Table 2.

225 Unfortunately, the low concentration of assimilable nitrogen (YAN) in the grape
226 must meant that the differences in nitrogen consumption were not as remarkable as
227 expected. Ammonium was completely consumed in all the conditions. The mixed
228 cultures consumed more amino acids than the pure cultures. Moreover, these mixed
229 cultures consumed more of certain groups (aliphatic and aromatic amino acids) than the
230 pure yeasts culture. They also consumed more glutamic acid, aspartic acid, glycine,
231 alanine, leucine and phenylalanine. However, the converse also happened, the mixed

232 cultures consumed fewer sulphur amino acids than the pure *S. cerevisiae* and *C.*
233 *zemplinina* cultures.

234 *Volatile compounds*

235 The most important aroma forming compounds were analysed in the final wines
236 (Table 3). The pure *H. uvarum* culture fermentation was not analysed because it did not
237 finish fermenting and its high concentration of acetic acid and ethyl acetate made the
238 analysis of other compounds very difficult. *S. cerevisiae* had the lowest production of
239 higher alcohols whereas *C. zemplinina* had the highest. The mixed fermentations
240 produced higher alcohols at levels between those of the pure *S. cerevisiae* and *C.*
241 *zemplinina*, although levels were closer to those of *S. cerevisiae*. The strong difference
242 between *C. zemplinina* and *S. cerevisiae* was due to a significant increase in each
243 detected compound, whereas the differences between mixed fermentations were mostly
244 due to the increases in 2 phenylethanol and 2 methyl-1-propanol.

245 The production of ethyl esters is also significantly higher in the presence of non-
246 *Saccharomyces* yeasts and especially in the pure cultures of *C. zemplinina*. In this case,
247 the difference was mostly due to the increase in ethyl octanoate, whereas in the mixed
248 fermentations it was related to the increases in ethyl lactate.

249 Although all the fermentations produced more acetate esters than the pure *S.*
250 *cerevisiae* culture, the only significant difference was in the mixed *H. uvarum* and *S.*
251 *cerevisiae* culture.

252 The production of short chain fatty acids (SCFA) was also higher in all the
253 fermentations than in the *S. cerevisiae* fermentation. This increase was higher in
254 fermentations which contained *C. zemplinina,* especially when it fermented alone. The
255 main contributor to this difference was isobutyric acid, which was highly synthesised by

256 *C. zemplinina*. On the other hand, medium chain fatty acids (MCFA) concentrations in
257 the *S. cerevisiae* fermentations were always higher than in the other wines, except for
258 dodecanoic acid, which was produced in higher quantities by the non-*Saccharomyces*
259 yeasts.

260 **Discussion**

261 The aim of this study was to analyze the effect of mixed *Saccharomyces* and non-
262 *Saccharomyces* cultures on amino acid consumption and aroma production in natural
263 grape must, and to determine the interactions among the different microorganisms
264 involved. These fermentations were inoculated with a *Saccharomyces* strain together
265 with a *C. zemplinina* strain and/or a *H. uvarum* strain that was selected according to its
266 fermentation performance. So far, the wine industry has only paid attention to the *S.*
267 *cerevisiae* strains as fermentative agents, and has ignored the possibility of using other
268 yeasts during fermentations. However, interest is growing in the possible contributions
269 of non-*Saccharomyces* yeasts to the fermentation process. Non-*Saccharomyces* species
270 can contribute to the aromatic properties and chemical composition of the resulting wine
271 because they produce more secondary metabolites which contribute to the taste and
272 flavour of the wines [20]. Garde-Cerdán and Ancín-Azpilicueta [34] already proved that
273 the best volatile composition of wine was obtained from mixed cultures Saccharomyces
274 and non-Saccharomyces than from pure cultures of a commercial S. cerevisiae strain.
275 Some authors have even reported that these yeasts produce extracellular enzymes that
276 may provide the wine with properties that are unique to the region where it is produced
277 [13]. In our opinion, however, further research is needed into how individual non-
278 *Saccharomyces* species and strains contribute to wine quality and into the synergy or
279 antagonism between *Saccharomyces* and non-*Saccharomyces* species in the final
280 resulting wines.

281 It is well-known that non-*Saccharomyces* yeast predominates in the first stages of
282 fermentation before disappearing in favour of *S. cerevisiae*, which has the highest
283 fermentative capacity. This phenomenon is generally ascribed to *Saccharomyces*' higher
284 capacity to withstand increasing concentrations of ethanol and organic acids, decreasing
285 pH and nutritional depletion [35]. However the predominant role of these classic
286 selective pressures is currently being questioned and other, as yet undefined, microbe–
287 microbe interactions are being put forward as potentially significant in influencing yeast
288 successions [10, 36, 37]. Our results clearly proved that *S. cerevisiae* has an antagonistic
289 effect upon *C. zemplinina* and *H. uvarum* strains. The presence of *S. cerevisiae* strongly
290 reduced the other species in the mixed cultures. To date, there have been only a few
291 thorough studies into the causes and the mechanisms underlying this antagonistic
292 phenomenon [37-40]. On one hand, Nissen et al. [38] concluded that the early death of
293 two wine-related yeasts (*Kluyveromyces thermotolerans* and *Torulaspora delbrueckii*)
294 during mixed fermentations with *S. cerevisiae* was not due to the presence of ethanol or
295 any other toxic compound but instead to a cell–cell contact-mediated mechanism. On
296 the other hand, Pérez-Nevado et al. [40] have studied the mechanism involved in the
297 cellular death of two *Hanseniaspora* wine strains (*H. guillermondii* and *H. uvarum*)
298 during mixed fermentations with *S. cerevisiae* under oenological growth conditions.
299 When *S. cerevisiae* reached cell densities of around 10^7 CFU ml^{-1}, a strong reduction in
300 the *Hanseniaspora* population was observed regardless of the ethanol concentration.
301 The authors hypothesised that one or more toxic compounds produced by *S. cerevisiae*
302 triggers the early death of the *Hanseniaspora* cells, though it has not yet been possible
303 to identify the nature of these compounds.

304 These yeast interactions had a clear impact on the fermentation kinetics. The presence
305 of *S. cerevisiae* guaranteed a fast fermentation. However, the fermentative behaviour

306 was very different between the pure culture of *C. zemplinina* and *H. uvarum*. Whereas
307 *C. zemplinina* ended the fermentation with a slight delay compared with the *S. cerevisae*
308 fermentations, the *H. uvarum* pure culture was unable to finish it. We did not expect this
309 strain to have such a poor fermentative capacity because in a previous experiment, it
310 was selected on the basis of predominance in synthetic grape must fermentation in
311 competition with other *H. uvarum* strains isolated from wine. This mixed culture of
312 different *H. uvarum* strains was able to consume all the sugars of the synthetic must
313 (data not shown). This controversial result might again be the result of interactions
314 between the yeasts, because a mixture of *H. uvarum* strains was able to end
315 fermentation whereas the predominant strain was unable to finish the fermentation when
316 it was alone.

317 The dominance of one species over the others may mean that it is better at using the
318 nutrients of the medium. In grape must, nitrogen is considered the main limiting nutrient
319 for optimized growth and good fermentation performance [41]. Several positive and
320 negative interactions have been reported regarding nutrient availability and nutrient
321 limitation [10]. Non-*Saccharomyces* species growing early in the fermentation could
322 strip the medium of amino acids and vitamins, limiting the subsequent growth of
323 *Saccharomyces* [41]. The proteolytic activity of some non-*Saccharomyces* together with
324 the early death and autolysis of these non-*Saccharomyces* could again enrich the
325 medium of nitrogen compounds [42]. In contrast to previous studies [28, 43], we
326 detected a higher consumption of amino acids in the mixed cultures than in the pure
327 cultures. *H. uvarum* pure culture presented the lowest consumption of assimilable
328 nitrogen but it should be taken into account that this yeast was unable to finish the
329 fermentation. However, the most remarkable result was the preferential use of some
330 groups of amino acids in the mixed fermentations compared with the pure cultures. The

331 presence of several yeast species might improve the uptake or consumption of some
332 amino acids by some kind of synergistic mechanism. The metabolism of these three
333 groups of amino acids with differential consumption (aliphatic, aromatic and sulphur
334 amino acids) has a great impact in the synthesis of aroma compounds [44, 45].

335 Most of the studies with co-inoculation or sequential inoculation of non-
336 *Saccharomyces*/*Saccharomyces* species have highlighted the differences in the aromatic
337 profiles obtained in these wines compared with monocultures of *S. cerevisiae*. Thus, *C.*
338 *stellata* (currently *C. zemplinina*) was associated with a higher production of glycerol,
339 which was confirmed by our data. Moreover, we detected that this strain of *C.*
340 *zemplinina* produced a huge amount of higher alcohols (approximately 5 times more
341 than the *S. cerevisiae* strain). These compounds can have both a positive and negative
342 impact on the aroma and flavour of a wine depending on the final concentration [44]. It
343 has been reported that concentrations below 300 mg l^{-1} add a desirable level of
344 complexity to wine, whereas concentrations that exceed 400 mg l^{-1} can have a
345 detrimental effect [46]. Both the monoculture of *C. zemplinina* and the mixed culture C.
346 *zemplinina/S. cerevisiae* clearly exceeded this concentration. However, the mixed
347 culture fermentations presented significant increases in compounds which can impact
348 positively on the aroma such as the β-phenyletanol, which contributes to a desirable
349 floral (rose) aroma [45], and thus the final result will be a higher complexity, yet further
350 studies including sensorial analysis should be performed. This strain also significantly
351 increased the synthesis of ethyl esters which impart fruity flavours to wine. This
352 increase correlated well with an important increase in short chain fatty acids, the
353 substrate for the synthesis of ethyl esters. To date, this high production of higher
354 alcohols and ethyl esters has not been described for strains of this species, in contrast to
355 *H. uvarum* strains, which have been widely described as great producers of esters [28,

29, 47]. However, this high production of esters goes together with a high volatile acidity production, which makes the wines unacceptable. This was the case with the *H. uvarum* monoculture, which produced such a large amount of acetic acid and ethyl acetate that was impossible to analyze the other minor compounds. All the mixed fermentations with *H. uvarum* presented a desirable increase in esters (especially the acetate esters), however the high production of acetic acid by this strain could jeopardise its use at industrial level. In any case, it should be tested at industrial or semi-industrial volumes because Beltran et al. [48] have already reported a higher production of acetate in small volumes and in less anaerobic fermentations.

Conclusions

The potential of using mixed cultures in industrial wine production is currently under scrutiny. However, detrimental results such as the production of acetic acid above acceptable levels counteract the benefits of high ester production, as observed in the present study. These benefits could justify the selection of appropriate non-*Saccharomyces* yeasts whose production of detrimental products is low and that they interact correctly with *S. cerevisiae*. Furthermore, a better understanding of the nutrient consumption in these mixed fermentations is required for industrial environments as our results suggest that these cultures use amino acids differently.

Acknowledgements

The present work has been financed by the projects AGL2007-66417-C02-02/ALI and AGL2007-65498-C02-02/ALI of the Spanish Ministry of Education and Science. The

authors thank the Language Service of the Rovira i Virgili University for revising the manuscript.

References

1. Ribereau-Gayon P, Dubourdieu D, Donèche B, Lonvaud A (2000) (eds) Cytology, Taxonomy and Ecology of Grape and Wine Yeast. In Handbook of Enology (vol. 1), John Wiley and Sons, West Sussex (England), pp 1–49
2. Fleet GH, Heard GM (1993). Yeasts growth during fermentation. In Fleet GH (ed) Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur (Switzerland), pp 27–54
3. Beltran G, Torija MJ, Novo M, Ferrer N, Poblet M, Guillamón JM, Rozès N, Mas A (2002) Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. Syst Appl Microbiol 25:287–293
4. Fleet GH (2008) Wine yeast for the future. FEMS Yeast Res 8:979-995
5. Sipiczki M, Ciani M, Csoma H (2005) Taxonomic reclassification of *Candida stellata* DBVPG 3827. Folia Microbiol 50:494–498
6. Hierro N, González A, Mas A, Guillamón JM (2006) Diversity and evolution of non-*Saccharomyces* yeast populations during wine fermentations: effect of grape ripeness and cold maceration. FEMS Yeast Res 6:102–111
7. Hierro N, Esteve-Zarzoso B, Mas A, Guillamón, JM (2007) Monitoring of *Saccharomyces* and *Hanseniaspora* populations during alcoholic fermentation by real-time quantitative PCR. FEMS Yeast Res 7:1340-1349
8. Andorrà I, Landi S, Mas A, Guillamón JM, Esteve-Zarzoso B (2008) Effect of oenological practices on microbial population using culture independent techniques. Food Microbiol 25:849-856

403 9. Esteve-Zarzoso B, Gostíncar A, Bobet R, Uruburu F, Querol A (2000) Selection
404 and molecular characterization of wine yeasts isolates from the "El Penedès"
405 area (Spain). Food Microbiol 17:553-562
406 10. Fleet GH (2003) Yeast interaction and wine flavour. Int J Food Microbiol 86:87-
407 99
408 11. Viana F, Gil JV, Genovés S, Vallés S, Manzanares P (2008) Rational selection
409 of non-*Saccharomyces* wine yeast for mixed starters based on ester formation
410 and enological traits. Food Microbiol 25:778-785
411 12. Fernandez MT, Ubeda JF, Briones AI (1999) Comparative study of non-
412 *Saccharomyces* microflora of musts in fermentation, by physiological and
413 molecular methods. FEMS Microbiol Lett 173:223-229
414 13. Charoenchai C, Fleet GH, Henschke PA, Todd BEN (1997) Screening of non–
415 *Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes.
416 Aust J Grape Wine Res 3:2–8
417 14. Mills DA, Johannsen EA, Cocolin L (2002) Yeast diversity and persistence in
418 botrytis-affected wine fermentations. Appl Environ Microbiol 68:4884–4893
419 15. Antunovics Z, Csoma H, Sipiczki M (2003) Molecular and genetic analysis of
420 the yeast flora of botrytized Tokaj wines. Bull OIV 76:380–397
421 16. Clemente-Jimenez JM, Mingronance-Cazorla L, Martínez-Rodríguez S, Las
422 Heras-Vázquez FJ, Rodríguez-Vico F (2004) Molecular characterization and
423 oneological properties of wine yeasts isolated during spontaneous fermentation
424 of six varieties of grape must. Food Microbiol 21:149-155
425 17. Divol B, Lonvaud-Funel A (2005) Evidence for viable but nonculturable yeasts
426 in botrytis-affected wine. J Appl Microbiol 99:85–93

427 18. Hierro N, Esteve-Zarzoso B, González A, Mas A, Guillamón JM (2006) Real-
428 time quantitative PCR (QPCR) and reverse transcription-QPCR for detection
429 and enumeration of total yeasts in wine. Appl Environ Microbiol 72:7148–7155
430 19. Ciani M, Ferraro L (1998) Combined use of immobilized *Candida stellata* cells
431 and *Saccharomyces cerevisiae* to improve the quality of wines. J Appl Microbiol
432 85:247-254
433 20. Soden A, Francis IL, Oakey H, Henschke PA (2000) Effects of co-fermentation
434 with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and
435 composition of Chardonnay wine. Aust J Grape Wine Res 6:21-20
436 21. Llauradó JM, Rozés N, Bobet R, Mas A, Constantí M (2002) Low temperature
437 alcoholic fermentation in high sugar concentration grape must. J Food Sci
438 67:268-273
439 22. Ciani M, Ferraro L (1996) Enhanced glycerol content in wines made with
440 immobilized *Candida stellata* cells. Appl Environ Microbiol 62:128–32
441 23. Ciani M, Maccarelli F (1998) Oenological properties of non-*Saccharomyces*
442 yeasts associated with winemaking. World J Microbiol Biotechnol 14:199–203
443 24. Jemec KP, Raspor P (2005) Initial *Saccharomyces cerevisiae* concentration in
444 single or composite cultures dictates bioprocess kinetics. Food Microbiol
445 22:293-300
446 25. Csoma H, Sipiczki M (2008) Taxonomic reclassification of *Candida stellata*
447 strains reveals frequent occurrence of *Candida zemplinina* in wine fermentation.
448 FEMS Yeast Res 8:328–336
449 26. Sipiczki M (2003) *Candida zemplinina* sp. Nov., an osmotolerant and
450 psychrotolerant yeast that ferments sweet botrytied wines. Int J Syst Evol
451 Microbiol 53:2079-2083

452 27. Sipiczki M (2004) Species identification and comparative molecular and
453 physiological analysis of *Candida zemplinina* and *Candida stellata*. J Basic
454 Microb 44:471–479
455 28. Ciani M, Beco L, Comitini F (2006) Fermentation behaviour and metabolic
456 interactions of multistarter wine yeast fermentations. Int J Food Microbiol
457 108:239-245
458 29. Moreira N, Mendes F, Hogg T, Vasconcelos I (2005) Alcohols, esters and heavy
459 sulphur compounds production by pure and mixed cultures of apiculate wine
460 yeast. Int J Food Microbiol 103:285-294
461 30. Angelo J, Siebert KJ (1987) A new medium for the detection of wild strains in
462 brewing culture yeast. J Am Soc Brew Chem. 45:135-140
463 31. Aerny J (1996) Composés azotés des moûts et vins. Rev Suisse Vitic Arboric
464 Hortic 28:161–165
465 32. Beltran G, Novo M, Rozès N, Mas A, Guillamón JM (2004) Nitrogen catabolite
466 repression in *Saccharomyces cerevisiae* during wine fermentations. FEMS Yeast
467 Res 4:625-632
468 33. Ortega C, López R, Cacho J, Ferreira V (2001) Fast analysis of important wine
469 volatile compounds development and validation of a new method based on gas
470 chromatographic-flame ionisation detection analysis of dichloromethane
471 microextracts. J Chromatogr A 923:205-214
472 34. Garde-Cerdán T, Ancín-Azpilicueta C (2006) Contribution of wild yeasts to the
473 formation of volatile compounds in inoculated wine fermentations. Eur Food
474 Res Technol 222:15-25
475 35. Pretorius I (2000) Tailoring wine yeast for the new millennium: novel
476 approaches to the ancient art of winemaking. Yeast 16:675–729

477 36. Ciani M, Pepe V (2002) The influence of pre-fermentative practices on the
478 dominance of inoculated yeast starter under industrial conditions. J Sci Food
479 Agr 82:573–578.
480 37. Nissen P, Arneborg N (2003) Characterization of early deaths of non-
481 *Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Arch
482 Microbiol 180:257-263
483 38. Nissen P, Nielsen D, Arneborg N (2003) *Saccharomyces cerevisiae* cells at high
484 concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed
485 cultures by a cell-cell contact-mediated mechanism. Yeast 20:331-341
486 39. Arneborg N, Siegumfeldt H, Andersen G, Nissen P, Daria V, Rodrigo P,
487 Glückstad J (2005) Interactive optical trapping shows that confinement is a
488 determinant of growth in a mixed yeast. FEMS Microbiol Lett 245:155-159
489 40. Pérez-Nevado F, Albergaria H, Hogg T, Girio F (2006) Cellular death of two
490 non-*Saccharomyces* wine-related yeasts during mixed fermentation with
491 *Saccharomyces cerevisiae*. Int J Food Microbiol 108:336-345
492 41. Bisson LF (1999) Stuck and sluggish fermentations. Am J Enol Vitic 50:107-
493 119
494 42. Hernawan T, Fleet GH (1995) Chemical and cytological changes during the
495 autolysis of yeasts. J Ind Microbiol 14:440-450
496 43. Mendoza LM, Manca de Nadra MC, Farías ME (2007) Kinetics and metabolic
497 behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces*
498 *cerevisiae* wine related strains. Biotechnol Lett 29:1057-1063
499 44. Beltran G, Esteve-Zarzoso B, Rozès N, Mas A, Guillamón JM (2005) Influence
500 on the timing of nitrogen additions during synthetic grape must fermentations on

501 fermentation kinetics and nitrogen consumption. J Agric Food Chem 53:996-
502 1002
503 45. Swiegers JH, Bartowsky EJ, Henschke PA, Pretorius IS (2005) Yeast and
504 bacterial modulation of wine aroma and flavour. Aust J Grape Wine Res 11:127-
505 138
506 46. Rapp A, Versini G (1991) Influence of nitrogen compounds in grapes on aroma
507 compounds of wine. In Seattle WA, Rantz J (eds) Proceedings of the
508 International Symposium on Nitrogen in Grapes and Wines,. American Society
509 for Enology and Viticulture, (Davis, California) pp. 15664.
510 47. Capece A, Fiore C, Maraz A, Romano P (2005) Molecular and technological
511 approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine
512 origin. J Appl Microbiol 98:136–144
513 48. Beltran G, Novo M, Guillamón JM, Mas A, Rozès N (2008) Effect of
514 fermentation temperature and culture media on the yeast lipid composition and
515 wine volatile compounds. Int J Food Microbiol 121:169-177

516 **Figure 1.** Fermentation kinetics (as density g l^{-1}) of the six experimental fermentations:
517 *C. Zemplinina* (–♦–), *H. uvarum* (–□–), *S. cerevisiae* (–▲–), *C. zemplinina-S. cerevisiae*
518 (--X--), *H. uvarum –S. cerevisiae* (-----), *C. zemplinina-H. uvarum- S. cerevisiae* (--●--).
519 Each point is expressed as the mean ± standard deviation.

520

521 **Figure 2.** Evolution of yeast population in two different plates, YPD and lysine-agar
522 (LYS) medium, in the different fermentations: *C. zemplinina*(–♦–), *H. uvarum* (–□–), *S.*
523 *cerevisiae* (–▲–), *C. zemplinina-S. cerevisiae* (--X--), *H. uvarum –S. cerevisiae* (-----),
524 *C. zemplinina-H. uvarum- S. cerevisiae* (--●--). Standard deviation was calculated on
525 each case, and was never higher than 20%.

526

527

528 **Table 1.** Principal oenological parameters at the end of the different fermentations.
529 Fermentations with *Saccharomyces* inoculum (S) were taken as the control. All values
530 are expressed as g l^{-1}, with the exception of the pH values, and are the mean ± standard
531 deviation of triplicate fermentations.

	Glycerol	Succinic acid	Acetic acid	pH	Glucose	Fructose
S	4.56 ± 0.19	1.52 ± 0.04	0.49 ± 0.03	2.94 ± 0.06	0	0.87
C	5.91 ± 0.21*	1.05 ± 0.02*	0.80 ± 0.05*	2.97 ± 0.02	0.60	1.49
H	3.37 ± 0.23*	0.50 ± 0.02*	37.50 ± 0.09*	2.95 ± 0.02	20	-
CS	5.79 ± 0.49*	1.82 ± 0.06*	1.76 ± 0.19*	2.96 ± 0.05	0.40	0.12
HS	5.31 ± 0.65	1.48 ± 0.05	1.58 ± 0.06*	3.05 ± 0.03*	0.00	0.55
CHS	5.41 ± 0.49	1.48 ± 0.02	1.53 ± 0.11*	3.01 ± 0.03	0.05	0.05

532 - Not detected

533 *Means statistically different from the control, $P \leq 0.05$

Table 2. Amino acids and ammonium consumed in the middle of the different fermentations. All values are expressed as mg l^{-1} and are the mean ± standard deviation of triplicate fermentations. Fermentations with *Saccharomyces* inoculum (S) were taken as the control.

Group	AA	Must	S	C	H	HS	CS	CHS
Acids &amides	**Asp**	51.77 ± 1.06	41.98 ± 1.41	41.24 ± 1.13	44.8 ± 0.85	48.76 ± 0.09*	48.23 ± 0.78*	47.64 ± 1.95*
	Glu	59.39 ± 1.52	49.67 ± 1.13	55.65 ± 2.12	56.53 ± 1.41*	57.94 ± 0.06*	55.21 ± 3.01	58.37 ± 0.67*
	Asn	13.71 ± 0.1	13.71 ± 0.0	13.71 ± 0.0	13.71 ± 0.0	13.71 ± 0.0	13.71 ± 0.0	13.71 ± 0.0
	Gln	166.35 ± 1.06	158.49 ± 7.55	158.57 ± 9.89	131.72 ± 9.20*	156.47 ± 7.36	153.95 ± 7.56	154.49 ± 2.72
	Total acids & amides	*291.22 ± 3.74*	*263.84 ± 10.09*	*269.177 ± 13.15*	*246.76 ± 11.46*	*276.888 ± 7.35*	*271.1 ± 11.08*	*274.21 ± 5.24*
Aliphatic	**Gly**	3.07 ± 0.08	2.30 ± 0.10	1.53 ± 0.14*	2.21 ± 0.13	2.8 ± 0.11*	2.73 ± 0.18*	2.74 ± 0.03*
	β-ala	1.75 ± 0.11	1.38 ± 0.13	1.45 ± 0.14	1.07 ± 0.08	1.45 ± 0.06	1.5 ± 0.12	1.22 ± 0.57
	α-ala	40.45 ± 0.51	36.94 ± 0.42	37.35 ± 0.28	36.79 ± 0.71	39.11 ± 0.76*	39.46 ± 0.32*	39.31 ± 0.19*
	Val	10.45 ± 0.53	9.26 ± 0.47	7.83 ± 0.54	9.02 ± 0.62	9.78 ± 0.35	9.35 ± 0.88	9.58 ± 0.37
	Ile	6.07 ± 0.85	5 ± 1.13	2.06 ± 0.48	3.32 ± 0.85	5.04 ± 0.74	4.22 ± 1.06	3.82 ± 0.59
	Leu	9.33 ± 0.54	3.77 ± 0.71	1.34 ± 0.58*	6.68 ± 0.48*	8.09 ± 0.48*	7.8 ± 0.41*	7.61 ± 0.67*
	Total aliphatic	*71.12 ± 2.62*	*58.66 ± 2.95*	*51.56 ± 2.16*	*59.09 ± 2.87*	*66.26 ± 1.97**	*65.03 ± 1.36*	*64.29 ± 1.42*
Aromatic	**Tyr**	5.15 ± 0.84	1.45 ± 0.57	3.99 ± 0.51	3.75 ± 0.97	4.2 ± 0.61	3.65 ± 1.69	4.03 ± 0.47
	Trp	9.65 ± 0.95	5.13 ± 1.10	2.63 ± 0.41*	4.15 ± 0.78	8.32 ± 0.53*	6.55 ± 0.92	6.79 ± 0.32
	Phe	15.93 ± 1.25	7.91 ± 1.42	4.33 ± 1.41	7.4 ± 0.99	14.63 ± 0.71*	13.22 ± 1.84*	14.1 ± 1.37*
	Total aromatic	*30.73 ± 3.04*	*14.49 ± 3.72*	*10.96 ± 2.33*	*15.3 ± 2.74*	*27.14 ± 0.91**	*23.42 ± 2.61**	*24.96 ± 1.18**
Hydroxyl	**Ser**	21.96 ± 0.52	21.08 ± 0.71	19.8 ± 0.56	21.96 ± 0.84	21.78 ± 0.31	21.54 ± 0.71	21.96 ± 0.0
	Thr	14.97 ± 0.09	14.95 ± 0.17	14.95 ± 0.11	14.89 ± 0.07	14.69 ± 0.04	14.77 ± 0.19	14.94 ± 0.01
	Total hydroxyl	*36.93 ± 0.61*	*36.04 ± 0.88*	*34.75 ± 0.68*	*36.85 ± 0.92*	*36.46 ± 0.28*	*36.311 ± 0.90*	*36.9 ± 0.01*
Sulphur	**Met**	3.01 ± 0.18	1.89 ± 0.25	3.01 ± 0.28*	0.8 ± 0.21*	1.13 ± 0.15*	1.97 ± 0.30	0.54 ± 0.08*
	Cyst	2.15 ± 0.25	2.15 ± 0.33	2.15 ± 0.26	1.05 ± 0.31*	1.75 ± 0.26	1.36 ± 0.13*	0.91 ± 0.18*
	Total sulphur	*5.16 ± 0.43*	*4.03 ± 0.59*	*5.16 ± 0.55*	*1.85 ± 0.52**	*2.88 ± 0.09**	*3.33 ± 0.17*	*1.53 ± 0.04**

Basic	**His**	6.24 ± 0.26	4.53 ± 0.41	3.91 ± 0.44	3.64 ± 0.37	4.69 ± 0.76	4.99 ± 0.34	5.55 ± 0.19
	Arg	120.06 ± 2.42	112.17 ± 2.39	111.39 ± 2.80	110.75 ± 2.21	116.9 ± 2.86	116.68 ± 0.54	114.61 ± 2.27
	Lys	7.77 ± 0.65	6.93 ± 0.85	7.08 ± 0.42	6.97 ± 0.28	7.43 ± 0.85	5.88 ± 1.56	7.35 ± 0.21
	Total basic	*134.07* ± 3.33	*123.63 ± 3.65*	*122.38 ± 3.66*	*121.36 ± 2.85*	*129.02 ± 2.91*	*127.55 ± 1.92*	*127.51 ± 2.12*
	Pro	127.38 ± 0.97	113.95 ± 0.74	114.96 ± 1.32	113.58 ± 0.86	105.21 ± 0.98	124.83 ± 1.64	124.55 ± 0.47
	NH4+	221.47 ± 1.73	209.69 ± 1.58	209.14 ± 2.03	210.03 ± 1.85	214.94 ± 2.25	217.85 ± 1.84	216.87 ± 0.48
	Total aas	*696.91*	*614.64*	*608.94*	*594.79*	*643.85*	*651.57*	*653.95*
	Total N	*154.07*	*141.58*	*140.54*	*136.40*	*147.04*	*146.05*	*145.64*
	N org	*96.12*	*86.71*	*85.81*	*81.44*	*90.79*	*89.05*	*88.89*
	N inorg	*57.95*	*54.87*	*54.73*	*54.96*	*56.25*	*57.01*	*56.75*

*Means statistically different from the control, P≤ 0.05

Table 3. Volatile compounds at the end of the different fermentations. All values are expressed as mg l^{-1} and are the mean ± standard deviation of triplicate fermentations. Fermentations with *Saccharomyces* inoculum (S) were taken as the control.

Group	Compound	S	C	CS	HS	CHS
Higher Alcohols	**1-Propanol**	9.55 ± 1.26	30.58 ± 0.96*	16.94 ± 4.38	11.47 ± 0.30	21 ± 1.40*
	2-Methyl-1-propanol	24.51 ± 1.53	468.86 ± 7.48*	93.46 ± 0.73	55.39 ± 0.75	77.4 ± 1.77
	Isoamyl alcohol	167.52 ± 4.33	334.63 ± 29.45*	213.13 ± 17.41	202.95 ± 15.50	199.86 ± 6.63
	β-Phenylethanol	30.63 ± 4.91	227.9 ± 15.26*	118.15 ± 3.00*	42.72 ± 2.69	61.22 ± 0.02*
	Total higher alcohols	*232.21 ± 9.51*	*1061.98 ± 29.15**	*441.68 ± 18.06**	*312.53 ± 12.35**	*359.49 ± 9.82**
Fatty acid ethyl esters	**Ethyl hexanoate**	0.03 ± 0.01	0.12 ± 0.01	0.09 ± 0.02	0.22 ± 0.11	0.1 ± 0.01
	Ethyl octanoate	0.22 ± 0.04	3.6 ± 0.06*	0.61 ± 0.19*	0.21 ± 0.02	0.25 ± 0.03
	Ethyl lactate	0.34 ± 0.04	0.89 ± 0.11	1.27 ± 0.22*	2.27 ± 0.05*	2.54 ± 0.28*
	Total Fatty acid ethyl esters	*0.6 ± 0.09*	*4.61 ± 0.05**	*1.97 ± 0.39**	*2.71 ± 0.04**	*2.89 ± 0.23**
Acetates esters	**Isoamyl acetate**	0.25 ± 0.08	0.15 ± 0.15	0.14 ± 0.01	0.70 ± 0.01*	0.2 ± 0.01
	Hexyl acetate	6.81 ± 0.17	13.98 ± 0.68	14.34 ± 0.95	23.22 ± 4.30*	9.47 ± 2.09
	2-Phenylethyl acetate	2.99 ± 0.14	1.09 ± 0.01*	2.79 ± 0.16	4.23 ± 0.34*	3.31 ± 0.07
	Total acetates	*10.06 ± 0.39*	*15.22 ± 0.55*	*17.27 ± 1.10*	*28.15 ± 3.97**	*12.99 ± 2.01*
SCFA	**Isobutyric acid**	1.77 ± 0.01	29.08 ± 2.38*	12.66 ± 1.26*	6.13 ± 0.47	4.35 ± 0.13
	Isovaleric acid	1.97 ± 0.13	1.01 ± 0.16*	2.48 ± 0.38	1.52 ± 0.07	1.64 ± 0.19
	Butyric acid	0.64 ± 0.02	0.34 ± 0.09	0.75 ± 0.20	0.77 ± 0.04	0.64 ± 0.12
	Total SCFA	*4.37 ± 0.14*	*30.32 ± 2.44**	*15.89 ± 0.68**	*8.42 ± 0.58*	*6.644 ± 0.06*
MCFA	**Hexanoic acid**	3.42 ± 0.46	0.31 ± 0.05*	2.89 ± 0.38	1.83 ± 0.19*	1.74 ± 0.01*
	Octanoic acid	2.62 ± 0.45	0.23 ± 0.07*	1.88 ± 0.04	0.92 ± 0.11*	1.62 ± 0.22*
	Decanoic acid	1.59 ± 0.32	0.12 ± 0.01*	0.84 ± 0.11*	0.69 ± 0.17*	0.56 ± 0.08*
	Dodecanoic acid	0.18 ± 0.05	1.2 ± 0.18*	0.56 ± 0.24	1.3 ± 0.09*	0.17 ± 0.01
	Total MCFA	*7.82 ± 0.26*	*1.86 ± 0.19**	*6.18 ± 0.55**	*4.74 ± 0.35**	*4.09 ± 0.15**

SCFA. Short Chain Fatty Acids, MCFA: Medium Chain Fatty Acids. *Means statistically different from the control, $P \leq 0.05$

Fig. 1.

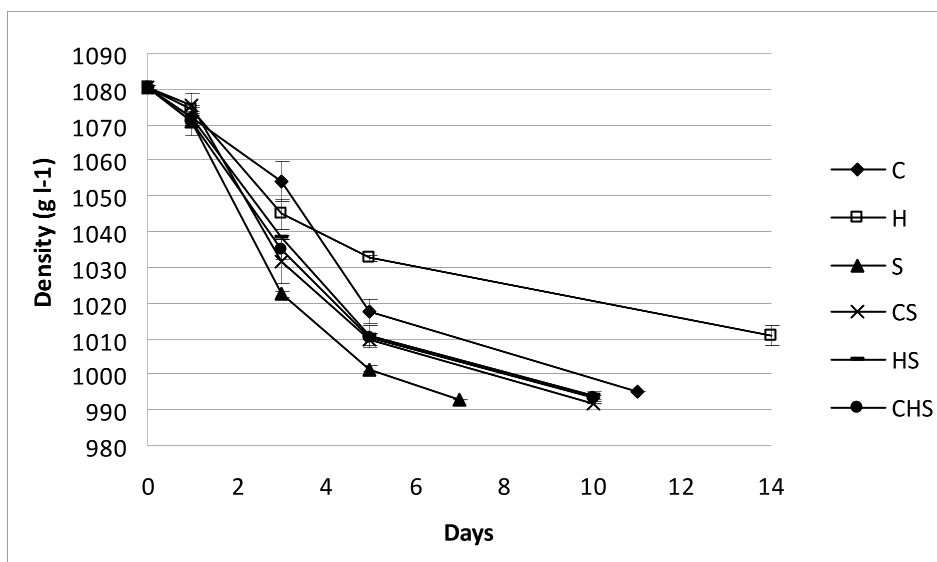

Fig 2. :