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Melissa Tan, Yanis Caro, Alain Shum Cheong Sing, Laurent Robert, Jean Marie François, et al.. Evaluation of mixed-fermentation of Saccharomyces cerevisiae with Saprochaete suaveolens to produce natural fruity beer from industrial wort. Food Chemistry, 2021, 346, 10.1016/j.foodchem.2020.128804 . hal-03170206

HAL Id: hal-03170206
https://hal.inrae.fr/hal-03170206

Submitted on 3 Feb 2023

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Version of Record: https://www.sciencedirect.com/science/article/pii/S0308814620326662
Manuscript_ce509485d26089f42f42201650d2322c

Evaluation of mixed-fermentation of *Saccharomyces cerevisiae* with *Saprochaete suaveolens* to produce natural fruity beer from industrial wort.

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Received: date; Accepted: date; Published: date

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Abstract:

Fruity beers can be promoted through production of flavoring compounds during fermentation by partial replacement of brewing yeast by non-conventional-yeasts with high aroma production abilities. We evaluated here the use of a wild *Saprochaete suaveolens* strain, producing atypical aroma compounds, to produce new natural fruity beer, while keeping classical production conditions used in brewing industry. *S. suaveolens* was inoculated as starter of culture during beer fermentation and the fermentation performance was evaluated through measurement of several physicochemical parameters. The aroma profile of the engineered beers was monitored using HS-SPME GC/MS. The results showed that high fruity aroma and low-ethanol content beers were obtained through single-fermentation using *S. suaveolens*. We also demonstrated that during mixed-fermentation, *S. suaveolens* maintained high metabolic activity and allowed production of beer enriched with fruity aroma. Production of high or low ethanol content fruity beer could be achieved by varying the composition of the starter of culture.

Keywords: Natural aroma; *Saprochaete suaveolens*; non-conventional yeast; beer; co-fermentation

Highlights:

- The unconventional yeast *Saprochaete suaveolens* (formerly *Geotrichum fragrans*) has been tested for the natural flavoring of beer produced in laboratory and industrial conditions.
- In mixed-fermentation of wort, *S. cerevisiae* dominated the fermentation of beer, but *S. suaveolens* maintained an active aromatic metabolism.
- Wort inoculated with 30% of *S. suaveolens* and 70% of *S. cerevisiae* generated beers with standard ethanol content.
- Wort inoculated with 90% of *S. suaveolens* and 10% *S. cerevisiae* produced beers with low-alcohol content.
- Beers produced using industrial recipe in mixed fermentation with at least 30% of *S. suaveolens* were significantly enriched with fruity aromas (including esters).

1. Introduction

The market of *fruity beers* has increased over the last decade with an estimated annual growth rate of over 4% from 2019 to 2023 (TechNavio, 2019). For a long time, the traditional manner to produce these fruity beers was to directly add fruity flavour additives during brewing process carried out as single-fermentation by yeast belonging to the *Saccharomyces* gender (mostly *S. cerevisiae, S. eubayanus, S. pastorianus or S. kudriavzevii*). This process which can reach high ethanol content beers (6-7% v/v) also generated a wide spectrum of more than 800 different volatile organic compounds (VOCs) in the form of esters, higher alcohols, organic acids, sulfur compounds, carbonyls or short-chain fatty acids (Bokulich and Bamforth, 2013; Lodolo *et al.,* 2008; Olaniran *et al.,* 2017; Stevens, 1960; Willaert and Nedovic, 2006). Despite this high amount of VOCs, standard beers mostly display poor *aromatic bouquet* as only a few part of them are flavor-active (Capece *et al.,* 2018; Gallone *et al.,* 2016, 2018; Gibson *et al.,* 2017; Kao, 2018; Libkind *et al.,* 2011; Lodolo *et al.,* 2008; Olaniran *et al.,* 2017; Petruzzi *et al.,* 2016; Stewart, 2016). Therefore, enhancing yeast VOCs with higher fruity character is of prime importance to satisfy this global trend of consumers for new and natural beverage.

A first manner to achieve this goal is through some genetic modifications of *Saccharomyces* spp. (Bellut and Arendt, 2019). For instance, Zhang and coworkers (Zhang *et al.*, 2013) engineered a brewer's *S. cerevisiae* strain by overexpressing *ATF1* gene (encoding for alcohol acyltransferase) and deleting *BAT2* gene (encoding for

74 cytosolic branched-chain amino acid aminotransferase), which led to an increase of
75 two of the major flavouring esters in beer, namely ethyl acetate (solvent-like aroma)
76 and 3-methylbutyl acetate (banana- flavour). Also Mertens and coworkers (Mertens
77 *et al.*, 2015) showed that created hybrids of *S. cerevisiae* and *S. eubayanus* strain
78 enriched the aroma profile of standard lager beer. However because of public's
79 scepticism regarding to genetically modified organisms (GMO), such engineered
80 yeasts have not or will never been used for commercial brewing (Gibson *et al.*, 2017;
81 Saerens *et al.*, 2010). Hence, a potential alternative to overcome this barrier is to
82 carry out single-fermentation using non-conventional yeasts (NCY) specifically
83 selected for such performance and harboring original fruity characters. The
84 *Brettanomyces* genus (e.g., *B. anomalus* or *B. bruxellensis*) have been the most popular
85 NCY involved in beer fermentation especially for the production of some
86 lambic-style beers in which they bring smoky, barnyard, spicy and fruity flavours
87 (Basso *et al.,* 2016; Bokulich and Bamforth, 2013; Daenen *et al.,* 2008; Gibson *et al.,*
88 2017; Serra Colomer *et al.,* 2019). More recently other yeast species such as *Lachancea*
89 *thermotolerans*, *Pichia kluyveri*, *Saccharomycodes ludwigii*, *Scheffersomyces shehatae*,
90 *Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *Williopsis saturnus*, *Lachancea*
91 *thermotolerans* and *Zygosshacaromycodes rouxii* have been investigated in
92 single-fermentation (Bellut and Arendt, 2019; Michel *et al.*, 2016; Petruzzi *et al.*, 2016;
93 Steensels and Verstrepen, 2014; Varela, 2016). These attempts mainly resulted in
94 nonalcoholic (0.00-0.50% alcohol by volume (ABV)) or low alcohol beer (0.60-3.50%

ABV) as NCY generally show low abilities for ethanol production as compared to conventional *Saccharomyces* species.

A third method to generate fruity beer is to carry out mixed-fermentation by combining a *Saccharomyces cerevisiae* strain with another yeast (NCY) that can provide flavours notes during the brewing process, while enhancing ethanol production compared to mono-culture of NCY. For instance, van Rijswijck and coworkers (van Rijswijck *et al.,* 2017) showed that co-cultures of *S. cerevisiae* with *Cyberlindnera fabanii* or *Pichia kudriavzevii* led to a reduced ethanol titer (3.80% and 3.55% ABV respectively) and a higher content of esters (0.43 and 0.60 relative abundance of total VOCs) compared to mono-culture of *S. cerevisiae* (4.19% ABV, 0.19 relative abundance of total VOCs) but increased ethanol titer compared to mono-culture of *C. fabanii* or *P. kudriavzevii* (0.49% and 0.53% ABV respectively). Likewise, assays with *T. delbrueckii* showed that co-cultures with *S. cerevisiae* cause an increase of 86% in ethanol content as compared to mono-culture of *T. delbrueckii,* and a raise of 44.6% of the main VOCs as compared to mono-culture of *S. cerevisiae* (Canonico *et al.,* 2017). While this mixed-fermentation is seen as a promising alternative to produce *fruity beers*, most brewing industries are reluctant to use them in industrial processes as their implementation would require important changes in the process conditions (Gibson *et al.*, 2017).

Taking into account these considerations, we report that the introduction of the NCY *Saprochaete suaveolens* (former *Geotrichum fragrans*) in combination with *S. cerevisiae* provided natural fruity beer from industrial wort that did not require any

117 major modification in the brewing process. The rational to investigate such
118 mixed-fermentation came from previous works from our group and others showing
119 that this NCY exhibits remarkable metabolic capacity to produce numerous esters
120 associated with tropical fruit flavors such as ethyl propanoate (banana flavor),
121 3-methylbutyl butanoate (pineapple flavor) or ethyl 2-methylbutanoae (apple
122 flavor) (Damasceno *et al.*, 2003; de Oliveira *et al.*, 2013; Grondin *et al.*, 2015a, 2015b,
123 2017; İşcan *et al.*, 2015; Zhu *et al.*, 2016). To get as close as possible to industrial
124 conditions, we managed our fermentation assays in industrial wort during 14 days.
125 We then investigated the fermentation performances and evaluated the influence of
126 ratio of *S. suaveolens* versus *S. cerevisiae* (30% and 90% cell/cell of *S. suaveolens*
127 completed to 100% with *S. cerevisiae)* in the process leading to best condition for
128 beers aroma production.

2. Materials and Methods

2.1. Yeast strains and preparation of the starter

Wild type *Saccharomyces cerevisiae* CEN.PK 112-2N (van Dijken JP *et al.*, 2000) and the wild strain of *Saprochaete suaveolens* (GEC0) previously isolated in our laboratory on Pitaya fruit (*Hylecereus polyrhisus*) from Reunion Island, France (Grondin *et al.*, 2015b) were used in this study. To prepare pure starter of culture, both *S. cerevisiae* and *S. suaveolens* strains were pre-grown in malt-peptone-yeast extract-glucose pH 5.0 (MPYG; 2% dextrose, 0.25% bacteriological peptone, 0.25% malt extract and 0.25% yeast extract (w/v)). Then 100 µL of this culture were used to inoculate 50 mL of sterile MPYG-broth (250 mL shake flask). Cultures were carried out at 30°C, while adding 50 mL of sterile MPYG-broth every 24h for 3 days. The mixed starters of culture were prepared by mixing a given volume of both pure starters set at an initial cell concentration of 1.10^7 cell/mL with a cell ratio of 0, 10, 20, 30, 40, 50, 60, 70, 90 or 100% of *S. suaveolens* completed to 100% with *S. cerevisiae*.

2.2. Beer fermentation set-up

Artisanal wort was prepared using the following preparation process: 5kg of malt were crushed and supplemented with 15L of water before heating (15 min at 45-50°C, 60 min at 60-65°C, 10 min at 78-80°C). Brew was then lautered and residues were rinsed with 1L of water and added to the initial brew before cooking (30 min boiling). Three grams of hops were then added before sterilization (30 min

149 ebullition) and filtration. The wort was then recovered in a 5L sterile bottle and
150 cooled at 12°C before use. The wort was characterized by pH 5.50 and a specific
151 gravity of 13° Plato. Industrial wort, characterized by pH 5.00 and specific gravity
152 of 13° Plato, was aseptically collected from local brewing industry (SOREBRA,
153 Saint-Louis, Reunion Island).

154 Pitching was performed by inoculating wort with 1% of starter (v/v) to reach a
155 concentration of 1.10^5 cell/mL (corresponding to 5 log CFU/mL). For lab scale
156 fermentation, artisanal wort was used. Assays were carried out in 500 mL
157 Erlenmeyer flasks containing 250 mL of wort by addition of 2.5 mL of desired
158 starter of culture and incubation at 20-25°C during 6 days. For large scale trials,
159 industrial wort was used. Then the fermentation was performed during 14 days in
160 6L sterile flasks containing 5L of wort and 50 mL of desired starter of culture.

161 *2.3. Total cell enumeration and determination of ratio between the two yeast species along*
162 *the brewing process.*

163 The total living yeast population was determined during fermentation by
164 spreading 100µL of beer-sample (including serial dilutions with peptone water) on
165 YPD-agar media (1% yeast extract, 2% peptone, 2% dextrose w/v) and incubation
166 during 48h at 30°C. Considering the ability of *S. suaveolens* to use branched-chain
167 amino acid as sole carbon source (Grondin *et al.,* 2015a), *S. suaveolens* cell
168 enumeration was done by spreading 100µL of beer-sample (including serial
169 dilution with peptone water) on YNB-Ile agar media containing 0.67 % yeast

nitrogen base without amino acids and 0.1% isoleucine w/v and incubation during 48h at 30°C. The *S. cerevisiae* cell count was thus estimated by subtracting *S. suaveolens* population from the total yeast population.

2.3. Physicochemical parameters and non-volatile compounds

Total soluble solids (°Brix) and pH were measured using a refractometer (Euromex) and a portable pH-meter (Checker by HANNA, HANNA Instruments) respectively. For ethanol, sugars and organic acids analysis, samples were firstly centrifuged and filtered through 0.45 μm membranes (Sartorius Sedi, Minisart) prior to analysis using high performance liquid chromatography (HPLC) (Ultimate® 3000, Dionex, Thermo Scientific). Isocratic elution using 1.25 mM H_2SO_4 mobile phase at 40 °C, with a 0.5 mL/min flow rate was performed through an ion exclusion column (300 * 7.8 mm, Biorad-Aminex HPX-87H 125-0.140). Ethanol and sugars were detected with a refractive index detector (Shimadzu, RID-20A) while organic acids were measured using a diode array detector (Ultimate 3000, Dionex, Thermo Scientific) at 210 nm. Ethanol content was confirmed by conversion of the difference of specific densities between beers and initial wort measured with a densimeter (Brewferm). Sucrose and maltose co-eluted with this method. Thus, for sucrose, samples were enzymatically hydrolyzed using 48 Units/L of invertase (SIGMA) during 30 min at 30°C prior to HPLC analysis. Then, glucose content of the hydrolyzed fractions was compared to non-hydrolyzed ones to determine

sucrose concentration and maltose content was then deducted by calculating the difference.

2.4. Volatile compounds analysis

5 mL of each sample were introduced in a 15 mL sealed vial before addition of 10 µL of octan-1-ol (1g/L in absolute ethanol) as internal standard prior to analysis. Then solid phase micro extraction (SPME) of the volatiles was performed using a 2 cm long fiber coated with 50/30 µm Divinylbenzene/Carboxen on polydimethylsiloxane (DVB/Car/PDMS) bonded to a flexible fused silica core (Supelco). The fiber was exposed to headspace for 10 min at 30°C and inserted into the injection port at 270°C for 2 min. Metabolites were separated by gas chromatography (GC), on a SPB5 columns (60m * 0.32mm * 0.25µm film thickness), coupled to a mass spectrometer (5973 Network mass selective detector, Agilent technologies). The carrier gas (He) was set at a flow rate of 1.4 mL/min. The column temperature was maintained at 45°C for 2 min, raised to 230°C at 4°C/min and finally kept at this temperature for 12 min. Volatile organic compounds (VOCs) were identified by comparing their mass spectra and their Kovats index (DB-5 columns) with the NIST database (www.chemdata.nist.gov).

2.5 Stastistical analysis

Means and standard deviations (SD) were determined based on triplicate independent fermentations and were presented as mean ± SD. Experimental data were subjected to one-way analysis of variance (ANOVA) using XL Stat Applied

Sensory software (2020.1.3.) at the 95% confidence level. Principal analysis component (PCA) was applied to discriminate among the means of the different volatile compounds and the ethanol content in the wort and the different beers after 7 and 14 days of fermentation. PCA was also carried out using the XL Stat Applied Sensory software (2020.1.3) and the data are presented as a biplot graph.

3. Results and discussion

The use of *S. suaveolens* (former *Geotrichum fragrans*) in the brewing field had not been studied yet even if this non-conventional yeast (NCY) is well described as good aroma producer with high proportion of fruity esters (Damasceno *et al.*, 2003; de Oliveira *et al.*, 2013; Grondin *et al.*, 2015a, 2015b, 2017; İşcan *et al.*, 2015). We therefore studied the capacity of *S. suaveolens* to impact on the aroma profile of beer. However, because *S. suaveolens* is known to display bad fermentative capacity, we investigated its performance in co-culture together with *S. cerevisiae* and compared it to mono-culture of *S. suaveolens* and of *S. cerevisiae*. Then, we first determined the best ratio of the two strains resulting in aromatic beers with satisfactory ethanol content under laboratory process condition.

3.1. Determination of optimal starter of culture under laboratory process condition

3.1.1. Validation of the artisanal wort

For laboratory scale assays, we produced artisanal wort using our laboratory brewing process. To validate this artisanal, we measured its mains physicochemical parameters, *i.e.* pH, total soluble solids content (TSS) and ethanol content. Results of these assays showed that the artisanal wort presented similar characteristics with standard wort with respect to pH (comprise within 5.6 and 5.8), TSS content (comprised within 12 and 15°Brix) and of course ethanol (0% ABV). Therefore, we

concluded that our process to produce the laboratory wort was fully acceptable to manage our study at laboratory scale.

3.1.2. Optimal starter of culture determination

Previous studies found in the literature (Canonico *et al.*, 2017; van Rijswijck *et al.*, 2017) have shown that the ratio of the brewing yeast *Saccharomyces* to another yeast in the starter strongly influences the final composition of the beer. Therefore, we first determined which ratios led to a final beer product with characteristics as closed as possible to a standard beer (classically fermented with mono-culture of *S. cerevisiae*). We therefore produced fermented beers from artisanal wort with yeast starter of culture including 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% cell/cell of *S. suaveolens* completed to 100% with *S. cerevisiae* and measured key physicochemical parameters, namely pH, total soluble solids (TSS) and ethanol content.

After six days of fermentation, the wort inoculated with mono-culture of *S. cerevisiae* (0-GEC beer) showed high reduction of TSS content (46.7 %) which lead to the production of beers with satisfying characteristics (pH 4.35, TSS content 8.0°Brix and ethanol content 6.34% ABV) comparable to average standard beers (4.00-5.00; 8-9 °Brix and 4.0-7.0% ABV respectively). Contrariwise, the wort fermented with mono-culture of *S. suaveolens* (100-GEC beer) showed much lower reduction of TSS content (21.3%) which lead to the production of low ethanol content beer (1.2 %ABV) (data not shown).

All the beers prepared using mixed-culture of *S. suaveolens* and *S. cerevisiae* led to beers with pH and TSS content comparable to those obtained for standard beer (0-GEC beer fermented with mono-culture of *S. cerevisiae*) but 30-GEC beer (fermented with 30% cell/cell of *S. suaveolens* c.s.p *S. cerevisiae*) and 90-GEC beer (fermented with 90% cell/cell of *S. suaveolens* c.s.p *S. cerevisiae*) showed the closest ethanol content with 0-GEC beer (5.98% and 6.20% ABV v.s 6.34% ABV respectively) while displaying more fruity-like odour. Consequently, this small lab scale study showed the possibility of aromatizing beers by fermenting wort with mixed-culture of *S. cerevisiae* and *S. suaveolens.*

3.2. Mixed-fermentation under industrial process condition

Once we set up the optimal fermentation conditions from the artisanal wort under laboratory conditions, we wanted to perform a complete mixed fermentation under industrial process condition using industrial wort (SOREBRA©) and in larger bioreactors. We therefore assessed this process with the starter conditions established previously (namely 30-GEC and 90-GEC beers) and compared it with beers generated from single fermentation with *S. cerevisiae* or *S. suaveolens* alone (0-GEC and 100-GEC beers respectively).

3.2.1. Monitoring cell population during mixed fermentation

Total yeast population and development of each yeast species was monitored during mixed-fermentation carried out with 30 and 90% of *S. suaveolens* at pitching with respect to *S. cerevisiae* (30-GEC and 90-GEC). As shown in Figure 1, a

276 significant increase of the total yeast population occurred mainly during the first
277 two days after pitching which reached a plateau at day 6 and remained constant
278 until the 14th day of process (Figure 1a). The increase of cell population in both cases
279 was actually due to *S. cerevisiae*, whose growth profile strictly matched that of the
280 whole cell population starting. However, at higher initial *S. suaveolens* inoculum
281 (90-GEC), the growth rate of cell population was slightly lower, although the *S.*
282 *cerevisiae* species represented more than 95 % of the whole population. In contrast to
283 *S. cerevisiae*, which showed a significant increase in cell population, *S. suaveolens*
284 alone was not able to grow during mixed-fermentation, and its specific cell
285 population slowly declined along the process at an average rate of 0.06 log CFU/mL
286 in 30-GEC fermentation and 0.04 log CFU/mL in 90-GEC fermentation. This
287 phenomena is consistent with assays of mixed-fermentation with other NCY and
288 could be explained by cell competition and physiological combined factors such as
289 oxygen limitation and ethanol toxicity in the wort (Bokulich and Bamforth, 2013;
290 van Rijswijck *et al.*, 2017).

291 However, we found that *S. suaveolens* still kept over 17% (during 30-GEC
292 mixed-fermentation) and 26% (during 90-GEC mixed-fermentation) of its
293 population alive after 14 days of fermentation. We therefore concluded that *S.*
294 *suaveolens* could maintain its metabolism throughout mixed-fermentation process.

295 3.2.2. Macro Kinetics studies of the 30 and 90-GEC mixed-fermentation

296 Cell population in single-fermentation with *S. cerevisiae* increased by about
297 three log within 4 days and then remained stable, whereas *S. suaveolens* barely grew
298 by less than one log over the entire period of the process (Figure 2a). Also, we found
299 that total yeast population increased more slowly when *S. suaveolens* was added in
300 the inoculum and the higher the proportion of *S. suaveolens* in the starter, the higher
301 was this inhibition of growth. Interestingly, we noticed an increase in yeast
302 population at the 8th day in non-inoculated raw wort. This result is not completely
303 surprising as most industrial processes are carried out with sterilized wort that are
304 brought into the fermentation tanks, which even they have been extensively clean
305 up, yet may contain residual living yeast cells from a previous fermentation (Atwell
306 et al., 2017; Salo et al., 2008) . This burst of yeast growth likely did disturb neither
307 the single nor the mixed-fermentation since cell population in all cases have already
308 reached a plateau at 4 to 6 days after pitching. Thus, it might be possible that such
309 spontaneous fermentation did only occur when the wort is not inoculated.

310 The TSS content of 0-GEC beers decreased by 44% up to the 6th day of
311 fermentation (from 12.7 to 7.0 °Brix) while a relative high TSS content was
312 maintained in the 100-GEC beer in the meantime (Figure 2b). The TSS content of the
313 30-GEC beer was comparable to what was observed for 0-GEC beer. On the other
314 hand, during 90-GEC beer fermentation, TSS content was intermediate between the
315 one obtained for 100-GEC beer fermentation and 0-GEC beer fermentation even if

316 total yeast population and its relative proportion of *S. suaveolens* and *S. cerevisiae*
317 were comparable to those of 30-GEC beer fermentation since the 2nd day of the
318 process (Figure 1). Thus, introducing *S. suaveolens* in high proportion in the initial
319 starter of culture *(i.e* 90-GEC) generates a delay in sugar consumption compared to
320 introducing *S. suaveolens* in low proportion in the starter of culture (*i.e* 30-GEC).
321 This delay subsists despite the inversion of proportion between *S. suaveolens* and *S.*
322 *cerevisiae* observed when initial starter of culture contains high amount of *S.*
323 *suaveolens* (*i.e.* 90-GEC).

324 The pH globally decreased (from 5.0 to 4.3 units) during the 14 days of
325 fermentation for all beer tested (Figure 2c). For the 0-GEC beer, this drop happened
326 during the first two days and pH remained stable until the end of fermentation
327 whereas for 100-GEC beer, the pH reached a plateau about 2 days later (day 4). This
328 was probably due to the slower growth rate of *S. suaveolens* in the wort media as
329 compared to *S. cerevisiae*. Adjunction of *S. suaveolens* in the starter of culture then
330 generated a delay of the pH drop during the fermentation which increased with the
331 proportion of *S. suaveolens* in the starter of culture (Figure 2d). However, this delay
332 did not affect the final beers as we found no significant difference in pH for all
333 tested beers after the 4th day of fermentation.

334 On the contrary, the reduction in sugar consumption with increasing of *S.*
335 *suaveolens* proportion in the starter of culture seemed to strongly affect the ethanol
336 production during the process, the ethanol production being much slower with a
337 high proportion of *S. suaveolens* (Figure 2d). Then, in accordance with other studies

338 (Canonico *et al.*, 2017; Liu and Quek, 2016), we noticed that the global fermentation
339 speed decreased with the amount of *S. suaveolens* in the starter of culture.
340 Considering the subsequent delay in ethanol production, this tends to show that
341 varying the ratio of *S. suaveolens* and *S. cerevisiae* in the starter of culture could lead
342 to production of beers with variable ethanol content.

343 3.2.3. Kinetics of sugar mobilisation in single and mixed fermentation

344 To better understand the metabolism differences between *S. suaveolens* and *S.*
345 *cerevisiae* which impart the fermentation, we monitored the utilization of glucose,
346 maltose, sucrose and fructose (Figure 3). In the initial wort, fructose and maltose
347 contents (1.8 g/L and 41.5 g/L respectively) were similar to the values reported in
348 literature (3 - 4 g/L and 45-50 g/L respectively), while sucrose content (25.4 g/L) and
349 glucose content (6.4 g/L) were respectively much higher and lower than the usual
350 amount (3-5 g/L and 20-25 g/L respectively) (Callejo *et al.,* 2019; Liu and Quek, 2016;
351 Toh *et al.,* 2018). The non-inoculated wort was relatively stable regarding maltose
352 and sucrose content but glucose and fructose slightly decreased along the 14 days of
353 fermentation (Figure 3). We concluded that this decrease can be attributed to the
354 spoilage of the industrial brewing yeast in the non-inoculated wort (Figure 2a).
355 In our standard beer inoculated with *S. cerevisiae* alone (0-GEC beer
356 fermentation), the glucose was almost completely consumed after 2 days of
357 fermentation while consumption of fructose and maltose required at least 4 days
358 before stabilizing at very low levels (Figure 3). TSS continued to decrease after the

359 4th day of fermentation and was in accordance with sucrose assimilation which
360 continued to slightly decrease after the 6th day of fermentation during 0-GEC beer
361 fermentation (Figure 3). The kinetic of sugars consumption by *S. cerevisiae* generally
362 correspond to our prediction according to which glucose would be consumed first,
363 before fructose and then maltose. However, the complete depletion of maltose in
364 the wort (day 4) surprisingly occurred before the end of sucrose consumption by *S.*
365 *cerevisiae.*

366 When compared to 0-GEC, beer inoculated with *S. suaveolens* alone (100-GEC
367 beer) showed a significant delay in all sugar consumption profiles. Indeed, even if
368 glucose and fructose uptake started from the first day of fermentation, the total
369 consumption of glucose required at least 6 days, while only 53% of fructose was
370 consumed in the same period (Figure 3). Contrary to what was observed during the
371 fermentation of 0-GEC beer, significant assimilation of sucrose and maltose did not
372 start until the 6th day of fermentation, although the fructose still remained in the
373 wort. Maltose (which constituted 70% of the TSS in the wort) assimilation by *S.*
374 *suaveolens* was very low when compared with *S. cerevisiae* and was probably the
375 first reason of the global fermentation speed decrease with addition of *S. suaveolens*
376 that we mentioned before (Figure 2). However, even if the decrease of maltose
377 during the fermentation of 100-GEC beer was low, this result was quite unexpected
378 because the yeast *S. suaveolens* is known to assimilate mono-carbohydrates in
379 priority and is not expected to consume this sugar. Moreover, decrease in the
380 maltose content of the fermented wort with a NCY in mono-culture is rarely

381 described in the literature, even when the NCY, such as *T. delbrueckii*, is known to
382 assimilate it (Callejo *et al.*, 2019; Liu and Quek, 2016; Kurtzman *et al.*, 2011; Toh *et al.*,
383 2018). In accordance with low ethanol production observed during 100-GEC
384 fermentation (Figure 2d), sucrose utilization by *S. suaveolens* was also very limited.
385 This was in accordance with other studies on the use of NCY in beer fermentation
386 which highlighted that yeasts with limited capacities to consume sugars led to low
387 alcohol content beers when inoculated alone but were particularly desirable for the
388 production of aromatic beers (Gibson *et al.*, 2017; de Francesco *et al.*, 2015; Saerens
389 and Swiegers, 2014; Branyik *et al.*, 2012; Budroni *et al.*, 2017).
390 The kinetics of sugars consumption of the co-fermented wort (30-GEC and
391 90-GEC beers) revealed intermediate profiles which were between those of 0-GEC
392 and 100-GEC beers, with 90-GEC beers displaying lower carbohydrate
393 consumption rates compared to 30-GEC beers. Likewise, these results highlighted
394 the influence of the initial starter of culture on maltose consumption which seems to
395 slow down with the increase of the *S. suaveolens* proportion in the starter of culture
396 (Figure 3d).

397 3.2.4. Changes in organic acids

398 We monitored the production of six common volatile organic acids (namely
399 α-ketoglutaric, lactic, pyruvic, succinic, acetic and malic acid) during fermentation
400 (Supplementary data A). In the initial wort, no succinic and lactic acid were
401 detected but pyruvic and α-ketoglutaric acids were present at very low levels (3.51

402 mg/L and 1.60 mg/L respectively) while malic acid content was much higher with
403 0.334 g/L. Finally, in accordance with the literature (Liu and Quek, 2016; Toh *et al.,*
404 2018), we found that acetic acid constituted the major organic acid as it content
405 reached up to 1.36 g/L in the initial wort.

406 For all the beer tested, no lactic and succinic acid were produced during the 14
407 days of fermentation. On the other hand, we found that the use of *S. suaveolens* in
408 co-culture with *S. cerevisiae* (30-GEC and 90-GEC) globally increased the production
409 of α-ketoglutaric acid during the fermentation of wort as compared to the use of a
410 mono-culture of *S. cerevisiae* (0-GEC), showing that *S. suaveolens* was favored the
411 production of this organic acids during the beer production. This was probably due
412 to the more extensive amino-acid catabolism of *S. suaveolens* than in *S. cerevisiae*
413 (Grondin *et al.,* 2015a), which produce part of the α-ketoglutaric acid (Yin et al.,
414 2017). On the other hand, pyruvic acid and acetic acid were produced at higher
415 level in 0-GEC than in 30-, 90- and 100-GEC beers, revealing the high impact of *S.*
416 *cerevisiae* in the production of these organic acids. With respect to malic acid, no
417 significant difference was found in all beers when compared to the initial wort,
418 confirming that malic acid content is not related to the composition of the starter of
419 culture (Li and Liu, 2015).

420 Finally, none of the organic acids reached their respective odor threshold
421 values, suggesting that they could impart the sourness of the final beer produced
422 without impacting their flavours whatever the composition of the starter of culture.

3.3. Contribution of S. suaveolens in the constitution of a new fruity aroma bouquet in beers after mixed fermentation using industrial wort

To assess the influence of *S. suaveolens* metabolism on the aromatic bouquet of the beers, we carried out large-scale batches (6L) of beers using industrial wort (recipe of SOREBRA©) and we analyzed the volatile organic compounds (VOCs) using headspace-solid phase micro-extraction (HS-SPME) of the initial wort and the beers obtained after 14 days of fermentation (0-GEC-D14, 30-GEC-D14, 90-GEC-D14 and 100-GEC-D14 beers) by gas-chromatography coupled with a mass spectrometry detector (GC-MS). Moreover, we also investigated VOCs produced in beers after 7 days of fermentation (0-GEC-D7, 30-GEC-D7, 90-GEC-D7 and 100-GEC-D7 beers). Based on this experiment, 22 major VOCs consisting of esters, alcohols and terpenoïds were detected in the beers.

3.3.1. Influence of the fermentation time on the aromatic bouquet of beers

To better illustrate the influence of the fermentation duration on the aromatic bouquet of the beers, a multivariate statistical analysis was carried out based on ethanol content and VOCs data for wort, and beers obtained after 7 and 14 days of fermentation (Figure 4). The first two principal components explained most of the variance (75.29%) with first principal component (F1) and second principal component (F2) covering 42.86 and 32.44 % of the variation in the data, respectively. Hierarchical ascendant classification allowed distinguishing one group (Group 1 including all the beers fermented during 14 days) and five independent samples,

444 namely non-inoculated wort, 0-GEC-D7, 30-GEC-D7, 90-GEC-D7 and 100-GEC-D7
445 beers. After 14 days of fermentation, beers obtained from single or mixed
446 fermentation (Group 1) had a tendency to converge into the center of the graph,
447 which indicates that the aromatic bouquets in these beers did not significantly differ
448 from beers prepared with *S. cerevisiae* alone. Moreover, the VOCs content of these
449 beers was between 38% (for 0-GEC beer) to 70% lower (for 100-GEC beer) than in
450 beers after 7 days of fermentation. This suggests that VOCs undergo volatilization
451 or are re-used by the yeast(s) in the second period of fermentation. Moreover, the
452 aromatic bouquets of beers fermented during 7 days were all significantly different
453 from each other, thus confirming that *S. suaveolens* have a strong impact on the
454 aromatic bouquet of the beers already 7 days after inoculation. Thus, to produce
455 beers with distinctive aromatic bouquet and that would benefit entirely of the
456 VOCs production from *S. suaveolens*, we suggest to stop the process of beers
457 production after 7 days of fermentation.

458 3.3.2. Influence of *S. suaveolens* metabolism on the aromatic bouquet of beers after 7
459 days of simple- or mixed-fermentation.

460 The beer obtained after 7 days of simple-fermentation of wort with *S. cerevisiae*
461 (0-GEC-D7 beer) was better described in the second principal component F2 where
462 principle components are mainly defined by VOCs from lipid metabolism and
463 glycolysis (such as ethyl caprate, ethyl 9-decenoate and 2-phenylethyl acetate
464 (Kobayashi *et al.*, 2008; Welsh *et al.*, 1989)) and a higher ethanol content (Figure 4).

On the contrary, the beers obtained after 7 days of fermentation with *S. suaveolens* alone (100-GEC-D7 beer) was set in the first principal component F1, and found to accumulate a majority of VOCs, such as ethyl 3-methylbutanoate, isoamyl alcohol, ethyl 2-methylbutanoate or ethyl tiglate from the amino acids catabolism (Brányik *et al.,* 2008; Chen, 1978; Dickinson *et al.,* 2003; Hazelwood *et al.,* 2008)). 7 days beers from the co-culture of *S. cerevisiae* with *S. suaveolens* (30-GEC-D7 and 90-GEC-D7 beers) displayed intermediates VOCs profiles characterized by metabolites which are specific to *S. suaveolens* metabolic network, such as ethyl tiglate or 2-methylpropyl acetate (Supplementary data B). This confirmed that *S. suaveolens* was metabolically active during mixed-fermentation of wort, although it did not grow under these conditions (Figure 1).

Major type of VOCs detected in 0-, 30-, 90 and 100-GEC beers obtained in this condition are presented in Table 1. 100-GEC beer displayed 150% more total VOCs as compared to the standard beer, whereas the 30-GEC and the 90-GEC beers displayed 23% and 15% more total VOCs respectively (Table 1). These results were quite surprising as it was expected that natural aromatization of beer in mixed-fermentation of wort would strictly require a higher ratio of the NCY in the starter of culture (van Rijswijck *et al.*, 2017). However this showed that varying the ratio between *S. suaveolens* and *S. cerevisiae* could positively influence the aromatic bouquet of the beers.

Generally, yeasts produce higher alcohols and their corresponding acids by transforming amino-acids *via* the Ehrlich pathway (Kobayashi *et al.,* 2008; Pires *et*

487 *al.*, 2014). In our assays, only two higher alcohols, namely 2-methylbutanol and
488 3-methylbutanol, were detected in beers whereas their corresponding acids were
489 not detected. This result suggests that a high rate of higher alcohol and acids
490 esterification occurs in the fermentation media. The odor threshold of
491 2-methylbutanol, which is estimated at 1.90 mg/L (Chen *et al.*, 2006; Czerny *et al.*,
492 2008; Leffingwell and Associates ; Nagata, 2003) was not reached in any beers
493 whatever the starter of culture used, suggesting that this compound was not
494 produced enough to impact the beers with its balsamic, wine and onion-like
495 off-flavor (Czerny *et al.*, 2008). With respect to 3-methylbutanol, its odor threshold
496 of 0.3 mg/L (Leffingwell and Associates) was reached only in beers containing *S.*
497 *suaveolens* (namely 30-GEC, 90-GEC and 100-GEC-D7 beers), while 100-GEC-D7
498 beers showed the highest amount of this compound. This suggested that *S.*
499 *suaveolens* participates actively to the production of 3-methylbutanol during the
500 fermentation and allows its production at level above its odor threshold. When
501 associated with *S. cerevisiae* which maintain a good fermentative activity in the
502 wort, the use of *S. suaveolens* was therefore successful to improve the aromas of the
503 beers and their drinkability because the heaviness of the flavor of the final product
504 increased with the concentration of 3-methylbutanol (Olaniran *et al.*, 2017).

505 In all beer produced after 7 days of fermentation, the esters, including acetate
506 and ethyl esters which define fruity aroma of beers (Engan, 1972; Michel *et al.*, 2016;
507 Peddie, 1990; Pires *et al.*, 2014; Verstrepen *et al.*, 2003; Willaert and Nedovic, 2006;
508 Zhang *et al.*, 2013), were the most abundant volatile compounds detected

509 (Supplementary data B). The concentration of these esters increased proportionally
510 with the increase of *S. suaveolens* in the initial starter of culture. This was in
511 accordance with the results of Zhu and collaborators (2016) who showed that some
512 strains of *Geotrichum* species and assimilates (such as *S. suaveolens*) were applicable
513 for increasing esters in fermented beverages including wine and liquors by
514 decreasing their higher alcohols content. Similarly, the beers produced after 7 days
515 of mono-culture with *S. suaveolens* (100-GEC-D7) displayed a significantly higher
516 amount of acetate esters as compared to standard beers made with *S. cerevisiae*
517 alone, confirming the higher esterification ability of *S. suaveolens* compared to *S.*
518 *cerevisiae* (Grondin *et al.,* 2015a; Zhu *et al.,* 2016). However, the overall acetate ester
519 production was lower in both mixed-fermentation beers (30-GEC-D7 and
520 90-GEC-D7) as compared to beers produced by mono-cultures (0-GEC-D7 and
521 100-GEC-D7). This result suggests that an inhibition effect occurs on acetate esters
522 production when both yeasts are present in the broth. Apart from ethyl acetate (that
523 could not be detected using this analytical system), the most important flavor active
524 acetate esters in beers were 3-methylbutyl acetate (banana-like), 2-methylpropyl
525 acetate (fruit-like) and 2-phenyl-ethyl acetate (flower-like) (Kobayashi *et al.,* 2008;
526 Peddie, 1990; Pires *et al.,* 2014; Verstrepen *et al.,* 2003). The concentration of
527 3-methylbutyl acetate (banana-like) was globally similar in all beers, despite the fact
528 that its alcohol precursor, namely 3-methylbutanol (malt-like) was higher in
529 30-GEC, 90-GEC and 100-GEC beers. 2-methylpopryl acetate (fruit-like) was only
530 detected in beers fermented by *S. suaveolens (3*0-GEC, 90-GEC and 100-GEC beers)

531 but the associated fruity and flowery flavours (Leffingwell and Associates) can
532 theoretically be detected in 100-GEC beer which obtained a concentration of this
533 compounds that was about 3 to 4 time higher (0.23 mg/L) than its odor threshold
534 (0.07 mg/L). Finally, 2-phenylethyl acetate (flower-like) was detected only in 0-GEC
535 and 30-GEC-D7 beers.

536 Ethyl esters, which are produced from esterification of medium chain fatty acid
537 (MCFA) in the presence of ethanol (Peddie, 1990; Stevens, 1960), were the most
538 abundant group of compounds detected in all the beers. This result is consistent
539 with literature in which most significant ethyl esters in beers are ethyl hexanoate
540 (apple-like) and ethyl octanoate (apricot-like) (Peddie, 1990; Verstrepen *et al.*, 2003).
541 In our study, both compounds were found above their odour thresholds in all beers
542 and could then confer their fruity flavour to them. Most of the medium-chain fatty
543 acid (MCFA) ethyl esters deriving from lipid metabolism (ethyl decanoate, ethyl
544 9-decenoate, ethyl dodecanoate and ethyl hexadecanoate) were more abundant in
545 beers fermented with a high ratio of *S. cerevisiae* (namely 0-GEC-D7 and 30-GEC-D7
546 beers). On the contrary, most of MCFA ethyl esters deriving from the metabolism of
547 amino acids (ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl tiglate) were
548 mainly more abundant in beers fermented with high ratio of *S. suaveolens* in the
549 starter of culture. Consistent with data from literature (Olaniran *et al.*, 2017), this
550 result showed that the major part of ethyl ester production occurred within the first
551 two days of fermentation, *i.e*, before *S. suaveolens* depletion (Figure 1 a and b and
552 Figure 2a).

553 Finally, esters were mostly found at concentration below their individual odor
554 threshold in final beers. But it is well known that even if their individual
555 concentration is well under their odor threshold, esters can have a synergic effect
556 and thus affect the overall flavor of the product (Olaniran *et al.,* 2017; Verstrepen *et*
557 *al.,* 2003). A preliminary hedonic analysis performed in the laboratory with beers
558 prepared with *S. suaveolens* alone (100-GEC) or in co-culture with *S. cerevisiae* (30-
559 and 90-GEC) tends to indicate that particularly high fruity aromas can be generated
560 in these beers (data not shown). This result, which will be confirmed by a thorough
561 sensory analysis, also suggests that the fruity taste increased proportionally with
562 the increase of *S. suaveolens* in the starter of culture, but at the same time the *alcoholic*
563 *note* of the standard beer was lost. The results of our experiments thus highlighted
564 the interest of (a) a mixed-fermenting wort with 30% cell/cell *S. suaveolens* and 70%
565 cell/cell of *S. cerevisiae* (30-GEC) during 7 days to produce fruity beers with
566 characteristics (pH, ethanol content, TSS content) comparable to standard beers and
567 (b) a mixed-fermenting wort with 90% cell/cell *S. suaveolens* and 10% cell/cell of *S.*
568 *cerevisiae* during 7 days to produce low ethanol content fruity beers, but associated
569 to higher residual sugars.
570
571 ## 5. Conclusions
572 In this study we demonstrated that the high aroma producer *S. suaveolens* strain
573 could be used in mixed-fermentation with *S. cerevisiae* to produce beers enriched
574 significantly in fruity flavors as compared to standard beers (*i.e.* produced in

mono-culture with *S. cerevisiae*), even when the ratio of the NCY was low as compared to *S. cerevisiae*. Beside its influence on VOCs production, we also demonstrated the possibility to control ethanol production in the final beer by varying the ratio of both strains in the starter of culture. A good compromise was found for wort inoculated with 30% of *S. suaveolens* and 70% of *S. cerevisiae* and fermented for 7 days which led to beers with ethanol content comparable to standard beers, but with an aromatic bouquet enriched in fruity flavours. Contrariwise, inoculating wort with 90% of *S. suaveolens* and 10% of *S. cerevisiae* for 7 days fermentation was shown as an interesting process to produce low-ethanol content fruity beers. This study confirms then that *S. suaveolens* is a good candidate to improve the aromatic properties of industrial beers. To complete these data, a sensorial comparative analysis of beers fermented during 7 days with simple or co-culture of *S. suaveolens* (30-, 90-, 100-GEC beers D7) with standard beer (0-GEC D7) will be published in another article specially turned to the sensorial properties of the novel beer.

Author Contributions: Conceptualization: MT, JMF and TP; Methodology: MT, YC, ASCS, LR, JMF and TP; Formal analysis, MT, YC, LR and TP; Investigation: MT, JMF and TP; Resources: LR and JMF ; Writing—original draft preparation: MT, YC, JMF and TP ; Writing—review and editing: MT, YC, JMF and TP; Supervision: JMF, ASCS and TP; Project administration: JMF and TP; Funding acquisition: MT, ASCS and TP. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Regional Council of Reunion Island.

Acknowledgments: The authors wish to thank the management of the local brewing company SOREBRA (Saint-Louis, Reunion Island, France) for kindly provide industrial wort for our fermentation assays.

Conflicts of Interest: The authors declare no conflict of interest.

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Figures

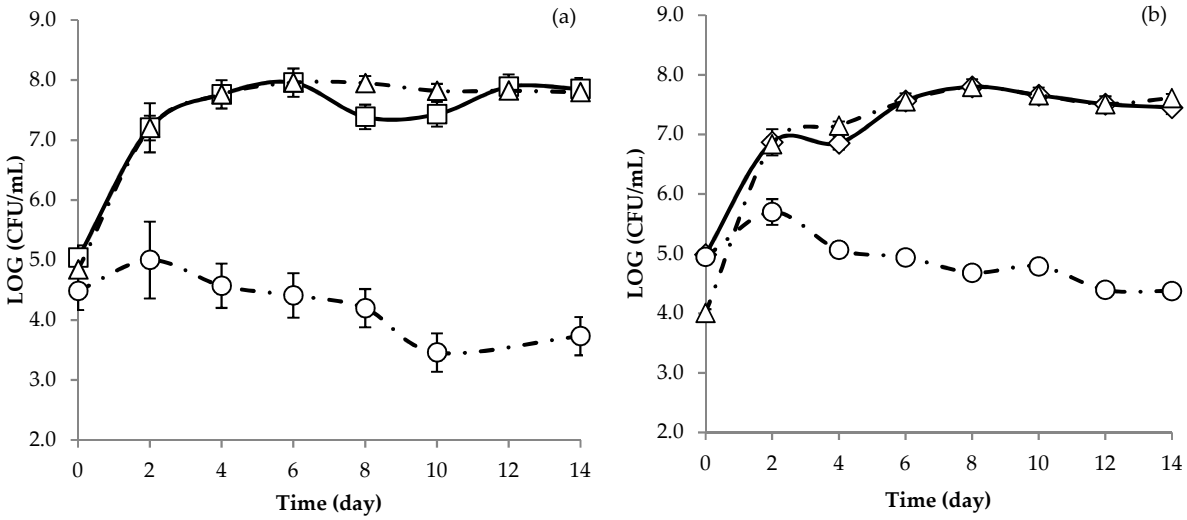


Figure 1. Cell growth of *S. suaveolens* and *S. cerevisiae* during wort mixed fermentation (a) 30-GEC (with starter of culture containing 70% of *S. cerevisiae* and 30% of *S. suaveolens* cell/cell) and (b) 90-GEC (with starter of culture containing 10% of *S.* cerevisiae and 90% of *S. suaveolens* cell/cell). □ : Total yeast growth; O: *S. suaveolens* growth; Δ : *S. cerevisiae* growth.

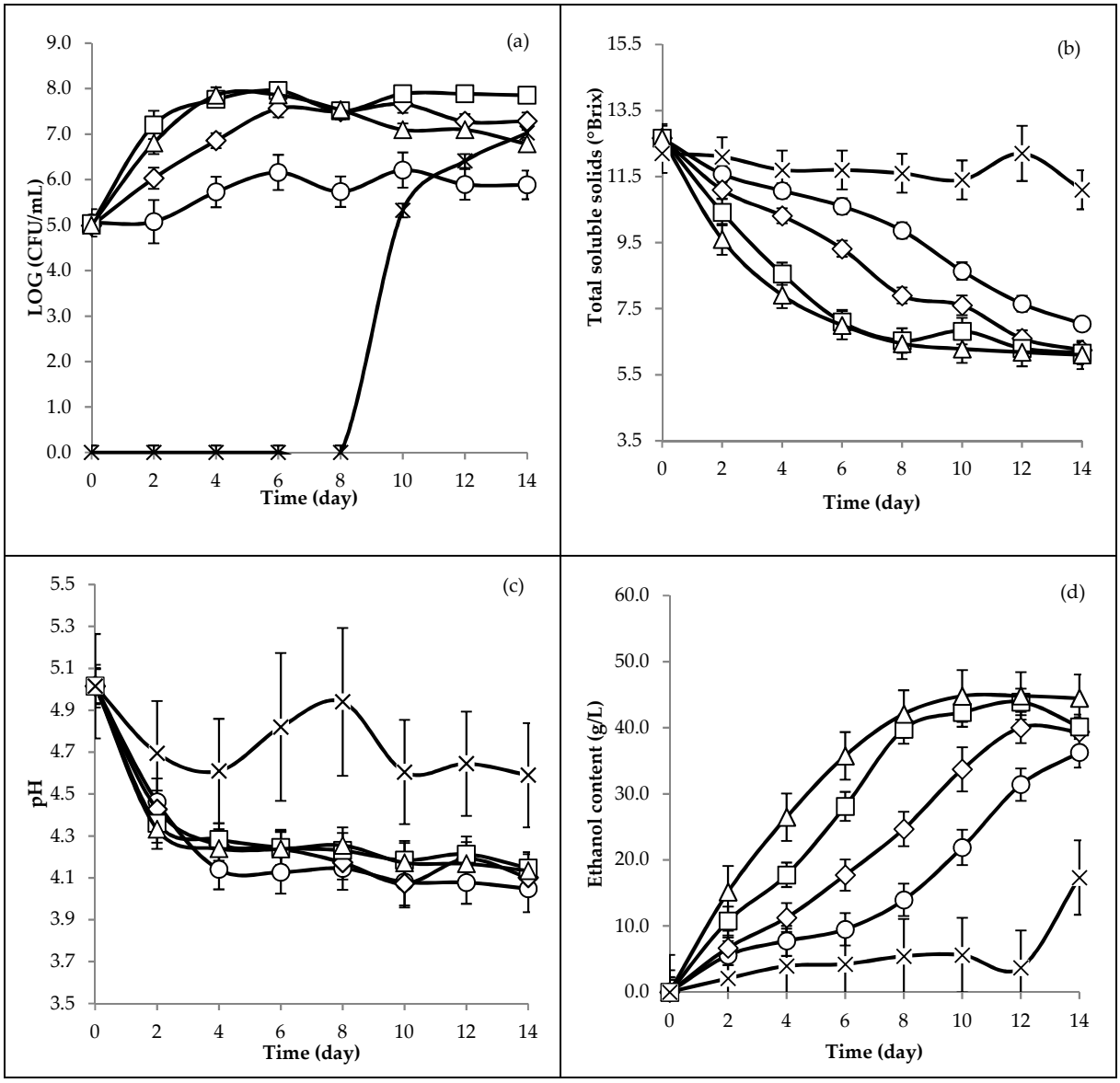


Figure 2. Kinetics of (a) total yeast content, (b) total soluble solids content, (c) pH and (d) ethanol content during fermentation of raw non inoculated wort (X), 0-GEC (Δ , single-fermentation with *S. cerevisiae*), 30-GEC (□ , mixed-fermentation with starter of culture containing 70% of *S. cerevisiae* and 30% of *S. suaveolens*, 90-GEC (◊ , mixed-fermentation with starter of culture containing 10% of *S. cerevisiae* and 90% of *S. suaveolens*) and 100-GEC (○ , single-fermentation with *S. suaveolens*).

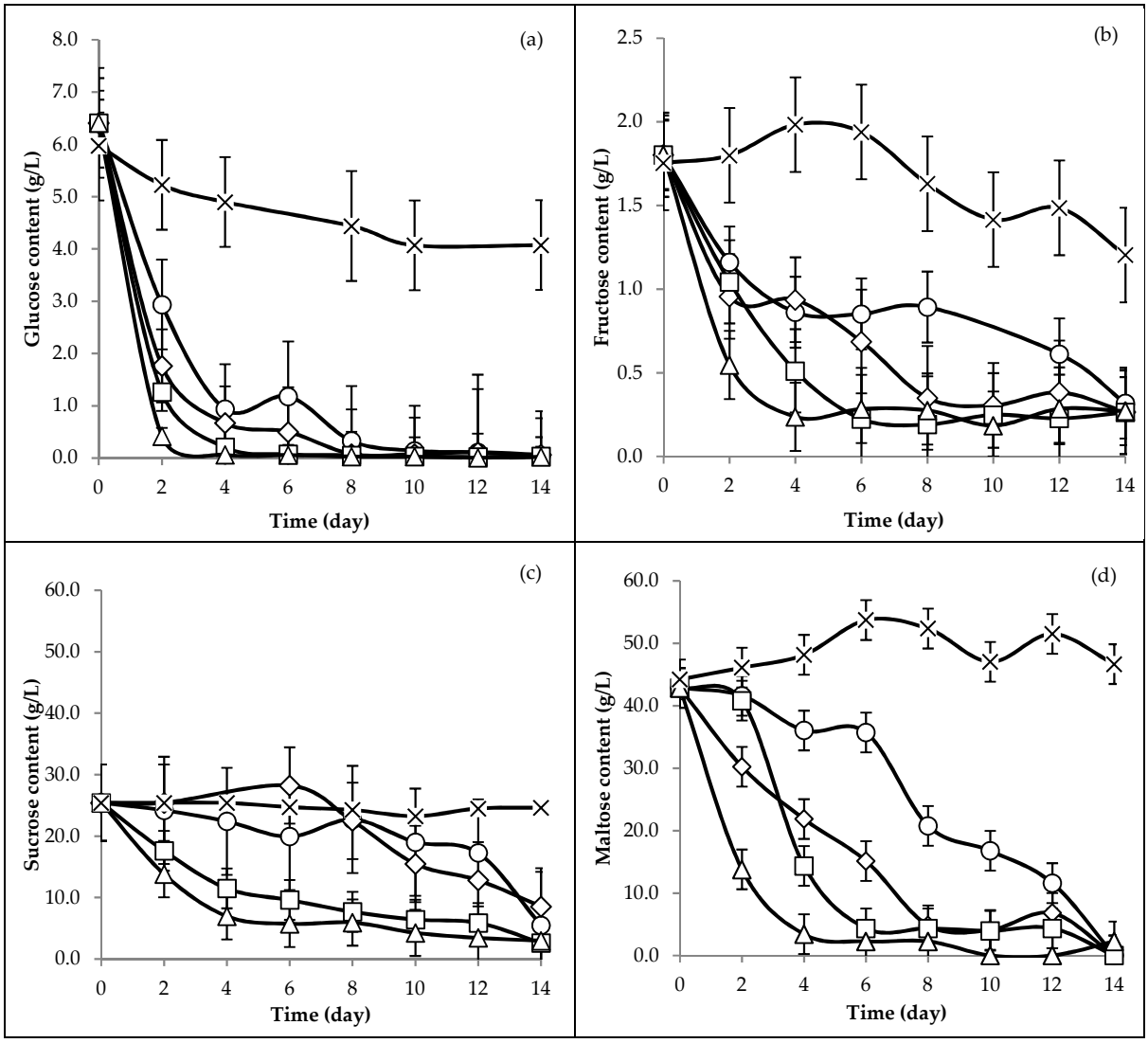
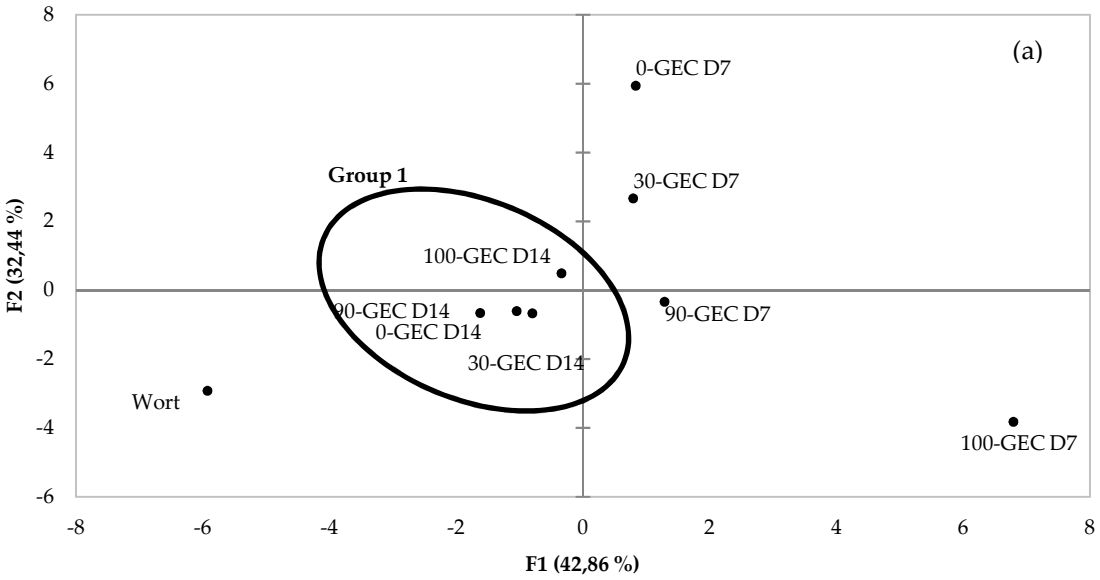


Figure 3. Kinetics of consumption of carbohydrates (in g/L) contained in the wort (a : glucose ; b : fructose; c : sucrose and d : maltose) content during fermentation of raw non inoculated wort (X), 0-GEC (Δ single-fermentation with *S. cerevisiae*), 30-GEC (□ , mixed-fermentation inoculated with 70% of *S. cerevisiae* and 30% of *S. suaveolens*, 90-GEC (◊ , mixed-fermentation inoculated with 10% of *S. cerevisiae* and 90% of *S. suaveolens*) and 100-GEC (○ , single-fermentation inoculated with *S. suaveolens* alone).



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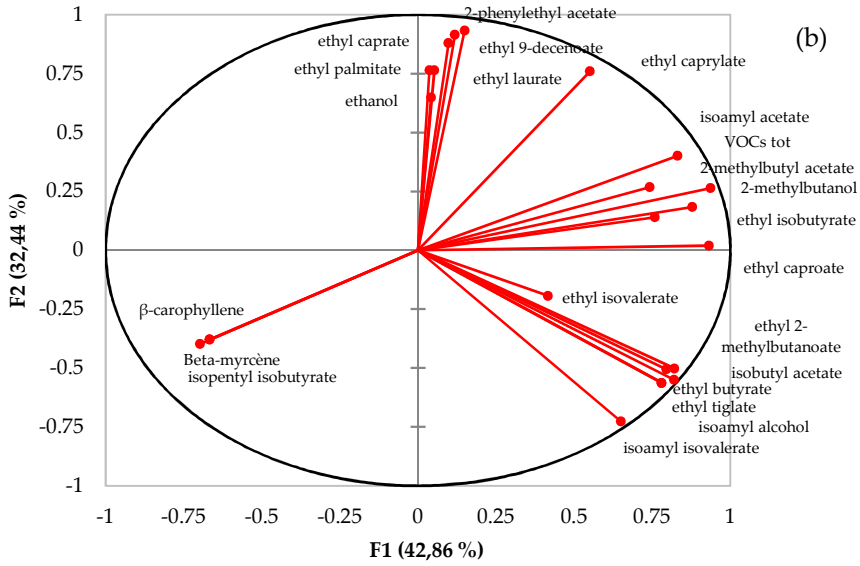

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22 **Figure 4.** Score plot of F2 versus F1 (4a and 4b) for the main organic volatile
23 compounds detected in initial and fermented wort analyzed after 7 days (D7) and
24 14 days (D14) of fermentation with 0-GEC (single-fermentation with *S. cerevisiae*),
25 30-GEC (mixed-fermentation inoculated with 70% of *S. cerevisiae* and 30% of *S.*
26 *suaveolens*, 90-GEC (mixed-fermentation inoculated with 10% of *S. cerevisiae* and
27 90% of *S. suaveolens*) and 100-GEC (single-fermentation inoculated with *S. suaveolens*
28 alone). Principal Component Analysis was performed using XLSTAT (Addinsoft).
29 Predicted groups were correlated to CAH clusters.

Tables

Table 1 : Concentration (mg/L) of the type of major volatile compounds (GC-MS) identified in wort and beers obtained after 7 days of mono-culture and mixed-fermentation of wort with *S. cerevisiae* and *S. suaveolens*.

Type of VOCs	**Wort**	**0-GEC beer**	**30-GEC beer**	**90-GEC beer**	**100-GEC beer**
Alcohols	0.04 ± 0.04	0.54 ± 0.94	1.08 ± 0.45	1.46 ± 0.29	1.62 ± 1.24
Esters	0.02 ± 0.02	3.06 ± 1.68	3.00 ± 0.88	4.20 ± 1.29	7.04 ± 4.40
Terpens and terpenoids	0.09 ± 0.06	-	-	-	-
Total volatiles	0.15 ± 0.12	3.61 ± 2.26	4.71 ± 1.02	4.29 ± 1.61	9.05 ± 5.20

Data are means ± standard deviations. **0-GEC-D7**, beer obtained after 7 days of wort inoculated with *S. cerevisiae* alone; **30-GEC-D7**, beer prepared during 7 days using a starter of culture containing 70% of *S. cerevisiae* and 30% of *S. suaveolens* (cell/cell); **90-GEC-D7**, beer obtained after 7 days of wort mixed-fermentation with starter of culture containing 10% of *S. cerevisiae* and 90% of *S. suaveolens* (cell/cell); **100-GEC-D7**, beer obtained after 7 days of wort inoculated with *S. suaveolens* alone